

Fundamentals of Food Biotechnology

Second Edition

Byong H. Lee



WILEY Blackwell

Fundamentals of Food Biotechnology

Fundamentals of Food Biotechnology

Second Edition

Byong H. Lee

Distinguished Professor, School of Biotechnology

Jiangnan University, Wuxi, China

Invited Distinguished Professor, Department of Food Science & Biotechnology, Kangwon

National University, Chuncheon, Korea

Adjunct Professor, Department of Food Science & Agric Chemistry McGill University, Montreal, Quebec, Canada

WILEY Blackwell

This edition first published 2015 © 2015 by JohnWiley & Sons, Ltd

Registered office: John Wiley & Sons, Ltd, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK

Editorial offices: 9600 Garsington Road, Oxford, OX4 2DQ, UK
The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK

For details of our global editorial offices, for customer services and for information about how to apply for permission to reuse the copyright material in this book please see our website at www.wiley.com/wiley-blackwell.

The right of the author to be identified as the author of this work has been asserted in accordance with the UK Copyright, Designs and Patents Act 1988.

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, except as permitted by the UK Copyright, Designs and Patents Act 1988, without the prior permission of the publisher.

Designations used by companies to distinguish their products are often claimed as trademarks. All brand names and product names used in this book are trade names, service marks, trademarks or registered trademarks of their respective owners. The publisher is not associated with any product or vendor mentioned in this book.

Limit of Liability/Disclaimer of Warranty: While the publisher and author(s) have used their best efforts in preparing this book, they make no representations or warranties with respect to the accuracy or completeness of the contents of this book and specifically disclaim any implied warranties of merchantability or fitness for a particular purpose. It is sold on the understanding that the publisher is not engaged in rendering professional services and neither the publisher nor the author shall be liable for damages arising herefrom. If professional advice or other expert assistance is required, the services of a competent professional should be sought.

Library of Congress Cataloging-in-Publication Data

Lee, B. H. (Byong H.)

Fundamentals of food biotechnology / Byong H. Lee. – Second edition.
pages cm

Includes bibliographical references and index.

ISBN 978-1-118-38495-4 (cloth)

1. Food–Biotechnology. I. Title.

TP248.65.F66L44 2015

664 – dc23

2014032719

A catalogue record for this book is available from the British Library.

Wiley also publishes its books in a variety of electronic formats. Some content that appears in print may not be available in electronic books.

Cover image: Golden wheat field © Jiri Vondracek/iStockphoto;

Fish © moremi/iStockphoto;

chicken farm © matteodestefano/iStockphoto;

Prescription Drugs © DNY59/iStockphoto;

Biotechnology © nicolas/iStockphoto;

Cows Feeding © 123ducu/iStockphoto; and

Hot pepper plants for disease testing © User9236883_407/iStockphoto

Typeset in 9/11pt TimesTenLTStd by Laserwords Private Limited, Chennai, India

Contents

Preface	xi
What Is Biotechnology?	xiii
What Is Food Biotechnology?	xvii
Part I New Trends and Tools of Food Biotechnology	1
1 Fundamentals and New Aspects	3
1.1 Biotechnological applications of animals, plants, and microbes	3
1.2 Cellular organization and membrane structure	6
1.3 Bacterial growth and fermentation tools	11
1.3.1 Classification and reproduction of biotechnologically important bacterial system	11
1.3.2 Bacterial growth	12
1.3.3 Environmental factors affecting bacterial growth	16
1.4 Fungal growth and fermentation tools	19
1.5 Classical strain improvement and tools	22
1.5.1 Natural selection and mutation	22
1.5.2 Recombination	27
Summary	30
1.6 Systems/synthetic biology and metabolic engineering	31
Summary	36
1.7 Bioengineering and scale-up process	36
1.7.1 Microbial and process engineering factors affecting performance and economics	38
1.7.2 Fermentor and bioreactor systems	39
1.7.3 Mass transfer concept	50
1.7.4 Heat transfer concept	53
1.7.5 Mass and heat transfer practice	57
1.7.6 Scale-up and scale-down of fermentations	71
1.7.7 Scale-up challenges	81
Summary	84
1.8 Molecular thermodynamics for biotechnology	85
1.8.1 Protein folding and stability	85
Summary	92
1.8.2 Downstream processes on crystallization and chromatography	93
Summary	96
1.9 Protein and enzyme engineering	96
Summary	100
1.10 Genomics, proteomics, and bioinformatics	100
Summary	108

1.11	Biosensors and nanobiotechnology	109
1.11.1	Biosensor	109
1.11.2	Nanobiotechnology and nanobiosensor	113
	Summary	116
1.12	Quorum sensing and quenching	116
	Summary	120
1.13	Micro- and nano-encapsulations	120
1.13.1	Microencapsulation	122
1.13.2	Nanoencapsulation	129
	Summary	138
	Bibliography	140
2	Concepts and Tools for Recombinant DNA Technology	147
2.1	Concepts of macromolecules: function and synthesis	147
2.1.1	DNA replication	147
2.1.2	Roles of RNA	150
2.1.3	Detailed aspects of protein synthesis	153
2.2	Concepts of recombinant DNA technology	161
2.2.1	Restriction endonucleases	162
2.2.2	Plasmid vectors	164
2.2.3	Purpose of gene cloning	168
2.3	DNA sequencing	180
2.4	Polymerase chain reaction (PCR)	180
2.5	Manipulation techniques of DNA	183
2.5.1	Isolation and purification of nucleic acids	183
2.5.2	Agarose gel electrophoresis	184
2.5.3	Blotting and hybridization	185
2.6	Gene cloning and production of recombinant proteins	186
2.6.1	Cloning and expression of bacterial β -galactosidase in <i>E. coli</i>	186
2.6.2	Cloning, expression, and production of bovine chymosin (rennet) in yeast <i>K. lactis</i>	188
	Summary	190
	Bibliography	191
Part I	Questions and Answers	193
Part II	Applications of Biotechnology to Food Products	205
3	Yeast-Based Processes and Products	207
3.1	Food yeasts and derivatives	207
3.1.1	Introduction	207
3.1.2	Industrial processes	207
	Summary	212
3.2	Alcoholic beverages	212
3.2.1	Introduction	212
3.2.2	Production and sales of major alcoholic beverages	212
3.2.3	Production processes	213
	Summary	225
3.3	Industrial alcohols	225
3.3.1	Introduction	225
3.3.2	Raw materials and microorganisms	226
3.3.3	Production processes	230
3.3.4	Economics	231
	Summary	232
3.4	Bread and related products	232
3.4.1	Introduction	232

3.4.2	Ingredients and formulations	233
3.4.3	Production processes	234
3.4.4	New developments	236
	Summary	237
	Bibliography	237
4	Bacteria-Based Processes and Products	241
4.1	Dairy products	241
4.1.1	Introduction	241
4.1.2	Basic knowledge of manufacture of dairy products	244
4.1.3	Metabolic systems in lactic acid bacteria	249
4.1.4	Genetic modification of lactic acid bacteria	252
4.1.5	Applications of genetic engineering	254
	Summary	262
4.2	Meat and fish products	262
4.2.1	Introduction	262
4.2.2	Fermented meat products	263
4.2.3	New developments	267
4.2.4	Fermented fish products	267
	Summary	270
4.3	Vegetable products	270
4.3.1	Introduction	270
4.3.2	Fermented vegetable products	271
4.3.3	Fermented soy products	275
4.3.4	New developments	280
	Summary	280
4.4	Vinegar and other organic acids	281
4.4.1	Introduction	281
4.4.2	Acetic acid	281
4.4.3	Citric acid	283
4.4.4	Lactic acid	284
4.4.5	Malic acid	285
4.4.6	Fumaric acid	286
	Summary	286
4.5	Bacterial biomass	287
4.5.1	Introduction	287
4.5.2	Microorganisms for the production of biomass	288
4.5.3	Raw materials for the production of biomass	289
4.5.4	Production process	292
4.5.5	Nutritional aspects	293
4.5.6	Economics and new developments	294
	Summary	295
4.6	Polysaccharides	296
4.6.1	Introduction	296
4.6.2	Microbial polysaccharides	297
4.6.3	Fermentation process	298
4.6.4	Bacterial polysaccharides	299
4.6.5	Other polysaccharides	304
	Summary	304
	Bibliography	306
5	Other Organism-Based Processes and Products	313
5.1	Enzymes	313
5.1.1	Introduction	313
5.1.2	Production of enzymes	315
5.1.3	Applications	317

5.1.4	New developments and protein engineering	326
5.1.5	Economics	328
	Summary	328
5.2	Sweeteners	329
5.2.1	Introduction	329
5.2.2	Nutritive sweeteners	329
5.2.3	High-intensity sweeteners	333
5.2.4	Low calorie sweeteners	337
5.2.5	New developments	338
	Summary	339
5.3	Flavors and amino acids	339
5.3.1	Introduction	339
5.3.2	Microbial flavors	340
5.3.3	Enzymatic flavor generation	347
5.3.4	Amino acids	348
5.3.5	Economics	350
	Summary	351
5.4	Vitamins and pigments	352
5.4.1	Introduction	352
5.4.2	Production of vitamins	352
5.4.3	Production of pigments	356
5.4.4	Economics	359
	Summary	359
5.5	Mushrooms	360
5.5.1	Introduction	360
5.5.2	Cultivation	361
5.5.3	Culture preservation	363
	Summary	363
5.6	Cocoa, tea, and coffee fermentation	364
5.6.1	Introduction	364
5.6.2	Cocoa fermentation	364
5.6.3	Coffee fermentation	367
5.6.4	Tea fermentation	369
	Summary	372
5.7	Bacteriocins	372
5.7.1	Introduction	372
5.7.2	Classification	373
5.7.3	Mode of action	375
5.7.4	Bioengineering of bacteriocins	376
5.7.5	Applications of bacteriocins	379
5.7.6	Commercial production of bacteriocins	382
	Summary	383
5.8	Functional foods and nutraceuticals	383
5.8.1	Probiotics and prebiotics	384
5.8.2	Health claim regulation	396
	Summary	397
	Bibliography	397
Part II Questions and Answers		411
Part III Other Potential Applications of the New Technology		431
6 Plant Biotechnology, Animal Biotechnology, and Safety Assessment		433
6.1	Plant biotechnology	433
6.1.1	Introduction	433
6.1.2	Plant cell and tissue cultivation	435

6.1.3	Plant breeding	437
6.1.4	Application of plant cell and tissue culture	441
	Summary	448
6.2	Animal biotechnology	449
6.2.1	Introduction	449
6.2.2	Transgenic animals	449
6.2.3	Animal cell culture	453
	Summary	463
6.3	Food safety issues of new biotechnologies	464
6.3.1	Introduction	464
6.3.2	Safety evaluation of novel food products	465
6.3.3	Genetically modified microorganisms and their products	467
6.3.4	Genetically modified plants and their products	469
6.3.5	Genetically modified animals and their products	473
6.3.6	Detection methods of GM crops	475
6.3.7	Detection methods of transgenic animals and fish	480
6.3.8	Containment: physical and biological	481
6.3.9	Promises and limitations	481
	Summary	482
	Bibliography	483
Part III Questions and Answers		491
	Index	497

Preface

In the past decade, major breakthroughs have happened and enormous progress has been made in all aspects of genetic engineering and biotechnology. This is clearly reflected in the voluminous publications of original research, patents, peer reviewed books, and symposia. However, an exciting account of how this new biotechnology can affect traditional methods of producing foods and beverages is the need of the hour. Many professional reference texts on food biotechnology are now available, but none of it is appropriate as classroom text. Most such volumes are the work of multiple contributors and the normal didactic criteria required to explain terms, flowcharts and frames of reference are lacking. No attempt has been made to explain the translation of basic scientific information into practical applications. Moreover, biotechnology has become a fashionable subject and, as one of the most abused buzz words of the decade, it now comprises a huge body of information. The very scope of this knowledge presents serious problems to instructors and students. Which facts are the most important for them to learn and which are less important? How can they assess the significance of food systems and food products? In writing this book, I have tried to keep these problems at the forefront and have therefore aimed at making the treatment of food biotechnology comprehensible rather than comprehensive. I see that separate pieces of a puzzle eventually fit together to form a picture that is clearer and more readily etched in memory than the design on the individual pieces. Experience in teaching this subject has made clear to me the importance of explaining the basic concepts of biotechnology, which is essentially multidisciplinary, to students who may have limited backgrounds in the scale up of bioengineering and rapidly developing new tools.

I hope that this book will prove valuable to both students and instructors as well as to research and industrial practitioners in specific aspects of the field who seek a broad view on food biotechnology. This book aims to give readers, general science students, and practicing researchers, an overview of the essential features of food biotechnology not covered in other institutions as typical science curriculum. The treatment of subjects is necessarily selective, but the volume seeks to balance the traditional biotechnologies with the new, and science and engineering with their industrial applications and potential. Because of the interdisciplinary nature of the subject and the overlapping nature of the principles of biochemistry, microbiology, and biochemical engineering, the second edition does not include this part. Instead, the New Trends and Tools of Food Biotechnology section in Part I (Fundamentals and New Aspects) has included Systems/Synthetic Biology and Metabolic Engineering, Bioengineering and Scale-Up Process, Molecular Thermodynamics for Biotechnology, Protein and Enzyme Engineering, Genomics, Proteomics and Bioinformatics, Biosensors and Nanobiotechnology, Quorum Sensing and Quenching, and Micro- and Nanoencapsulations. For the Concepts and Tools for Recombinant DNA Technology (Chapter 2), examples of Gene Cloning and Production of Recombinant Proteins have been included. In Chapter 5 on Other Microorganisms-Based Processes and Products, a new section on Bacteriocins and Functional Foods and Nutraceuticals was

supplemented and the Waste Management and Food Processing section was deleted; it will be included in my forthcoming book entitled: “Advanced Fermentation Technology.” In Part III, Chapter 6 included Plant Biotechnology, Animal Biotechnology, and Safety Assessment and Detection Methods and other sections were detailed. Up-to-date reading materials as well as questions and answers have been included in all parts.

I must, of course, thank all those students who have helped me by compiling materials used in the class to produce this book. I greatly appreciate the contribution of many scientists who have embellished this book by permitting me to reproduce their tables and figures, which have been illustrated in the pages of this book. I must accept my ignorance and limitations naturally imposed on a book of this scope when it is written by a single individual.

A special note of thanks also goes to my previous research associates and students for the first edition at the McGill University, Dr. S. Y. Park, Dr. J. L. Berger who helped me in typing and drawing the figures, and other associates, G. Arora, M. Torres, M. B. Habibi-Najafi, and graduate students, M. Bellem, M. Daga, J. James, and T. Wang who helped me in many ways.

Most of all, my thanks go to Prof. Jian Chen, the President, and Prof. Guchang Du, the Dean for their support during my stay in the School of Biotechnology at Jiangnan University in China and the other staff in the 9th floor: Dr. F. Fang, Dr. Z. Kang, Dr. L. Song, Dr. J. Zhang, Dr. J. Zhou, Dr. L. Liu, and Prof. J. Li for their friendship during my absence. I would like to specially thank Dr. Gazi Sakir for his comments on a part of the bioengineering/scale up section, as well as my students, Dr. Zixing Dong, Yousef Mahammad, and Nestor Ishimwe, and all international students who took my courses on Food Biotechnology and Advanced Fermentation Technology at the Jiangnan University.

Last but not the least, I thank my wife Young for her love and encouragement; I also thank and appreciate my sons, Edward in Toronto and David in New York, for their patience and support during the preparation of this second manuscript.

December, 2013

Byong H. Lee
Wuxi, China

What Is Biotechnology?

We are in the middle of another industrial revolution in which biotechnology, depending mainly on microbes, plays a major role in the production of exotic drugs, industrial chemicals, bioingredients, fuel, and even food. Although biotechnology involves the potential use of all living forms, microorganisms have played a major role in the development of this discipline because of the ease of mass growth, the rapid growth that occurs in media consisting of cheap waste materials, and the massive diversity of metabolic types. These characteristics in turn allow for a diverse selection of potential products and facilitate genetic manipulation to improve strains for new products.

The *bio* in “biotechnology” means *life* and refers to microbes and other living cells including animal and plant cells. The *technology* comprises the growth of living cells in vats (fermentors or bioreactors) containing nutrients and oxygen (if needed) at the specified conditions, and the processing of biological materials produced by the cells through process integration and optimization at top efficiency for achieving commercialization. Biotechnology has arisen through the interaction between various parts of biology and engineering, employing techniques derived from three well-recognized disciplines: biochemistry, microbiology, and biochemical engineering.

The term *biotechnology* is not a new one, although it has certainly become fashionable in recent years. It had its origin in prebiblical times but was not widely used until the postwar university boom in the 1950–1960s, when the volume of scientific and engineering research output rose dramatically. New disciplines emerged out of increasing specialization. Thus in the early 1960s, research groups and university departments as well as journals arose with titles such as *BioTechnology*, *Biochemical Engineering*, and *Bioengineering*. “Biotechnology” is the term that has commonly survived. Table I.1 shows that prior to the twentieth century, biotechnology consisted almost solely of spontaneous processes. The introduction of the fed batch in the production of baker’s yeast is probably the starting point of *controlled biological processes* designated as biotechnological. Biotechnology thus includes many traditional processes such as brewing, baking, wine making, and cheese making; and the production of soy sauce, tempeh, many secondary metabolites (antibiotics, steroids, polysaccharides, etc.), and numerous food ingredients (amino acids, flavors, vitamins, and enzymes). Traditionally, the biotechnological process based on classical microbial fermentation has been augmented by simple genetic manipulation using a mutagenic agent to improve microorganisms for food fermentation and to enhance the production of bioingredients. However, it is not possible to predetermine the gene that will be affected by a given mutagen, and it is difficult to differentiate the few superior producers from the many inferior producers found among the survivors of a mutation treatment.

The potential of fermentation techniques was dramatically increased in the late 1960s and early 1970s through achievements in molecular genetics, cell fusion, and enzyme technology. A new biotechnology was founded based on these methods. However, additional completely novel, very powerful biotechnology techniques were developed

Table I.1 Biotechnology Milestones

Date	Milestone
	<i>Old biotechnology</i>
Before 6000 B.C.	Leavening of bread, alcoholic beverages, and vinegar from fermented juice
Before fourteenth century	Beer and wine production, vinegar industry (Orleans)
1650	Cultivation of mushrooms (France)
1680	Yeast cells first seen by Anton van Leeuwenhoek
1857–1876	Fermentative ability of microbes demonstrated by Pasteur
1881	Microbiological production of lactic acid
1885	Artificial growth of mushrooms (U.S.A.)
Nineteenth century	Ethanol, acetic acid, butanol, acetone production, sewage treatment, baker's yeast, sulfite process for glycerol, citric acid
1940s	Introduction of sterility to the mass cultivation of microbes for antibiotics (penicillin, streptomycin, chlorotetracycline) and bioingredients (amino acid, enzymes, vitamins, steroids, polysaccharides) and vaccines
1953	Discovery of the structure of DNA by Watson and Crick
1957	Manufacture of glutamic acid by Kinoshita et al.
1955–60	Manufacture of citric acid by the submerged culture process
	<i>New Biotechnology</i>
1970–1972	Bacterial plasmid DNA and transformation of <i>E. coli</i>
1973	Genetic barriers breached (restriction enzymes, ligase)
1974	Expression of heterologous gene in <i>E. coli</i>
1975	Hybridoma made (monoclonal antibody)
1978	Somatostatin (first recombinant DNA product)
1982	Recombinant human insulin (Humulin [®])
1983	Heterologous plant gene expression
1984	Cohen/Boyer Patent
1985	Recombinant human growth hormone (Protropin [®])
1986	Recombinant hepatitis B vaccine (Recombivax HB), Recombinant α -interferon (Roferon A [®])
1987	Recombinant tissue plasminogen activator (Activase [®]), Recombinant tryptophan
1989	Recombinant interleukin-2 (Proleukin [®]), Recombinant γ -interferon (Immuneron [®])
1989–1991	Recombinant rennet (Gist-Brocades, Genencor, and Pfizer)
	Recombinant vitamin C (Genencor), bacteriophage-resistant lactic starters
1990	Maltase-enhanced baker's yeast (Gist-Brocades)
1992	Lipase (Unilever), Amylase (Novomil [®])
1994	Flavr Savr tomato (Calgene), Recombinant bovine somatotrophin, BST (Eli Lilly; Monsanto), Brewing yeast (Carlsberg; British Brewing Research Institute), Acetolactate decarboxylase (Novo Maturex)
2004	47 genetically modified crops on market

out of experiments conducted in bacterial genetics and molecular biology: the field now called *genetic engineering*. The discovery of genetic engineering via *recombinant DNA technology* is responsible for the current biotechnology boom. Recombinant DNA technology was an outgrowth of basic research on restriction enzymes and enzymes involved in DNA replication. Not only do these techniques offer the prospect of improving existing processes and products, but also they enable us to develop totally new products and new

processes that were not possible using standard mutation techniques. This new technology has spawned a new industry and prompted a dramatic refocusing of the research directions of established companies.

Biotechnology is not itself a product or range of products like microelectronics; rather, it is a range of enabling technologies, which will find application in many industrial sectors. It has been defined in many forms, but in essence it implies the use of microorganisms and animal and plant cells:

- for the production of goods and services (Canadian definition)
- for the utilization of biologically derived molecules, structures, cells, or organisms to carry out a specific process (U.S. definition)
- for the integrated use of biochemistry, microbiology, and chemical engineering to achieve industrial application of microbes and cultured tissue cells (European Federation definition).

What Is Food Biotechnology?

Food biotechnology is the application of modern biotechnological techniques to the manufacture and processing of food. Fermentation of food, which is the oldest biotechnological process, and food additives, as well as plant and animal cell cultures, are included. New developments in fermentation and enzyme technological processes, genetic engineering, protein engineering, bioengineering, and processes involving monoclonal antibodies have introduced exciting dimensions to food biotechnology. Although traditional agriculture and crop breeding are not generally regarded as food biotechnology, *agricultural biotechnology*, i.e., of animal and plant foods, is expected to become an increasingly important “engine” of development for the agri-food industry. Nevertheless, food biotechnology is a burgeoning field that transcends many scientific disciplines.

How do these new technologies ultimately affect our food supply? Biotechnology will influence the production and preservation of raw materials and the planned alteration of their nutritional and functional properties. It also affects the development of production/processing aids and direct additives that can improve the overall utilization of raw materials. This illustrates the diverse nature of the field of food biotechnology. The new aspects of modern biotechnology will not necessarily revolutionize the food industry, but certainly they will play an increasingly useful and economic role. Techniques such as enzyme/cell immobilization and genetic engineering are now beginning to have a considerable impact on raw material processing. The potential for developing rapid, inexpensive, and highly sensitive biosensor kits for food analysis is considerable. New developments in biochemical engineering will also be of advantage to industries using traditional mechanical or physical methods, which will be replaced by modern unit operations in product recovery and advanced fermentation control. There are great difficulties in precisely forecasting economic opportunities arising from technical progress. The annual value of biotechnologically related products in the food and drink industries is expected to reach U.S.\$35 billion dollars by the year 2000, compared with that of the pharmaceutical industries (U.S.\$24 billion) and commodity chemicals (U.S.\$12 billion).*

The technology must be economically effective, yet preserve the capacity of the world's largest industry to generate wealth. It has also to meet the changing fashions in food, without disturbing the traditional virtues of wholesomeness and natural appeal. Thus clear and rational policies are needed regarding the regulatory status of bioengineered products. Regulatory provisions follow the same procedures used to establish the safety of conventionally derived food products but are still undergoing clarification with respect to the safety of genetically cloned system. Because of the recognition that some rDNA products

* Throughout the text, all dollar amounts refer to U.S. dollars.

without any side effects are already on the market, the initial concerns over possible health hazards have been relaxed, particularly for single constituents or defined chemical mixtures. The safety issue of whole foods is more difficult than that of single ingredient products, however. For example, recombinant chymosin produced by microorganisms is used to replace calf rennet in cheesemaking. It has been used in 60% of all cheese manufactured since 1990. Benefits include purity, reliable supply, a 50% cost reduction, and high cheese yield. In 1994 the transgenic Flav Savr tomato was marketed by Calgene in the United States after a lengthy regulatory process. The Flav Savr tomato offers improved flavor and extended shelf life. Calgene argues that the use of biotechnology per se poses no specific risks and that products should not be discriminated against on the grounds of their method of production. On the other hand, a number of issues such as allergenicity, labeling of all recombinant foods, and consumer perception, as well as ethical and moral issues, will need further regulatory clearances and public debate.

Part I

New Trends and Tools of Food Biotechnology

1

Fundamentals and New Aspects

1.1 Biotechnological applications of animals, plants, and microbes

In transgenic biotechnology (also known as genetic engineering), a known gene is inserted into an animal, plant, or microbial cell in order to achieve a desired trait. Biotechnology involves the potential use of all living forms, but microorganisms have played a major role in the development of biotechnology. This is because of the following reasons: (i) mass growth of microorganisms is possible, (ii) cheap waste materials which act as the media for the growth of microorganisms can be rapidly grown, and (iii) there is massive diversity in the metabolic types, which in turn gives diverse potential products and results in the ease of genetic manipulation to improve strains for new products. However, mass culture of *animal cell lines* is also important to manufacture *viral vaccines* and other products of biotechnology. Currently, *recombinant DNA (rDNA) products* produced in *animal cell cultures* include *enzymes, synthetic hormones, immunobiologicals (monoclonal antibodies, interleukins (ILs), lymphokines), and anticancer agents*. Although many simpler proteins can be produced by recombinant bacterial cell cultures, more complex proteins that are glycosylated (carbohydrate-modified) currently must be made in animal cells. However, the cost of growing *mammalian cell cultures* is high, and thus research is underway to produce such complex proteins in *insect cells* or in *higher plants*. *Single embryonic cell* and *somatic embryos* are used as a source for direct gene transfer via *particle bombardment*, and *analyze transit gene expression*. *Mammalian cell-line products* (expressed by *CHO, BHK (baby hamster kidney), NSO, myeloma cells, C127, HEK293*) account for over 70% of the products in the biopharmaceutical markets including therapeutic monoclonal antibodies.

Biopharmaceuticals may be produced from microbial cells (e.g., recombinant *Escherichia coli* or yeast cultures), mammalian cell culture, plant cell/tissue culture, and moss plants in bioreactors of various configurations, including photo-bioreactors. The important issues of cell culture are cost of production (a low-volume, high-purity product is desirable) and microbial contamination by bacteria, viruses, mycoplasma, and

so on. Alternative but potentially controversial platforms of production that are being tested include whole plants and animals. The production of these organisms represents a significant risk in terms of investment and the risk of nonacceptance by government bodies due to safety and ethical issues.

The important animal cell culture products are monoclonal antibodies; it is possible for these antibodies to fuse normal cells with an immortalized tumor cell line. In brief, lymphocytes isolated from the spleen (or possibly blood) of an immunized animal are fused with an immortal myeloma cell line (B cell lineage) to produce a hybridoma, which has the antibody specificity of the primary lymphocyte and the immortality of the myeloma. Selective growth medium (hyaluronic acid (HA) or hypoxanthine–aminopterin–thymidine (HAT)) is used to select against unfused myeloma cells; primary lymphocytes die quickly during culture but only the fused cells survive. These are screened for production of the required antibody, generally in pools to start with and then after single cloning, the protein is purified. As mammals are also a good bioreactor to secrete the fully active proteins in milk, several species since 1985 have been cloned including cow, goat, pig, horse, cat, and most recently dog, but the most research has been on cloning of cattle. Genetically modified (GM) pigs, sheep, cattle, goats, rabbits, chickens, and fish have all been reported.

The main potential commercial applications of cloned and GM animals include production of food, pharmaceuticals (“pharming”), xenotransplantation, pets, sporting animals and endangered species. GM animals already on sale include cloned pet cats, GM ornamental fish, cloned horses, and at least one rodeo bull. Two pharmaceutical products from the milk of GM animals have completed (Phase III and Phase II) clinical trials, respectively, and may be on the market in the EU in the next few years. Cloned livestock (especially pigs and cattle) are widely expected to be used within the food chain somewhere in the world, though it would not be economical to use cloned animals directly for food or milk production, but clones would be used as parents of slaughter pigs, beef cattle, and possibly also milk-producing dairy cows. The first drug manufactured from the milk of a GM goat was ATryn (brand name of the anticoagulant antithrombin) by GTC Biotherapeutics in 2006. It is produced from the milk of goats that have been GM to produce human antithrombin. A goat that produces spider’s web protein, which is stronger and more flexible than steel (BioSteel), was successfully produced by a Quebec-based Canadian company, Nixia.

Faster-growing GM salmon developed by a Canadian company is also awaiting regulatory approval, principally for direct sale to fish farming markets. Canada has also approved the GM pig (trade named “Enviropig”) developed by University of Guelph and it is designed to reduce phosphorus pollution of water and farmers’ feed costs. Enviropig excretes less phosphorous manure and is a more environmentally friendly pig. It will be years before meat from genetically engineered pigs could be available for human consumption. Molecular pharming can also produce a range of proteins produced from cloned cattle, goats, and chickens. An ornamental fish that glows in the dark is now available in the market. It was created by cloning the deoxyribonucleic acid (DNA) of jellyfish with that of a zebra fish. GM fish may escape and damage the current ecosystem by colonizing waters. Some tropical fish, like piranhas, could be engineered to survive in the cold and this could lead to major problems. These details will be covered in the section on Animal Biotechnology.

Recently, the production of foreign proteins in transgenic plants has become a viable alternative to conventional production systems such as microbial fermentation or mammalian cell culture. Transgenic plants are now used to produce pharmacologically active proteins, including mammalian antibodies, blood product substitutes, vaccines, hormones, cytokines, a variety of other therapeutic agents, and enzymes. Efficient biopharmaceutical

production in plants involves the proper selection of a host plant and gene expression system in a food crop or a nonfood crop. Genetically engineered plants, acting as bioreactors, can efficiently produce recombinant proteins in larger quantities than mammalian cell systems. Plants offer the potential for efficient, large-scale production of recombinant proteins with increased freedom from contaminating human pathogens. During the last two decades, approximately 95 biopharmaceutical products have been approved by one or more regulatory agencies for the treatment of various human diseases including diabetes mellitus, growth disorders, neurological and genetic maladies, inflammatory conditions, and blood dyscrasias. None of the commercially available products are currently produced using plants mainly because of the low yield and expensive purification costs; however, DNA-based vaccines are potential candidates for plant-based production in the future. After the cell is grown in tissue culture to develop a full plant, the transgenic plant will express the new trait, such as an added nutritional value or resistance to a pest. The transgenic process allows research to reach beyond closely related plants to find useful traits in all of life's vast resources. The details of transgenic plants will be covered in the section on Plant Biotechnology.

All the biopharmaceutical products are mostly manufactured commercially through various fermentation routes on using genetically engineered microorganisms like *E. coli*, yeast, and fungi. Some of the biopharmaceutical products produced commercially through fermentation routes are human insulin, streptokinase, erythropoietin, hepatitis B vaccine, human growth hormone, IL, granulocyte-colony stimulating factor (GCSF), granulocyte-macrophage colony stimulating factor (GMCSF), alpha-interferon, gamma interferon, and so on. All three domains – animals, plants and microbes – are not only involved in production of biopharmaceuticals but also find their application in manufacture of food products (Figure 1.1). Although there is a high level of public support for the development of new biotech, that is, for the production of new medicines (insulin, interferon, hormone, etc.), diagnostics (cancer detection kits), and food enzymes (recombinant

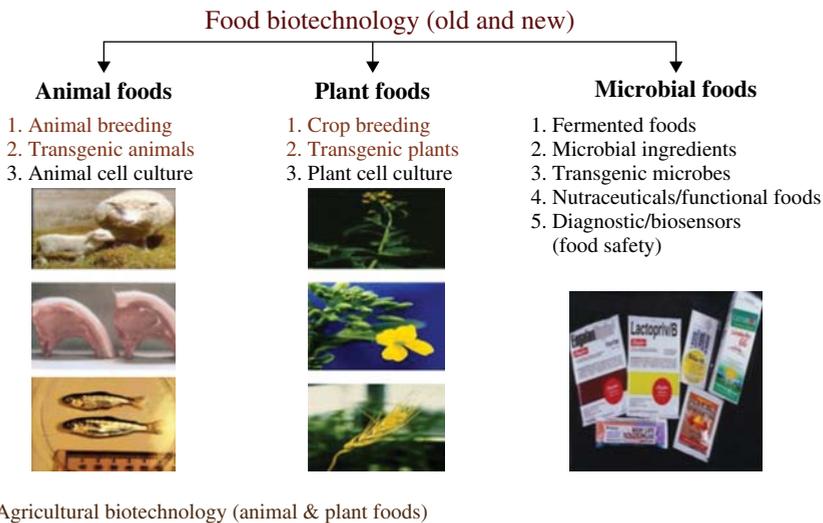


Figure 1.1 Concept of food biotechnology.

rennet, etc.), there is no support for the production of GM whole foods. This is because of the safety factor that is involved in the consumption of food. This is covered in detail in the section on Food Safety and New Biotechnology.

1.2 Cellular organization and membrane structure

Cellular organization comprises three levels of organization that exist within each cell. Cells are composed of organized organelles, which are unique structures that perform specific functions within cells. Organelles themselves are made up of organized molecules, and molecules are forms of organized atoms, which are the building blocks of all matter.

Most organisms share (i) a common chemical composition, their most distinctive chemical attribute being the presence of three classes of complex macromolecules: DNA, ribonucleic acid (RNA), and proteins (ribosomes, enzymes), and (ii) a common physical structure, being organized into microscopic subunits, termed cells. Cells from a wide variety of organisms share many common features in their structure and function. All cells are enclosed by a thin *cytoplasmic membrane*, which retains various molecules, necessary for the maintenance of biological function, and which regulates the passage of solutes between the cell and its environment. These generalizations apply to all living organisms, except for the virus because they cannot maintain life and reproduce by themselves.

Dissatisfaction with the existing classification of the biological kingdom led Haeckel (1866) to propose a third kingdom, the *Protists* (protozoa, algae, fungi, bacteria), besides the *plants* and *animals*. Observation with the electron microscope (developed in about 1950) revealed two radically different kinds of cells in the contemporary living world. Although the various groups of organisms are still linked by certain common features, we can distinguish two major groups of cellular organisms: the *Prokaryotes* (or *Prokaryotes*) and the *Eucaryotes* (or *Eukaryotes*). As scientists learn more about organisms, classification systems change. Genetic sequencing has given researchers a whole new way of analyzing relationships between organisms. In recent years, the evolutionary relationships of prokaryotes are quite complex, in that the taxonomic scheme of life has been revised. The current system, the *Three Domain System*, groups organisms primarily based on differences in the structure of the ribosomal RNA, that is, a molecular building block for *ribosomes*. Under this system, organisms are classified into *three domains* and *six kingdoms*. The domains are Archaea, Bacteria, and Eukarya. The kingdoms are *Archaeobacteria* (*ancient bacteria*), *Eubacteria* (*true bacteria*), *Protista*, *Fungi*, *Plantae*, and *Animalia*. The *Archaea* and *Bacteria* domains contain prokaryotic organisms. These are organisms that do not have a membrane-bound *nucleus*. Eubacteria are classified under the Bacteria domain and archaeobacteria are classified as Archaeans. The Eukarya domain includes eukaryotes, or organisms that have a membrane-bound nucleus. This domain is further subdivided into the kingdoms Protista, Fungi, Plantae, and Animalia.

Figure 1.2 illustrates the relationship between the three domains. Archaea are sometimes referred to as *extremophiles*, inhabiting in extreme environments such as hot springs, hydrothermal vents, salt ponds, Arctic ice, deep oil wells, and acidic ponds that form near mines. In fact, many extremophiles cannot grow in ordinary human environment. Compared to eukaryotes, prokaryotes usually have much smaller genomes and an eukaryotic cell normally has 1000 times more DNA than a prokaryote. The DNA in prokaryotes is concentrated in the *nucleoid*. The prokaryotic chromosome is a double-stranded DNA molecule arranged as a single large ring. Prokaryotes often have smaller rings of extra-chromosomal DNA termed *plasmids* in which most plasmids consist of only a few genes.

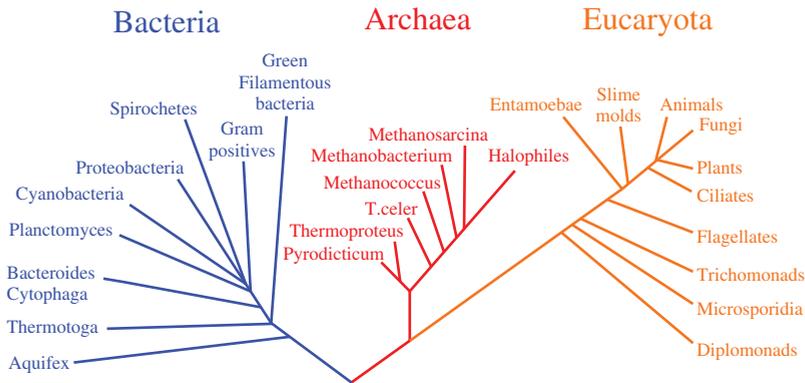


Figure 1.2 Concept of three life domains based on rRNA data, showing the separation of bacteria, archaea, and eukaryotes. *Source:* Wikipedia (June, 2007); http://en.wikipedia.org/wiki/Three-domain_system. (See insert for color representation of this figure.)

Plasmids are not required for survival in most environments because the prokaryotic chromosome programs all of the cell's essential functions. However, plasmids may contain genes that provide resistance to antibiotics, metabolism of unusual nutrients, and other special functions. Plasmids replicate independently of the main chromosome, and many can be readily transferred between prokaryotic cells. Prokaryotes replicate via binary fission, that is, simple cell division whereby two identical offsprings each receive a copy of the original, single, parental chromosome. Binary fission is a type of asexual reproduction that does not require the union of two reproductive cells, and that produces offspring genetically identical to the parent cell. A population of rapidly growing prokaryotes can synthesize their DNA almost continuously, which aids in their fast generation times. Even as a cell is physically separating, its DNA can be replicating for the next round of cell division.

Membranes are large structures that contain lipids and proteins as their major components, along with a small amount of carbohydrates. The ratio of lipid to protein can range from 4:1 in the myelin of nerve cells to 1:3 in bacterial cell membranes, though many have a similar lipid to protein ratio (1:1) as in human erythrocytes. The predominant lipids in cell membranes are *phospholipids*, *sterols*, and *glycolipids* (*sphingolipids*). The long-nonpolar hydrocarbon tails of lipids are attracted to each other and are sequestered away from water. Membrane proteins contain a high proportion of hydrophobic and acidic amino acids, but the study of membrane proteins are difficult, mainly due to loss of biological activity. However, it became apparent from earlier studies that protein was layered on both sides of a lipid bilayer which was confirmed by electron microscopy using OsO_4 (*Osmic acid*) staining. Several difficulties were encountered in explaining the properties of cell membranes in terms of this structure. Later several micellar models suggested that the nonpolar tails of the lipids formed a close association within the micelles with their polar carboxyl heads on the outside and surrounded by protein. However, the stability of this system was difficult to explain because highly nonpolar compounds must pass through the polar protein layer. Controversy continued about the exact location of the protein in the membranes. The cell membrane functions as a semipermeable barrier, allowing very few molecules across it, while fencing the majority of organically produced chemicals inside the cell. Electron microscopic examinations of cell membranes have led to the development of the lipid

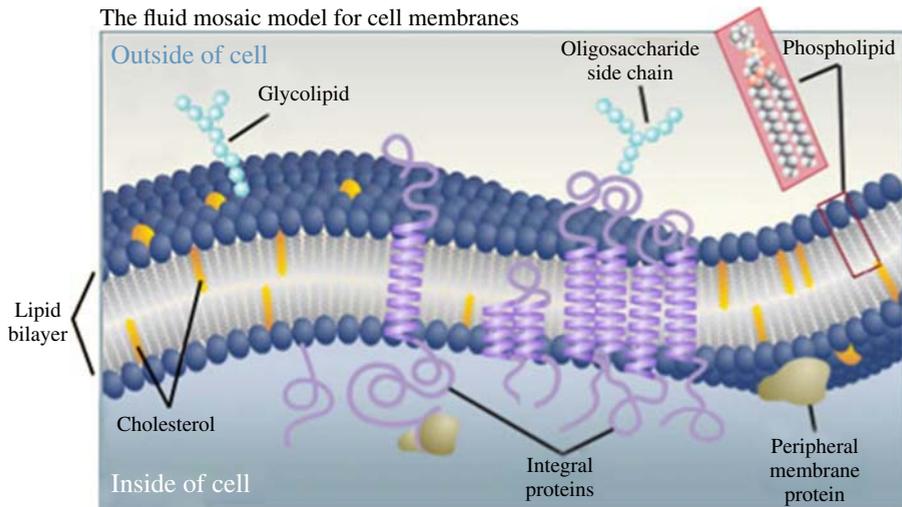


Figure 1.3 Fluid mosaic model of the structure of a membrane. *Source:* http://www.biology.arizona.edu/cell_bio/problem_sets/membranes/fluid_mosaic_model.html. (See insert for color representation of this figure.)

bilayer model (referred to as the *fluid mosaic model* proposed by Singer and Nicolson in 1972). This model suggested that the integrated proteins are located within the lipid bilayer in a number of ways. The hydrophobic amino acid residues of the protein are in close contact with the hydrophobic side chains of the phospholipids and the hydrophilic amino acid residues are on the surface in contact with water (Figure 1.3).

The oligosaccharide side chains of glycoproteins and glycolipids are always present on the outer membrane surface and never on the inside of the cell. The lipid bilayer is fluid at physiological temperatures, so that the phospholipid molecules are more mobile in the membrane plane to flow laterally and membranes are distinctly asymmetric. Membranes perform a variety of important functions, where their principal role is to control the flow of ions, metabolites, and other foreign compounds into and out of the cell and between the various cellular compartments. Membrane transport can occur by *diffusion* (nonmediated transport) or by means of a carrier (carrier-mediated transport). Transport can also be described as either passive or active. Further references on the structure and transport of membranes are listed. Figure 1.4 shows the differences of typical three cells.

Animal cells are typical of the eukaryotic cell, enclosed by a plasma membrane and containing a membrane-bound nucleus and organelles. Unlike the eukaryotic cells of plants and fungi, animal cells do not have a cell wall. This feature gave rise to the kingdom *Animalia*. Most cells, both animal and plant, range in size between 1 and 100 μm and are thus visible only with the aid of a microscope. The lack of a rigid cell wall allowed animals to develop a greater diversity of cell types, tissues and organs. The animal kingdom is unique among eukaryotic organisms because most animal tissues are bound in an *extracellular matrix* by a triple helix of protein known as *collagen*. Plant and fungal cells are bound in tissues or aggregations by other molecules, such as *pectin*. Animals are a large and incredibly diverse group of organisms. Making up about three-quarters of the species on Earth, they run the gamut from corals and jellyfish to ants, whales, elephants, and, of course, humans. Unlike plants, however, animals are unable to manufacture their own food, and therefore,

Some typical cells

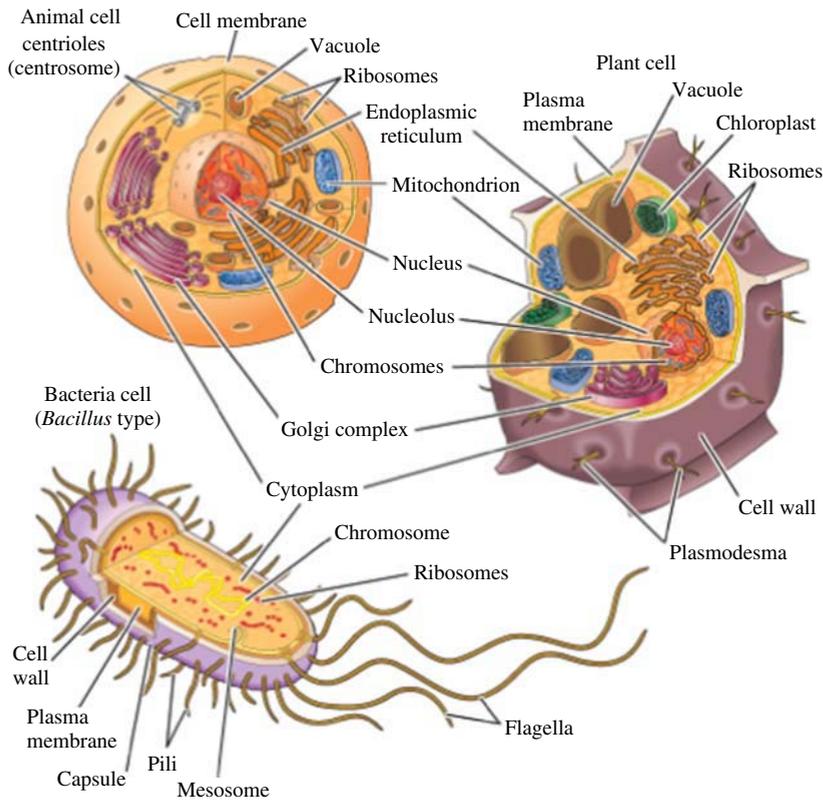


Figure 1.4 Anatomy differences of typical animal, plant, and bacterial cells. *Source:* Reprinted with permission from Encyclopædia Britannica, ©2010 by Encyclopædia Britannica, Inc. (See insert for color representation of this figure.)

are always directly or indirectly dependent on plant life. Most animal cells are *diploid*, meaning that their chromosomes exist in homologous pairs. Different chromosomal ploidies are also, however, known to occasionally occur. For the proliferation of animal cells in sexual reproduction, the cellular process of *meiosis* is first necessary so that haploid daughter cells, or *gametes*, can be produced. Two haploid cells then fuse to form a diploid *zygote*, which develops into a new organism as its cells divide and multiply.

Animal cells have a similar basic structure like bacteria in that there is a nucleus surrounded by cytoplasm contained in a cell membrane. As animals are multicellular organisms, there is a centrosome that splits in two when the cells divide during a process called mitosis. Lysosome has a similar job to chloroplasts in plant cells as they are responsible for absorbing and digesting.

Similarities and differences among cells are shown in Table 1.1 (www.k12.de.us/richardallen/science/comparing_cells/). The most striking difference among plant cells and other cells is the uniform shape. Each plant cell is roughly square or rectangular in shape, whereas an animal cell varies in shape. Around the nucleolus of the plant cell is a

Table 1.1 Comparison of features in bacterial, plant, and animal cells

Cell feature	Bacterial cells	Plant cells	Animal cells
1. Cell size (μm)	1–10	10–100	10–100
2. Cell wall	Yes (murein)	Yes (cellulose)	No
3. Cell nucleus	No	Yes (double nucleus membrane)	Yes (d.n.m)
4. Plasmids	Yes (double stranded, circular)	No	No
5. Chromosome (DNA)	Single circular without histone	Multiple linear with histone	Multiple linear with histone
6. Mitochondria	No	Yes (double membrane)	Yes (d.m)
7. Ribosomes	Small (70S)	Large (80S)	Large (80S)
8. Chloro plasts	No	Yes	No
9. Golgi	No	Yes	Yes
10. Vacuoles	No (except for blue-green bacteria)	Yes	No
11. Flagella	Yes	No	No
12. Capsule	Some	No	No
13. Cell division	Fission or budding	Mitosis	Mitosis
14. Cytoplasm (cytoskeleton)	No	Yes	Yes

d.n.m, double nucleus membrane; d.m, double membrane

layer of chromatin, which is a DNA protein complex nourishing and protecting the cells and is the most important element of the plant cell. Another vital element of a plant's cell structure is the chloroplasts, which are responsible for photosynthesis. Contained in the chloroplast are the granum, stroma, and thylakoid. The peroxisome is another unique plant cell element that removes hydrogen from the air and facilitates water absorption during photosynthesis. Plant cells also possess a cell wall and a membrane. The cell wall does roughly the same job as the membrane but its solid nature allows plant cells to maintain a ridged shape. Bacteria are single-celled organisms with a basic cellular structure that has a nucleolus, which is the brain of the cell; it is surrounded by cytoplasm, a jelly-like substance containing nutrients and a cell membrane. Although animals, plants and bacteria may seem vastly different, there are more similarities among the cell's structures than differences. All cells have a nucleus and most of the body space is taken up by the cytoplasm. Plants and animals then share more components than bacteria due to more complex structures. The vacuole is a sack filled with water within the cell. It is much larger in plants and sometimes comprises 90% of the total cell. It contains ions, sugars, and enzymes. The Golgi body contains proteins and carbohydrates and helps maintain the cell membrane. Mitochondrions produce energy for the cell by converting glucose into adenosine triphosphate (ATP). The rough and smooth endoplasmic reticulum (ER) can be seen as the intestines of the cell as they transport proteins through the cell. These are covered in ribosomes, which are small grains of cytoplasmic material responsible for protein synthesis.

Many transgenic (or genetically modified) microorganisms are particularly important in producing large amounts of pure human proteins for use in medicine. GM bacteria are now used to produce the protein, insulin, to treat diabetes. Similar bacteria have been used to

produce clotting factors to treat hemophilia and human growth hormone to treat various forms of dwarfism. These microbial recombinant proteins are safer than the products they replaced because the products obtained earlier were purified from cadavers and could transmit diseases. In fact, the human-derived proteins caused many cases of AIDS and hepatitis C in hemophiliacs and the Creutzfeldt–Jakob disease from human growth hormone. Recombinant proteins derived from microorganisms will be discussed in the section of microbial products.

1.3 Bacterial growth and fermentation tools

Growth and applications of animal cells and plant cells will be separately covered in the chapters on Animal Biotechnology and Plant Biotechnology.

Microbes are the *tools of fermentation* because they produce enzymes, amino acids, vitamins, biogums, other valuable recombinant proteins, and organic acids. This discussion will thus mainly focus on the growth of unicellular bacteria as they are ideal objects for study of the growth process, current scale-up process for the manufacture of industrial products, and many aspects of food biotechnology. Negative aspects of microorganisms are also the most common causes of food-borne illness and food spoilage and thus the detection of pathogens, and so on, using biosensors and nanobiotechnology will also be covered in a different section.

Fermentation technology is becoming increasingly important in the production of various bulk chemicals, fine chemicals, and pharmaceuticals. Compared to the chemical manufacturing processes of various compounds, the fermentative production process is a very promising technology to produce enantiomer pure chemicals with low environmental burden. High conversion efficiencies are often achieved in fermentative production processes. For this reason, chemical industries are now investigating the field of biotechnology as a more economic alternative for the chemical synthesis of compounds. Moreover, by means of fermentation, it is possible to convert abundant renewable raw materials or waste materials to produce high-value products.

1.3.1 Classification and reproduction of biotechnologically important bacterial system

In contrast to the taxonomy of plants and animals, which show a diversity of cell types, a bacterial system is very simple and is classified based on artificial criteria such as structure, shape, motility, nutrition, propagation and immunological reactions. Tables 1.2 and 1.3 summarize the most important bacterial species that are involved in biotechnology processes on the basis of the classification in *Bergey's Manual of Systematic Bacteriology*. This familiar reference work differentiates the bacteria into the 19 parts listed in Table 1.2, each of which is subdivided into orders, families, genera and species. These classifications show differences in many characteristics of energy and nutritional requirements, growth and product release rates, method of reproduction, motility, and habitats. All these factors are of great practical importance in applications of biotechnology. Other differences in the morphology or the physical form and structure are also important in the calculation of the rate of nutrient mass transfer and the fluid mechanics of a suspension containing microbes. Table 1.3 lists some bacteria of technological importance by group, family, genus and process. The detailed fermentation processes and tools related to the important food fermentations are described in Part II.

Table 1.2 The important bacterial family in biotechnological processes

Part	Type
1	Photosynthetic bacteria
2	Gliding bacteria
3	Sheathed bacteria
4	Budding and/or appendaged bacteria
5	The spirochetes
6	Spiral and curved bacteria
7	Gram-negative aerobic rods/cocci
8	Gram-negative facultative anaerobic rods
9	Gram-negative anaerobic rods
10	Gram-negative cocci and coccobacilli
11	Gram-negative anaerobic cocci
12	Gram-negative chemolithotrophic bacteria
13	Methane-producing bacteria
14	Gram-positive cocci
15	Endospore-forming rods and cocci
16	Gram-positive asporogeneous rod-shaped bacteria
17	Actinomycete and related organisms
18	The rickettsias
19	The mycoplasmas

Source: Adapted from *Bergey's Manual of Systematic Bacteriology*, Vol. 3, J. T. Staley, Ed. Baltimore: Williams & Wilkins, 1989.

The basic unit is the species, which is characterized by a high degree of similarity in physical and biochemical properties, and significant differences from the properties of related organisms. The Gram-positive bacteria are those that retain the purple stain of crystal violet/iodine after it is washed with ethanol, while Gram-negative species are those that decolorize. The Gram stain developed by Christian Gram in 1884 reflects an important chemical property of the cell wall and has proved to be a valuable taxonomic criterion.

Most prokaryotes reproduce by asexual means in the haploid state. The asexual process involves simple fission, in which DNA replication is followed by the formation of a septum, which divides the cell into two genetically identical clones (i.e., descendants of a single bacterial cell). Sexual reproduction involves the fusion of two reproductive cells (i.e., gametes), each of which contains a complete set of genetic material, producing more individuals. Therefore, only incomplete sets of genetic material can be transferred between bacteria. Sexual reproduction, which is characteristic of many eukaryotes (persistent diploidy), rarely occurs in prokaryotes. Genetic transfer among prokaryotes always occurs by means of a unidirectional passage of DNA from a donor cell to a recipient. This can be mediated either by conjugation, which involves direct cell-to-cell contact, or by transformation and transduction. However, genetic exchange of prokaryotes is rather an occasional process, but it occurs quite frequently in eukaryotes.

1.3.2 Bacterial growth

This discussion focuses mainly on the growth of unicellular bacteria, which are ideal objects for study of the growth process. In an adequate medium to which microorganisms have

Table 1.3 Some bacteria of biotechnological importance among the 19 bacterial groups

Group and family	Genus	Process
<i>7. Gram-negative aerobic rods/cocci</i>		
<i>Pseudomonadaceae</i>	<i>Pseudomonas</i>	Single cell protein (SCP) from methanol, oxidation of steroids/hydrocarbon, polysaccharides (alginate); oxidation of alcohols
<i>Methylomonadaceae</i>	<i>Methylomonas</i>	SCP from methanol
	<i>Methylococcus</i>	Oxidation of methane
<i>Azotobacteriaceae</i>	<i>Azotobacter</i>	Nonsymbiotic binding of nitrogen
<i>8. Gram-negative facultative anaerobic rods</i>		
<i>Enterobacteriaceae</i>	<i>Escherichia</i>	Many different processes, productions of amino acid (lysine)
	<i>Aerobacter</i>	Nucleotides, 2-ketoglutaric acid, pullulanase, 6-aminopenicillanic acid, recombinant rennet
<i>12. Gram-negative chemolithotrophic bacteria</i>		
	<i>Thiobacillus</i>	Leaching of copper, zinc, iron, manganese, other sulfides
<i>13. Methane-producing bacteria</i>		
<i>Methanobacteriaceae</i>	<i>Methanobacterium</i>	Methane from sewage
	<i>Methanococcus</i>	
<i>Nocardiceae</i>	<i>Nocardia</i>	Oxidation of hydrocarbon, steroids
<i>14. Gram-positive cocci</i>		
<i>Micrococcaceae</i>	<i>Micrococcus</i>	Oxidation of hydrocarbon, meat starter culture
<i>Streptococcaceae</i>	<i>Streptococcus (Lactococcus)</i>	Production of lactic acid, diacetyl; cheese and fermented dairy product starter
	<i>Pediococcus</i>	Meat starter
	<i>Leuconostoc</i>	Dextran production; cheese starter, wine starter (heterofermentation)
<i>15. Endospore-forming rods/cocci</i>		
<i>Bacillaceae</i>	<i>Bacillus</i>	Antibiotics, many enzymes, amino acids, and vitamins (B ₂ , B ₁₂)
	<i>Clostridium</i>	Butanol, acetone, butyric acid, botulins
<i>16. Gram-positive asporogeneous rod-shaped bacteria</i>		
<i>Lactobacillaceae</i>	<i>Lactobacillus</i>	Lactic acid, fermented milk products, fermented sausage and vegetables; silage, spoilage of foods
	<i>Bifidobacterium</i>	Bifidoyogurt, bifidotablets
<i>17. Actinomycete-related organisms</i>		
<i>Coryneform group</i>	<i>Corynebacterium</i>	Oxidation of hydrocarbon, amino acids
	<i>Arthrobacter</i>	Transformation of steroids
	<i>Cellulomonas</i>	Cellulose fermentation
<i>Propionibacteriaceae</i>	<i>Propionibacteria</i>	Vitamin B ₁₂ , propionic acid, cheese fermentation
<i>Mycobacteriaceae</i>	<i>Mycobacterium</i>	Oxidation of hydrocarbons and steroids

become fully adapted, cells are in a state of balanced growth. Cultures undergoing balanced growth maintain a constant chemical composition with an increase of the biomass. In higher organisms, growth is defined as an increase either in size or in organic matter. In unicellular microbes, however, increases in number (population) or mass of cells normally are used as indicators of growth.

The rate of increase in bacteria at any given time is proportional to the number or mass of cells present, which is similar in many aspects to first-order chemical reaction kinetics. The velocity of a chemical reaction is determined by the concentration of the reactants, but the growth rate of bacteria remains constant until the limiting nutrient of the medium is almost exhausted. This can be explained by the action of carrier proteins known as permeases, which are capable of maintaining saturating intracellular concentrations of nutrients over a wide range of external concentrations.

In *batch culture*, a pure culture is grown in a suitable medium containing the substrate, and incubation is continued until transformation of the substrate ceases. In this process, the biocatalyst is used only once and then discarded. The procedure is useful for screening purposes. If the concentration of one essential medium constituent is varied, while the other medium components are kept constant, the growth curves to nutrient concentration are typically hyperbolic and fit the *Monod equation*:

$$\mu = \frac{V_{\max}[S]}{K_s + [S]}$$

where μ is the specific growth rate at limiting nutrient concentration, μ_{\max} is the maximum growth rate achievable when $[S] > K_s$ and all other nutrient concentrations are unchanged, and K_s is the value analogous to the Michaelis–Menten constant of enzyme kinetics, being equal to the concentration supporting a growth rate to $\mu_{\max}/2$. The K_s values for glucose and tryptophan utilization by *E. coli* are 1×10^{-6} and 2×10^{-7} M/mL, respectively. These very low values can be attributed to the high affinities characteristic of bacterial permeases. In the following equation, the constant of proportionality K is an index of the growth rate, which often is called the growth rate constant, and t_d is the mean generation or doubling time:

$$K = \frac{\ln 2}{t_d} = \frac{0.693}{t_d}$$

For example, the mean doubling time t_d of the culture may be $0.693/2.303 = 0.3$ h (≈ 18 min), which is a relatively high growth rate for a bacterium. In a typical batch growth, the cell numbers vary with time, as shown in Figure 2.7. The lag period of adjustment, where no increase in cell numbers is evident, is extremely variable in duration depending on the period of the preceding stationary phase. After this lag phase, a straight-line relationship is obtained between the log of cell number and time, with a slope equal to $K/2.303$ and an ordinate intercept of a log N_0 . This stage of batch growth is called the *exponential* (or *logarithmic*) phase.

Bacterial growth in a closed vessel is normally limited either by the exhaustion of available nutrients or by the accumulation of toxic by-products. As a consequence, the growth rate declines and growth eventually stops; at this point, however, the population has achieved its maximum size. This stage is called the stationary phase. The transition between the exponential phase and the stationary phase involves a period of unbalanced growth during which the various cellular components are not synthesized at equal rates. Eventually, bacterial cells held in a nongrowing state die; this is the death phase. Death results from a number of factors, such as depletion of the cellular reserve of energy.

The death rate of bacteria is highly variable, depending on the environment as well as the particular species, and the age and size of the transferred inoculum.

Each phase is of potential importance in a biotechnological process. The general objective of a good fermentation design is to minimize the length of the lag phase and to maximize the rate and length of the exponential phase for achieving the largest possible cell density at the end of the process. When cells switch rapidly to a new environment, an adaptive period is required for the synthesis of the new enzymes and cofactors needed for assimilation; thus a lag will appear. Multiple lag phases can sometimes be observed when the medium contains multiple carbon sources. This phenomenon, called diauxic growth, is carried out by a shift in metabolic patterns in the middle of growth. For example, during the growth of *E. coli* in the presence of glucose and lactose, glucose is consumed during the first phase of exponential growth and lactose in the second. The enzymes for glucose utilization are constitutive, which means that the enzymes are always present, while those for lactose utilization are inducible in that they are produced only in the presence of lactose.

The net amount of bacterial growth is the difference between cell mass or number used as an inoculum and cell mass obtained at the end of culture. When growth is limited by a particular nutrient, a linear relationship between nutrient and the net growth results. The cell mass produced per unit of limiting nutrient is a constant called the growth yield (Y), and the value of Y can be calculated by the following equation.

$$Y = \frac{X - X_0}{[N]} \quad \text{or} \quad Y = \frac{X - X_0}{[S]}$$

where X is the dry weight per milliliter of culture at the beginning of stationary growth, X_0 is the initial cell mass immediately after inoculation, and the concentration of limiting nutrient (organic substrate) is $[N]$ ($[S]$).

In the case of chemoheterotrophic bacteria, which use the organic substrate as the sole source of carbon and energy, the growth yield can be measured in terms of the organic substrate and biomass resulting. Many microorganisms utilizing sugars as the sole source of carbon reveal that the ratio of the sugars to cellular carbon varies between 20% and 50%. The microbes usually use about half the carbon source to make cells and metabolize the other half to CO_2 or other by-products. The differences in conversion of efficiency probably reflect differences in the efficiency of generating ATP through catabolism of the substrate.

In batch cultures discussed so far, nutrients are not renewed and growth remains exponential for only a few generations. Thus, the physiological state of cells in batch cultures varies continuously throughout the growth cycle. In continuous cultures, however, cells can be maintained in a steady physiological state for long periods of time by adding fresh medium continuously and removing equal amounts of spent medium. Although exponentially growing cells in batch cultures may suffice for some studies, many studies on microbial physiology require a cell that is not constantly changing. A batch fermentation can be extended by feeding, either intermittently or continuously, nutrients containing a substrate that limits cell growth. This so-called *fed-batch* operation can forestall the inevitable accumulation of too much cell mass; but since there is no built-in provision for product removal, at some point the cell mass will become unsustainable. Growth may be prolonged, but depletion of selected nutrients and accumulation of metabolic by-products change the environment.

In the absence of genetic selection, continuous culture offers the means of obtaining a cell population that grows indefinitely in an unchanged environment. This is accomplished by feeding a complete medium to a fermentation and removing whole broth to maintain a fixed volume. The turbidostat and the chemostat are the two most widely used devices for

promoting growth at the maximal rate. The cell density is controlled by washing the cells out of the vessel to maintain a certain turbidity, as ascertained by optical density measurements of the medium.

Chemostatic operation involves maintenance of the microbial culture density by exhaustion of either a limiting substance or the nutrient. The flow rate is set at a particular value and the growth rate of the culture adjusts to this flow rate. Thus cell growth is limited by a selected nutrient, and the rate at which the medium is supplied dictates the growth rate of the organism. Continuous culture systems offer a few valuable features:

1. They provide a constant source of cells in an exponential growth phase.
2. They allow cultures to be grown continuously at extremely low concentrations of substrate, which is valuable in studies on the regulation of synthesis or catabolism of the limiting substrate, or in the selection of various classes of mutants.
3. They offer an increase (over batch or fed-batch systems) in productivity per unit of product manufactured and a reduction of scale-up and capital costs.

Nevertheless, continuous culture is not widely used as an industrial process, mainly because of the problems of chance contamination, and the danger of strain degeneration by spontaneous mutation, which produces a new strain of low product formation.

In the Monod chemostat model (Figure 2.8), the concentration of the limiting nutrient remains constant. Thus, the rate of addition of the nutrient must equal the rate at which it is utilized by the culture together with that lost through the overflow. The flow rate F is measured in culture volumes V per hour. The expression F/V is the dilution rate D . Thus

$$\mu = D$$

$$\mu_{\max} = \frac{[S]}{K_s + [S]} = D \quad \text{and} \quad C = \mu \frac{D}{\mu_{\max} - D}$$

solving for X (cell concentration)

$$X = Y(C_r - C)$$

where Y = yield factor.

In the relationship between cell concentration (X), limiting nutrient concentration (C), and the dilution rate (D), cell number and the concentration of limiting nutrient change little. As D_{\max} approaches μ_{\max} , it is near washout. It is equivalent to μ , and the concentration of the limiting nutrient approaches its concentration in the reservoir (C_r).

1.3.3 Environmental factors affecting bacterial growth

The growth of microorganisms is influenced by various factors, including nutrients, which have already been discussed, and the interactions between the microbial cell and its environment, which are shown in Figure 1.5.

1.3.3.1 Solutes Transport mechanisms play two essential roles in cellular function. First, they maintain the intracellular concentration of all metabolites at levels high enough to operate both catabolic and anabolic pathways at near-maximal rates, even when nutrient concentration of the external medium is low. This is known to be true because the

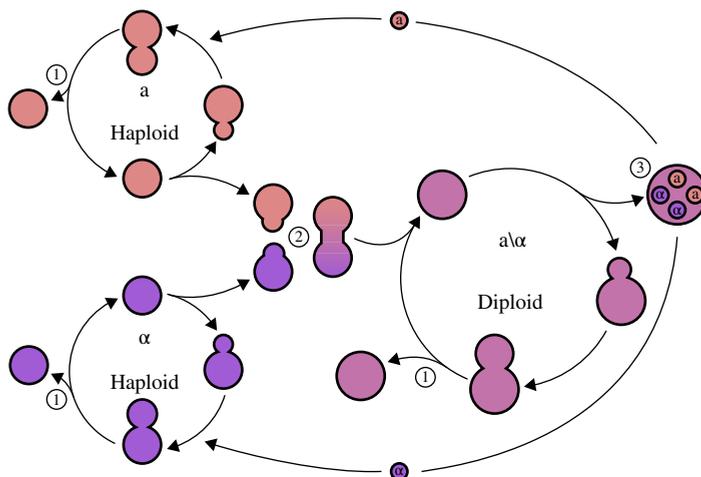


Figure 1.5 Sexual reproduction in the yeast life cycle. *Source:* http://en.wikipedia.org/wiki/File:Yeast_lifecycle.svg. (See insert for color representation of this figure.)

exponential growth rate of a microbial population remains constant until one essential nutrient in the medium falls to zero. Second, transport mechanisms function in osmoregulation, which maintain the solutes (principally small molecules and ions) at levels optimal for metabolic activity, even under a wide range of the osmolarity (i.e., the osmotic pressure exerted by any solution). If the internal osmotic pressure of the cell falls below the external osmotic pressure, water leaves the cell and the cytoplasmic volume decreases with accompanying damage to the membrane. Thus, the lysis of cells can be achieved by osmotic shock. In Gram-positive bacteria, this pressure causes plasmolysis: the pulling away of the cell membrane from the wall. Plasmolysis can be induced in a strong solution of sodium chloride.

Bacteria vary widely in their osmotic requirements. Microorganisms that can grow in solutions of high osmolarity are called osmophiles. Halophiles are microbes that grow in saline environments. Halophiles such as *Pediococcus halophilus* can tolerate high concentrations of salt in the medium but can also grow without salt. Other bacteria, such as marine bacteria and certain extreme halophiles, require NaCl for growth.

1.3.3.2 Temperature Temperature has a marked effect on microbial growth. Note given in Chapter 1 (Figure 1.18) in the Arrhenius plot that a plot of log velocity V of chemical reaction, as a function of temperature T , yields a straight line with a negative slope. This relationship can be expressed as follows:

$$\log_{10} V = \frac{-\Delta H}{2.303RT} + C$$

where V is the reaction velocity, ΔH the activation energy of the reaction, R a gas constant, and T the temperature in Kelvin's.

Unlike chemical reactions, however, the growth curve is linear only over the upper limit for survival of the microbes. Within certain limits, the rate of growth increases with a rise in temperature; but the cells die if the temperature is too high. Most microbes have preferred

Table 1.4 Temperature ranges of bacterial growth

Group	Temperature (°C)			Organism examples (temperature range, °C)
	Minimum	Optimum	Maximum	
Psychrophiles	−5 to 5	15–30	19–35	<i>Bacillus globispolus</i> (−10 to 25) <i>Micrococcus cryophilus</i> (−8 to 25) <i>Vibrio marinus</i> (−8 to 20) <i>Xanthomonas pharmiticola</i> (0–40) <i>Pseudomonas rinicola</i> (3–40)
Mesophiles	10–15	30–45	35–47	Many species
Thermophiles	40–45	55–75	60–110	<i>Bacillus thermophilus</i> (45–60) <i>Thermus aquaticus</i> (65–100) <i>Pyrococcus</i> spp. (100–103)

temperature ranges, reflecting the chemical and physiological properties of their proteins and membranes. The range of temperature that is capable of supporting life lies roughly between -5 and 95°C (up to 110°C). Bacteria are often divided into three main broad groups: psychrophiles, which grow well at 0°C , mesophiles, which grow well between 30 and 45°C , and thermophiles, which grow at elevated temperatures above 55°C .

Psychrophiles that grow at temperatures above 20°C are often called facultative psychrophiles, while the ones that cannot grow above 20°C are called obligate psychrophiles. The psychrophiles maintain the fluid nature of the membranes and are active at low temperatures. However, most bacteria stop growing at a temperature well above the freezing point of water. Some isolates from a cold environment can grow at temperatures as low as -10°C , since high solute concentrations prevent the medium from freezing. Some bacteria isolated from hot springs such as *Pyrococcus* are capable of growth at temperatures as high as 110°C , that are called extremophiles. Fluidity in psychrophiles is believed to be a function of the length and structure of the fatty acids in the phospholipid bilayer of the cell membrane. In *E. coli*, as the temperature decreases, the increase of unsaturated fatty acids (hexadecenoic and octadecenoic) is observed, and there is an increase in the amount of saturated fatty acids, such as palmitic, in membrane lipids. At low temperatures, all proteins also undergo slight conformational changes, attributable to the weakening of their hydrophobic bonds, which are important in determining the three-dimensional structure. Therefore, mutations that decrease the temperature at which growth can occur are likely to be present in genes encoded in these proteins.

Similarly, the adaptation of a thermophile to its thermal environment can be achieved through mutations affecting the structure of most proteins of the cell. Carbohydrates in glycoproteins and the rigidity of the protein structure in the presence of salts are considered to be the causes of increased thermoresistance. A large number of enzymes (e.g., α -amylase from thermophiles) depend on calcium for their high thermotolerance. Thermostability in thermophiles is controlled by plasmid DNA (Table 1.4).

1.3.3.3 Oxygen The oxygen requirements among bacteria are remarkably variable, and the fermentation conditions are decisively affected by whether the organism is aerobic or anaerobic. For aerobes, an adequate amount of dissolved oxygen (DO) must always be available in the medium. Facultative aerobes (or anaerobes) tolerate a wide range

of oxygen tensions. Anaerobes cannot utilize O_2 , and there are two types: the *obligate anaerobes* (e.g., *Clostridium*), which will grow only in its absence and for which O_2 is toxic, and the aerotolerant anaerobes, which are not killed by exposure to O_2 . Some obligate aerobes (e.g., lactic acid bacteria) show optimum growth at low oxygen levels (2–10% v/v); these organisms are called *microaerophiles*.

Some bacteria contain certain enzymes capable of eliminating O_2 toxicity. The oxidations of flavoproteins by O_2 produce a toxic compound H_2O_2 , but most aerobes and aerotolerant anaerobes contain the enzyme catalase, which decomposes hydrogen peroxide to oxygen and water. In these organisms, a more toxic compound, superoxide, is decomposed by superoxide dismutase, which catalyzes its conversion to oxygen and hydrogen peroxide. Members of the other bacterial group that are able to grow in the presence of air, the microaerophiles, do not have catalase but contain peroxidases, which decompose H_2O_2 . All strict anaerobes so far studied lack both superoxide dismutase and catalase. Thus, these three enzymes play roles in protecting the cell from the toxic consequences of oxygen.

Many enzymes of strict anaerobes are rapidly and irreversibly denatured by exposure to O_2 , and thus their purification and study must be conducted under anaerobic conditions. A notable example is nitrogenase, responsible for nitrogen fixation (e.g., *Azotobacter*). In most filamentous nitrogen-fixing cyanobacteria, however, nitrogenase is protected from oxygen inactivation by specialized cells (heterocytes) lacking photosystem II. The primary metabolic function of O_2 in strict aerobes is to serve as a terminal electron acceptor; but it also serves as a cosubstrate for enzymes like oxygenases, which catalyze some steps in the dissimilation of aromatic compounds and alkanes. Many aerobic pseudomonas can grow anaerobically using nitrate in place of O_2 as a terminal electron acceptor.

1.3.3.4 pH Since protein structure and enzyme activity are pH dependent, we expect cellular transport mechanisms, reactions, and growth rates to depend on pH. Bacterial growth usually is maximum in the pH range of 6.5–7.5, as exemplified by the effects of medium pH on the growth rates of *E. coli* and *Methylococcus capsulatus*. Most microbes are able to tolerate a variation of about 1–2 pH units on either side of a definite optimum. There are exceptions, however, including acidophiles, which grow at pH 2.0, and *Thiobacillus thiooxidans*, which can grow below pH 1 for generation of sulfuric acid. At the other extreme, the urea splitters can tolerate pH values greater than 10.

Other factors – such as ultraviolet (UV) irradiation, which causes lethal mutations, and biotic factors, which require the production of secondary metabolites – can also affect microbial growth.

1.4 Fungal growth and fermentation tools

Among eucaryotic organisms, the most frequently known species in biotransformation work are the subgroups of *fungi*, namely the *yeasts* and *molds*. Most fungi are aerobic microbes that form long filamentous, nucleated cells known as *hyphae*. The cell sizes are larger than bacteria, being 4–20 μm wide and $>100 \mu\text{m}$ long. Hyphae grow intertwined to form mycelia. Fungal classification is based more on morphological characteristics than on dye staining and biochemical reactions.

Based on the nature of their life cycle, fungi are classified into (i) *Zygomycetes* (or *Phycomycetes*), (ii) *Ascomycetes*, (iii) *Basidiomycetes*, and (iv) *Fungi imperfecti*. Two characteristics are common to all fungi: *heterotrophic* and *saprophytic*. *Heterotrophic* fungi require a source of organic carbon for growth. Many also require particular amino acids

and vitamins. *Saprophytic* fungi grow on dead organisms and are parasitic and others are mutualistic. The second feature of fungi is that they are true eucaryotes that possess nuclei, and many cytoplasmic organelles such as an ER, cytoskeletal components, and mitochondria. The cell wall is mostly composed of chitin and rarely cellulose, and thallus consisting of hyphae. They are aerobic, rarely facultative anaerobic. There are about 70,000 species.

Yeasts form one of the important subgroups of fungi which have lost the mycelial habit of growth. Although most of the fungi have a relatively complex morphology, yeasts are distinguished by their usual existence as unicellular, small cells (5–30 μm long \times 1–5 μm wide). Yeasts are classified in all three classes of higher fungi; *Ascomycetes*, *Basidiomycetes*, and *F. imperfecti*. The well-known yeast, *Saccharomyces cerevisiae*, is an ascomycetous yeast; budding ceases at a certain stage of its growth and the vegetative cells become transformed into asci, each containing four ascospores. The various paths of reproduction of yeasts are *asexual* (*budding* and *fission*) and *sexual* (Figure 2.6). In budding, a small offspring cell begins to grow on the side of the original cell, and physical separation of mature offspring from the parent and formation of clumps of yeast cells involving several generation are then achieved.

Although budding is the predominant mode of multiplication in yeasts, there are a few that multiply by *binary fission*, much like bacteria. Fission occurs by division of the cell into two new cells. Sexual reproduction occurs by conjugation of two *haploid* cells (each having a single set of chromosomes) with dissolution of the adjoining wall to form a diploid (two sets of chromosomes/cell) *zygote*. The nucleus in the *diploid zygote* may undergo one or several divisions and form *ascospores*, and each of these eventually becomes a new haploid vegetative individual which may then undergo subsequent reproduction by budding, fission, or sexual fusion again. Besides playing an important role in the manufacture of wine and beer and in the leavening of bread, yeast supplies flavoring ingredients, nucleic acids, protein supplements, and other useful chemicals is described (Chapter 2).

A typical representative of the haploid yeasts is the fission yeast, *Schizosaccharomyces pombe*, in which the diploid phase is restricted to the *zygote*. Other example is the alkane yeasts, *Saccharomycopsis lipolytica* for the production of *single cell protein (SCP)* from paraffins and *Saccharomyces fibrigera* for the SCP from starch.

Molds are higher fungi with a vegetative structure called a *mycelium*, which is a highly branched system of tubes. Within these tubes is a mobile mass of cytoplasm containing many nuclei. The long, thin filaments of cells within the mycelium are called *hyphae*. Molds do not contain chlorophyll, are nonmotile and reproduction, which may be sexual or asexual, is accomplished by means of spores. The mycelium (which is very dense), coupled with the mold's oxygen-supply requirements for normal function, can cause complexities in their cultivation, as the mycelium offers a substantial mass-transfer resistance. The most important classes of molds industrially are *Aspergillus* and *Penicillium*. Major useful products of these organisms are antibiotics, organic acids (oxalic acid, citric acid), and biological catalysts (enzymes). The fungi of biotechnologically importance are summarized in Table 1.5. Filamentous fungi are also large-scale producers of pigments and colorants for the food industry and some fermentative food grade pigments from filamentous fungi exist in the market are: *Monascus* pigments, Arpink redTM from *Penicillium oxalicum*, riboflavin from *Ashbya gossypii*, lycopene and *b*-carotene from *Blakeslea trispora*. The production yield in the case of *b*-carotene could be as high as 17 g/L of the *B. trispora* culture medium. For more detailed information about this group of organisms, the reader should consult specialized books dealing with this subject.

Table 1.5 Some of recognized biotechnologically important fungi

Genus	Products
<i>Mucor</i>	Organic acids, enzymes
<i>Rhizopus</i>	Organic acids, enzymes
<i>Blackeslea</i>	β -Carotene
<i>Choanephora</i>	β -Carotene
<i>Ashbya</i>	Riboflavin
<i>Cryptococcus</i>	Riboflavin
<i>Candida</i>	Citric acid
<i>Rhodotorula</i>	Lipids
<i>Saccharomyces</i>	Ethanol, wine, beer
<i>Saccharomycopsis</i>	Proteins
<i>Torulopsis</i>	Citric acid
<i>Aspergillus</i>	Enzymes, antibiotics, organic acids
<i>Cephalosporium</i>	Antibiotic
<i>Penicillium</i>	Antibiotic, organic acid, enzymes
<i>Fusarium</i>	Protein, fat
<i>Gibberella</i>	Hormone like substances, Gibberellins

Fungal growth is often carried out in *solid-state fermentation (SSF)*, in which microbial growth and product formation occur on solid, normally organic materials such as cereal grains, wheat bran, legumes, and lignocellulosics, and so on. Traditionally SSF has been used in Oriental foods to produce *Koji* using rice in order to manufacture alcoholic beverages such as *Sake* or *Koji* using soybean to produce soy sauce or temph, sufu, and so on. In western countries, the traditional manufacturing process of many foods requires SSF for fermented bakery products such as bread or for the maturing of mold-ripened cheese and sausage. SSF is also widely used to prepare raw materials necessary for some of our daily food or cacao bean and coffee bean fermentation.

One important application of SSF is the manufacture of industrial enzymes; that is, SSF is particularly well suited for the production of various enzymatic complexes composed of many different enzymes. SSF enzymes are widely used in fruit and vegetable transformation (pectinases), baking (hemicellulases), animal feeding and bioethanol (hemicellulases, cellulases), brewing and distilling (hemicellulases), and in the production of digestives (crude form of mixed enzymes). SSF has several advantages in lower cost and simple technology, and higher and reproducible product yields, but have problems with heat buildup, slower microbial growth, and high power requirement in continuous agitation as well as the risk of bacterial contamination.

Liquid-, submerged-, and SSF have been used for the preservation and manufacturing of foods. However, *liquid-state fermentation* has been developed on an industrial scale to manufacture vital metabolites such as antibiotics. Economic changes and the growing awareness for environmental criteria generate new perspectives for SSF. The renewal of SSF has now become possible with a new generation of industrial equipments dedicated to that technology. The Fujiwara company in Japan can transform substrate volumes up to 400m² for the production of soy sauce or sake. SSF companies for the production of enzymatic complexes can be found at LYVEN (France) for the manufacturing pectinases and hemicellulases on beet pulp and wheat bran. LYVEN has been taken over by SOUFFLET group in 2003.

1.5 Classical strain improvement and tools

After an organism is chosen for a particular fermentation, the next step is to increase its yield. The aim of strain improvement is to block the regulatory mechanisms of an organism so that maximum metabolic energy is devoted to a single product. The major aim is to achieve economic viability because the metabolite concentrations produced by wild strains are usually too low for use in economical processes.

1.5.1 Natural selection and mutation

The oldest method, *screening*, does not require complicated biochemical and genetic information on the strain. The screening process is often the most direct and least expensive means of improving most industrial microorganisms. Improvement in the quality of agricultural animals and plants has also for many years relied on the selection of desirable characters from natural variants or the hybridization of related species. Natural variants are often the products of chance mutations. Another selection technique, the so-called *enrichment* procedure, frequently uses special environmental conditions that are toxic to a majority of cell types but less or nontoxic to a desired minority of cells, to enrich a cell population for the desired mutants. Many enrichment procedures take advantages of the evolutionarily conserved natural regulatory mechanisms that control primary metabolism of microorganisms. By applying *analogs* of amino acids and vitamins, which regulate their own synthesis, mutants that lack feedback regulation can be selected to overproduce these metabolites. In the wild type cell, the analog prevents synthesis of an adequate amount of the primary metabolite. The primary metabolite is required for growth and maintenance. Thus, most normal cells die and the remaining population is enriched for *deregulated mutants*. Selection of deregulated mutants has been applied extensively to the microbial production of amino acids, vitamins, and nucleic acid precursors (Table 1.6).

The enrichment procedures of *auxotrophic mutants* can also be valuable tools for strain improvement. Blockage of a known metabolic pathway represents a simple and direct method of shunting metabolism in predictable patterns. Many economically important primary metabolites such as amino acids and nucleotides have been formed through branching biosynthetic pathways (Table 1.7). As auxotrophs lack one or more enzymatic step in one or several biosynthetic pathways, one or several end products of the pathway are not synthesized and the missing metabolites must be fed to grow a mutant. When the unsynthesized metabolite is a repressor, such auxotrophic mutants (lacking repressor synthesis) can be made to overproduce an intermediate metabolite.

By keeping the concentration of repressor low in the medium, feedback inhibition and repression of pathway enzymes are minimized. The intermediate, normally substrate for that enzyme which is absent from the mutant, will then achieve much higher concentrations than in the native organism. As compared with an auxotroph, which requires the nutrient, a strain that synthesizes its own nutrient is said to be *prototroph* (parent strain).

The role of auxotroph in commercial *L-lysine* production using *Corynebacterium glutamicum* is illustrated in Figure 1.6. A mutant of *C. glutamicum* lacks *homoserine dehydrogenase*, so that the inhibition of end product, threonine on lysine synthesis by *asparto (aspartate) kinase* does not occur. As the auxotrophic mutant does not synthesize threonine or methionine, these amino acids must be added to the growth medium. In *E. coli* having similar biosynthetic pathways, the aspartokinase system differs from *C. glutamicum* in that lysine inhibits its own production through the reaction leading to dihydropicolinate.

Table 1.6 Examples of analog-resistant deregulated mutants which overproduce primary and secondary metabolites

Product	Analog resistance	Microbe
L-Tryptophane	5 or 6-Methyltryptophane	<i>Bacillus subtilis</i>
L-Threonine	α -Amino- β -hydroxyvalerate	<i>Brevibacterium flavum</i>
L-Arginine	D-Arginine, arginine hydroxamate	<i>Corynebacterium glutamicum</i>
L-Isoleucine	α -Amino- β -hydroxyvalerate α -Methylthreonine	<i>Brevibacterium flavum</i>
L-Histidine	Triazolealanine	<i>Corynebacterium glutamicum</i>
L-Glutamate	Anthracycline	<i>Corynebacterium</i> sp.
Inosine	8-Azoguanine	<i>Bacillus subtilis</i>
Guanosine	8-Azoxanthine	<i>Bacillus subtilis</i>
Biotin	5-Valeric acid, actithiazate	<i>Bacillus</i> sp.
Thiamine	Pyriothiamine	<i>Propionibacterium</i> sp.
Cellulase	2-Deoxyglucose	<i>Trichoderma</i> sp.
Amylase	Tunicamycin	<i>Bacillus subtilis</i>

Source: Author's compiled data.

Table 1.7 Examples of auxotrophic mutants that overproduce primary metabolites

Product	Organism	Auxotrophic requirement	Substrate	Yield (g/L)
L-Lysine	<i>Corynebacterium glutamicum</i>	Threonine, methionine	Glucose (double mutant)	50
	<i>Brevibacterium flavum</i>	Homoserine, threonine	Acetate	75
L-Phenylalanine	<i>Arthrobacter paraffineus</i>	Tyrosine	<i>n</i> -Alkanes	15
	<i>Brevibacterium lactofermentum</i>	Methionine	Glucose	25
L-Tryptophane	<i>Brevibacterium flavum</i>	Tyrosine	Glucose	19
L-Ornithine	<i>Corynebacterium glutamicum</i>	Arginine	Glucose	26
L-Leucine	<i>Brevibacterium lactofermentum</i>	leucine, methionine	Glucose	28
L-Threonine	<i>Escherichia coli</i>	Lysine, methionine, isoleucine	Glucose	20
L-Tyrosine	<i>Corynebacterium glutamicum</i>	Phenylalanine	Glucose	18
L-Valine	<i>Corynebacterium glutamicum</i>	Isoleucine	Glucose	11
Inosine	<i>Brevibacterium ammoniagenes</i>	Adenine, guanine	Glucose	30
Guanosine	<i>Bacillus subtilis</i>	Adenine, histidine	Glucose	16

Source: Author's compiled data.

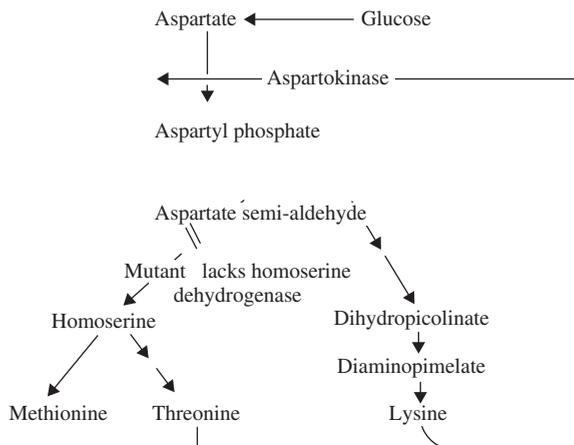


Figure 1.6 Pathway and control of lysine production in auxotrophic mutant of *Corynebacterium glutamicum*.

Similar strategies using an auxotrophic mutant of *Brevibacterium ammoniagenes* have been successfully used to overproduce the flavor enhancing *ribonucleotides* such as *inosine monophosphate (IMP)* and *guanine monophosphate*.

Almost half of increased crop yields and dramatic improvements in the efficiency of livestock production have come about through conventional genetic improvements, such as bulk breeding and selection techniques. These techniques have been extended to microorganisms and have contributed to the development of strong fermentation technologies. The biotechnological process based on classical microbial fermentation has been augmented by single genetic manipulation using chemical or physical mutagens to improve microorganisms for food fermentation and to enhance the production of bioingredients.

Mutants of *C. glutamicum* and *Brevibacterium flavum* can accumulate in the medium even in the presence of high concentrations of *biotin* (up to 100 g/L). Similarly, *α-amylase* yields from *Bacillus subtilis* have been improved a 1000-fold by mutation and screening. Pathways for the synthesis of antibiotics are often more complex than others and the effects of mutation are less predictable. Nevertheless the progressive selection of *high-yielding mutants* has been the basis for the development of today's highly efficient commercial strains. The yields of penicillin have been significantly increased from 0.15 to 7 g per liter by both *spontaneous* and *induced mutations*.

As a result of normal chromosomal replication or exposure to certain chemicals or physical agents, called *mutagenic agents (mutagens)*, the nucleotide sequence of a gene occasionally changes. Any such change is called a *mutation*. If the mutant protein differs functionally from the wild type (the unmutated form), then it may result in a corresponding change in an observable character and produce a mutant organism. The difference could be one of enzyme activity or stability or one which affects the role of protein as a carrier or as a structural component of the cell. If the mutation affects the DNA of the reproductive cells, it is expressed as a heritable variation and is subject to natural selection. Such mutations provide the process for evolutionary change. Mutations in non-reproductive cells are called *somatic mutations* that produce local, non-heritable changes such as the pigmented cells in human cancer and possibly in aging.

Mutations may be due to different types of change in the DNA sequence and these are described by a number of specialized terms. *Point mutations* result from a single base

change in the DNA sequence and show a characteristic tendency to back mutation, that is, to revert to the wild type. This is because a further mutation at the same site has a one in three chance of restoring the original base sequence of wild type. Four types of single base change, transition, transversion, insertion, and deletion are known. *Transitions* are changes from a pyrimidine to a pyrimidine bases ($T \rightarrow C$ or $C \rightarrow T$) or from a purine to a purine base ($A \rightarrow G$ or $G \rightarrow A$). *Transversions* refer to the substitution of a pyrimidine with a purine or vice versa (T or $C \rightarrow A$ or G ; A or $G \rightarrow T$ or C). A transition or transversion may change the codon for an amino acid residue to another codon for the same amino acid residue (same-sense mutation), to the codon for a different amino acid residue (mis-sense mutation), or to a termination codon (nonsense mutation).

Frameshift mutations, another category of change, results when one nucleotide or more is inserted or deleted, thus altering the reading frame in the following transcription and translation processes. Although genome mutations are important in plant genetics, mutations used in microbial strain improvement usually are point mutations. Chromosome mutations also occur (e.g., deletions, duplications) but are of minor significance. Multisite mutations that affect more than one base do not back-mutate; that is, they are stable. This stability can be an advantage in industrial microbial strains which must retain the same characteristics over long periods. However, an extensive deletion will completely alter the resultant protein, and even a short deletion will have a severe effect if it alters the reading frame.

Spontaneous and induced mutations To some extent, mutation is a spontaneous process that is constantly occurring but the rate of spontaneous mutation is rather low. The naturally occurring error rate is as low as about one error in 10^{10} bases. The spontaneous mutation rate means that one in 10^7 cells will contain a point mutation, depending on the growth conditions of the organism. The causes of spontaneous mutations are likely due to the existence of *tautomeric* forms of all four bases in DNA, the integration and excision of *transposons*, along with errors in the functioning of several enzyme DNA polymerases, recombinant enzymes, DNA repair enzymes. *Tautomer* is a chemical that exists as a mixture of two interconvertible forms (keto or enol; amino or imino). Tautomerization can lead to mispairing during replication. *Transposon* is a DNA sequence (several kilobases in length) that can insert copies of itself into any DNA molecule in the same cell and hence disrupt the transcription and translation of any gene in which they insert. The movement of transposons constitutes transposition. Because of such low frequency of spontaneous mutations, it is not a cost-effective approach to isolate such mutants for the industrial strain improvement. The mutation frequency of cells can be significantly increased by using the chemical mutagens such as *nitrosoguanidine*, which interferes with DNA function. The chemical or physical agents work in many different ways but basically all interfere with DNA replication or repair. The agents may increase to 10^{-5} to 10^{-3} for the isolation of improved secondary metabolite producers or even up to 10^{-2} to 10^{-1} for the isolation of auxotrophic mutants. Commonly used chemical mutagens and their roles are shown in Table 1.8.

Except for UV radiation, the alkylating agents are the most potent mutagens for practical application. The alkylating agents add methyl or ethyl groups to the heterocyclic nitrogen atoms of the bases and are known to cause transition, transversion, and -1 (but not $+1$) frameshift mutations. Because of their structural similarity, base analogs such as 5-bromouracil or 2-aminopurine are incorporated into replicating DNA in place of the corresponding bases, thymine, and adenine. The analogs tautomerize more frequently than the natural bases. Base analogs are of less importance for practical applications because of the costs required to set up the optimal conditions and of the complications in strain development.

Table 1.8 Examples of chemical mutagens and their mode of action

Chemical agent	Action
Mutagens that affect nonreplicating DNA	
<i>Alkylating agents</i>	
Nitrosoguanidine (<i>N</i> -methyl- <i>N</i> -nitro- <i>N</i> -nitrosoguanidine, NTG)	Transition, transversion, deletion, and frameshifts
Ethyl or methylmethanosulfonate	
<i>Other DNA modifiers</i>	
Hydroxylamine	Hydroxylates six amino groups of cytosine, causing G–C to A–T transitions
Nitrous acid (HNO ₂)	Deamination of purine and pyrimidine bases of DNA
Mutagens that associate with or become incorporated into replicating DNA	
<i>Base analogs</i>	
5-Bromouracil	Incorporates into DNA
2-Aminopurine	Causing transition mutations
<i>Intercalating agents</i>	
Acridine dyes (acridine orange)	Frameshift mutations
Proflavine/acriflavine	

Intercalating agents (frameshift mutagens) such as acridine dyes, proflavine, and acriflavine are planar molecules that insert between the stacked pairs of bases. Such insertion distorts the backbone of the double helix, causing errors that result in the formation of faulty protein or no protein. Although acridines are useful for research, they are not very practical for a routine isolation of mutants. They are strong mutagens for bacteriophages (T₂ and T₄) but they have little mutagenic effect in bacteria. Various types of radiations are also powerful mutagens. UV light at a short-wavelength (200–300 nm) is strongly absorbed by DNA and the energy released causes dimerization between adjacent thymine residues, so that replication of the DNA cannot occur. UV light rapidly kills most cells, but the surviving cells exhibit a higher rate of mutation. However, there is one enzymatic repair mechanism that is specific for thymine dimers and can cleave them back to the monomer. Unlike the general repair mechanisms, this is totally error free and is light dependent. To maximize the yield of mutants, *in vitro* UV-treated cells should be kept in the dark for a few hours. In the presence of light, photoreactivating enzyme splits thymine dimers into monomer pyrimidines. Up to 80% of the thymine dimers in the genome (1000 pyrimidine dimers) can be repaired.

A different repair mechanism after UV damage is *excision repair*, which is possible with the complementary strand. Repair of DNA-containing pyrimidine dimers is achieved by nucleotide excision using specific endonucleases, DNA polymerase I and polynucleotide ligase. Excision repair can be partially prevented by inhibitors such as caffeine, acriflavine, and 8-methoxypsoralen. Ionizing radiations such as X-ray, γ -ray, and β -ray act by causing ionization of the medium and breaking single and double strands. Ninety percent of the single-strand breaks are repaired by nucleotide excision. Double-strand breaks result in major structural changes such as translocation, inversion, or similar chromosome mutations. Thus, in industrial strain development, UV radiation or chemical agents are normally preferable for mutations.

For most antibiotics from the discovery, the titer of the producing strain has increased by the order of magnitude by classical mutations. Although medium development and process engineering have been successful, strain improvement by radiation and chemical agents has been the key to improve the final antibiotic titer in fermentation. A typical strain improvement involved first generating genotype variants in the cells by physically or chemically induced mutations or by recombination among strains, that was followed by selection or screening of those with improved phenotype properties. The most popular agent of these, because of its very high mutant to survivor ratio and multiplicity of mutations, has been nitrosoguanidine. Many possible mechanisms to contribute toward enhanced antibiotic production may be due to (i) the increased flux of a precursor primary metabolite, (ii) the increased resistance of the strain to the antibiotics, and (iii) the enhanced gene expression and the resulting concentration of biosynthetic pathway enzymes. Besides random screening for mutants with the desired phenotype properties, selection of auxotrophe mutants resistant to analogs involved in *de novo* synthesis.

Phenotypic expression of mutations Many mutations that result in increased formation of metabolites are recessive. When a recessive mutation takes place in a uninuclear, haploid cell (e.g., bacteria, actinomyces spores, asexual conidia of fungi), the mutant phenotype can be expressed only after further growth and reproduction have taken place. In diploid or eucaryotes, recessive mutations are allowed to undergo phenotypic expression after meiosis or mitotic recombination. Delays in expression are not directly the result of genetic effects. A regime can be selected in which the lethal mutation is not expressed (or the wild type phenotype is expressed) and the organism survives, the so-called permissive condition. The mutations cause death only under certain conditions, termed the restrictive condition, in which the mutant phenotype is expressed.

Temperature sensitive mutations (t^s) are an example of conditional lethals that can be understood in biochemical terms. t^s mutations cause the gene to become nonfunctional at either high (heat sensitive) or low (cold sensitive) temperatures. The biochemical basis of t^s mutation is probably changes in the amino acid sequence of a protein which affects thermostability rather than activity because they affect the overall secondary or tertiary structure of the protein rather than an active site. Osmotically, remedial mutations cause the gene product to be particularly sensitive to the osmotic strength of the growth medium. The protein is usually stable only in the presence of higher concentrations of solutes. Streptomycin-remedial mutants express a wild-type phenotype in a medium that contains low levels of aminoglycoside antibiotic (Streptomycin, Neomycin, or Kanamycin). Restrictive condition denotes growth in a medium that lacks the antibiotic by altering the translation mechanism rather than the gene products.

A gene activity that has become lost through mutation can be restored at least partially through a second mutation, called a *suppressor mutation*. Suppressor mutations act in several different ways. Suppressor mutation like streptomycin changes the translation mechanism, thereby producing some gene products that are functionally active, but often only as a somewhat abnormal pseudo wild-type. In contrast, error-free repair restores the DNA sequence to give a normal wild-type.

1.5.2 Recombination

The genetic information from two genotypes can be brought together into a new genotype through genetic recombination. This is another effective way of increasing the genetic variability of a cell. The advantages of genetic recombination are (i) different alleles of the

parent strains with increased metabolic production can be brought together in one strain, so that the cumulative effect of these mutation is greater than the effect of the single mutation. However, a significant yield increase by recombining two high-yielding mutants has only been successful in a few instances and in most cases, the productivity of the recombinants is intermediate between the values of the parent strains; (ii) there is frequently a decline in the increase in yield and in the development of inapparent mutations, which prevent a further increase in the metabolite production by pleiotrophic influences. With genetic recombination, these unfavorable mutant alleles can be replaced with alleles of one of the parents in the cross; and (iii) high-yielding mutants can actually increase the cost of the fermentation because of the changed physiologies such as greater forming, nutrient requirements and others. By crossing back to the wild type strains, high-yielding strains with improved fermentation properties may be obtained.

Sexual and parasexual recombination in eucaryotes When a sexual cycle is known, *nuclear fusion (karyogamy)* results after fusion of hyphae, leading to a recombination of nuclei in the heterokaryotic mycelium. After diploid formation in some fungi (*Aspergillus*, *Sacharomyces*, etc.), recombination takes place during the subsequent meiosis process. A new genotype results either from the combination of parent chromosomes or from crossover between two paired homologous chromosomes both of which are *replicons* (called general recombination). This type of recombination is known to be catalyzed by the *recA* gene. The main role of the product of the *recA* gene is in the process of recombination, which creates crossover exchange between DNA molecules by binding single-strand DNA in order to unwind double-stranded DNA. The so-called *tetrad analysis* is often applied to eucaryotes, especially for the yeast, *S. cerevisiae*. This method consists in the possibility of isolating the four products (four spores) and cultivating them separately. The attainment of recombinants through the sexual process has been confined to commercial mushrooms and yeasts. Some of the most economically important fungi, such as *Penicillium chrysogenum* and *Cephalosporium acremonium*, which are producers of penicillin and cepharosporin antibiotics do not have a sexual cycle.

In parasexual reproduction, the fusion of two hyphae of equal or different polarity results in a mycelium with nuclei of both parent strains. This heterokaryon is normally stable with the nuclei mingling but not interacting. In rare cases, nuclear fusion occurs and a diploid nucleus is formed. In such mitotic crossover between homologous chromosomes, genetic recombination can be obtained. To obtain a recombinant, haploid cells or spores must be formed, but spontaneous haploid formation is relatively rare. It can be induced with *p*-fluorophenylalanine.

Recombination in bacteria Although the parasexual mechanisms such as conjugation, transformation, and transduction in bacteria are established, only a fragment of the genome of the donor cell is transferred into a recipient cell, becoming a partial diploid. After homologous pairing, recombination occurs, but the rate of recombination is far lower than when the perfect sexual cycle is used.

Conjugation generally involves the participation of plasmid and single-stranded DNA is transferred from the donor cell to the recipient cell after the two cells have come into contact. In transformation, short pieces of DNA are taken up by the competent recipient cells. In *generalized transduction*, *temperate phage* particles, which have lost a piece of their own genomes, transfer a chromosome DNA fragment of the host bacteria at the rate of 10^5

per phage. In specialized transduction, recombination occurs within homologous segments shared by the phage (phage) and the chromosome at the attachment site of the phage. The insertion of the prophage into the chromosome results in further incorporation of the attached piece of DNA into the genome of the host cell. This is an example of site-specific recombination, which does not require the participation of the *recA* protein.

Transposable genetic elements, termed *insertion sequences*, and *transposons* have the ability to transpose to various sites on the bacterial genome. Insertion sequences are small elements (~1 kb) that encode only their capacity for replicative recombination. Transposons are larger (up to 10 kb in length) composite elements terminated by insertion sequences. The discovery of transposons that undergo replicative recombination at relatively high frequency (Tn_5) provided an explanation for the spread throughout a bacterial population of certain genes. Transposon Tn_5 contains the gene for resistance to an aminoglycoside antibiotic (Kanamycin), which can be expressed in a wide variety of prokaryotes and eukaryotes. Several transposons have been integrated into plasmids (Tn_1 and Tn_3) and others in either plasmids or chromosomes (Tn_5). Thus, transposons can form multiple drug-resistant plasmids that can then be transferred to other strains. Thus, transposons are available for a wide variety of purposes in gene technology.

Protoplast fusion Recently developed methods such as protoplast fusion have extended the number of organisms in which two genotypes can be recombined. *Protoplasts* are wall-less cells, which retain full respiratory activity and can synthesize protein and nucleic acids. Under certain special conditions of cultivation, they can even be induced to regenerate cell walls and again assume a rod shape. The artificial production of protoplasts can be prepared by subjecting cells to the actions of cell wall-lysing enzymes (lysozyme for bacteria, chitinase, or cellulase for fungi) in isotonic sucrose solution. Protoplast fusion is normally rare because of the strong negative charge of the protoplast surface. However, in the presence of polyethylene glycol (PEG), the protoplasts fuse relatively easily accompanied by DNA exchange. Many yeast species like *Saccharomyces lipolytica*, it is not necessary to degrade the cell wall completely. Such cells in which cell wall residues still adhere to the plasmalemma are called *sphaeroplasts*.

Besides the use of PEG to bring about fusion, the method of electric-field-induced fusion (electrofusion) of protoplasts has also been developed. With electrofusion, two or more protoplasts can be fused under microscopic control of several cells into one giant cell. Protoplasts can also be induced to fuse artificial phospholipid vesicles, called liposomes. The fusion rate is about 60% using PEG and 80–90 % with electrofusion. Protoplast fusion can be used for intraspecific recombination of strains, which lack sexual or parasexual systems or whose frequency of recombination is too low, and interspecific hybridization to obtain completely new organisms capable of synthesis of new or modified metabolites. Protoplast fusion has been achieved with the filamentous fungi, yeasts, *Bacillus* sp., *Brevibacterium* sp., *Streptomyces*, *Aspergillus*, *Penicillium*, mucor, lactic acid bacteria, and many more strains.

In vitro rDNA technology made possible for the development of methods for transformation of protoplasts with plasmid, chromosomal, or viral DNA. Protoplasts are usually treated with DNA in the presence of PEG and Ca^{2+} and in this way, all of the numerous technologies of genetic engineering become available for use with the industrial strains. Even microorganisms for which conventional host-vector systems are not available can be transformed. For example, protoplast transformation is inefficient in dairy lactic acid

bacteria such as *Lactococcus* and *Lactobacillus* strains. Most recently, however, electroporation has developed into a very efficient method for physically introducing DNA into the Gram-positive microorganisms. Electroporation uses short electric pulses of a certain field strength, which alters the permeability of membranes such that DNA molecules can enter the cell. Using virus vectors, transfection (or transformation) systems have also been described for *Streptomyces*, *Thermomonospora*, *Mycobacterium*, and *Brevibacterium lactofermentum*.

Summary

Biotechnology involves the potential use of all living organisms including cells of animals, plants, and microorganisms. However, microorganisms have played a major role in this field for millennia, since the discovery of fermentation, and will continue to do so more extensively for the foreseeable future. There are many reasons for the choice of microbes: their rapid growth rate, ease of mass cultivation in many cheap substrates, diverse metabolic types (which give rise to many different products), and the tremendous possibility of genetic improvement for new products. Of the many thousands of microbial species known, relatively few are currently exploited. Microbes are responsible for the photosynthesis of energy from the sun, and they participate in the cycles of nitrogen, oxygen, sulfur, and other elements essential for life. They are also responsible for many diseases of humans, animals, and plants.

Among the vast contents in the science of microbiology, we have concentrated primarily on the cellular organization, reproduction and classification, genetics of useful microorganisms for foods and commodity products. Except for the viruses, most organisms share a common chemical composition, the presence of three complex macromolecules (DNA, RNA, proteins), and common physical structural and functional properties.

Two major groups of cellular organisms, the prokaryotes and the eukaryotes, were compared. The prokaryotes are the Eubacteria and Archaeobacteria. The rapid growth and the biochemical versatility of prokaryotes make them important tools for biological and genetic research, and for biochemical scale-up processing. Eukaryotes are the unit of structure in plants, animals, and protists (algae, fungi, protozoa). The eukaryotic cells possess several unique organelles: the nuclear envelope, the endoplasmic reticulum, and the Golgi apparatus. A basic knowledge of eukaryotic cells and the function of organelles is assumed.

The microbial classifications are based on many different factors, which are of great practical importance in applications of biotechnology. With the exception of the Actinomycetes, most biotechnology-related bacteria belong to the Eubacteria.

The fungi are nonphotosynthetic, aerobic, filamentous, and nucleated cells known as hyphae. Fungal classification is based more on morphological and habitat characteristics than on staining and biochemical reactions. The fungi can be classified into zygomycetes, ascomycetes, basidiomycetes, and fungi imperfecti. Of these, the most frequently known species in biotransformation work are the yeasts and the molds.

The principal macromolecules of all cells are proteins and nucleic acids, and the biochemical reactions leading to their formation are similar among prokaryotes and eukaryotes. However, a greater diversity is formed in the synthesis of other classes of cell constituents such as polysaccharides and lipids, which are the group-specific substances. The reactions of biosynthesis and polymerization common to all organisms were discussed. Also classical strain improvement and tools were described.

1.6 Systems/synthetic biology and metabolic engineering

Classical strain improvement is too labor intensive for the improvements. In classical strain improvement, cells are treated with a chemical or physical mutagen that kills off most of the cells, and screen the survivors for that rare clone which produces more of the interested product. This new strain becomes the starting point for the improvement, but it is not easy to know why the strain is improved. In the era of metabolic engineering (ME), more specifically targeted in the way that one can improve strain productivity more efficiently. That might have been true 20 years ago, but with the use of miniaturized cultivation along with automated colony picking and liquid handling, a large number of colonies can be screened regardless of whether their genetic variability was introduced by ME or chemical mutagenesis. Now biology companies are producing biofuels and renewable chemicals using classical strain improvement as a supplement to synthetic biology or other ME approaches. The term *synthetic biology* was used to describe concepts that would be classified today as ME, but in the last 10 years, terms such as “unnatural organic molecules,” “unnatural chemical systems,” “artificial, biology-inspired systems,” and “functions that do not exist in nature” have been used to describe synthetic biology. Synthetic biology can thus be defined as “the design and construction of new biological components, such as enzymes, genetic circuits, and cells, or the redesign of existing biological systems” as well as “reconstruction of a complete bacterial genome.” Synthetic biology has application to many fields, including cell-free synthesis, tissue and plant engineering and drug discovery, but in industrial biology, synthetic biology offers some tremendous opportunities to create cell factories that are tailor-made for efficient production of commodity chemicals and fuels from renewable resources. In most cases, the design and construction of cell factories for use in industry requires both synthetic biology and ME (Figure 1.7).

In Figure 1.7a, the first approach is a traditional biotech step where a naturally producing organism is selected as the cell factory for production of the desirable product. Typically, the flux toward the product is naturally low but through the use of classical strain improvement or the use of directed genetic modifications (ME), the increase of the sufficient flux toward the product is possible. Economically feasible processes are currently on the production of industrial enzymes, antibiotic (adipoyl-7-amino-3-deacetocephalosporanic acid: adipoyl-7-ADCA), a precursor for cephalexin using fungal and bacterial cell factories, and fuel production using *S. cerevisiae*. In Figure 1.7b, the platform cell factory does not naturally produce the product of interest, but through insertion of a synthetic pathway in the organism (illustrated by the red pathway), the cell factory can often produce small amounts of products initially, but through pathway optimization, the flux through this synthetic pathway can be increased to ensure a high flux toward the product. As all metabolites produced in nature are derived from a set of only 12 precursor metabolites that are intermediates of the central carbon metabolism, when a new synthetic pathway is inserted into a designated cell factory, a drain of one of these precursor metabolites will occur to the unadapted cells, resulting in a low yield initially. However, through engineering of the central carbon metabolism, the flux can be redirected toward the precursor metabolite for the desired product, thus enabling enhanced yield and productivity. Once engineering of the central carbon metabolism is successful, many different synthetic pathways can be inserted into the cell platform with the intermediate result of high-level synthesis of the target products. This approach clearly applies concepts from

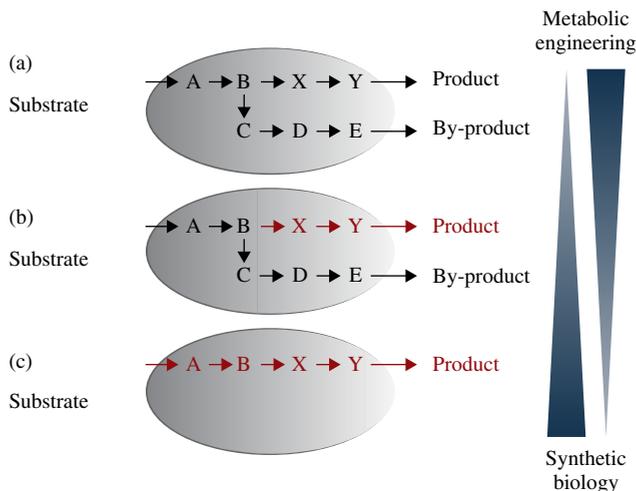


Figure 1.7 (a–c) Illustration of the overlap between metabolic engineering and synthetic biology by the use of three different approaches to produce a desirable product. *Source:* Nielsen and Keasling 2011. Reproduced with permission of Nature Publishing Group.

both ME and synthetic biology. If the cell factory that efficiently converts different sugars to acetyl-coA is developed, such a cell factory can be a good platform for producing many ranges of products including polyketides (antibiotics, anticancer drugs, immunosuppressors), lipids (dietary supplements, pharmaceuticals, biodiesels), isoprenoids (perfumes, antimalarial drugs, antibiotics, dietary supplements, food ingredients, vitamins), and so on. In Figure 1.7c, a complete synthetic cell is constructed to produce the desirable product. In this approach, the limited platform cell factories like *E. coli* and *S. cerevisiae* will function as the important model organisms for prokaryotic and eukaryotic cells. These two organisms already have the detailed mathematical models *in silico* representation, that are the most probable cells to study in terms of *systems biology* that seeks to integrate biological data as an attempt to understand how biological systems function. By studying the relationships and interactions between the various parts of a biological system (e.g., organelles, cells, physiological systems, organisms, etc.), it is hoped that an understandable model of the whole system can be developed. In this kind of *in silico* representation, mathematical modeling of the interaction between all the components in the system is carried out to obtain a predictive model for the system. Our knowledge is currently quite limited in the field of engineering of entire cells (that is, the ultimate goal of *synthetic biology*), but through advances in systems biology, the necessary knowledge and mathematical models can be obtained. In a short term, advance of systems biology will affect our ability to reengineer cells (that is, the goal of *ME*), which further speed up to develop more efficient cell factories. Therefore, there are synergies between systems biology, synthetic biology, and *ME* (Figure 1.8). Although the term “synthetic biology” has been around since the mid-1970s, the definition has been very vague. The activities related to synthetic biology are considered by some to be just extensions of already existing fields, like molecular biology, genetic engineering, and microbiology, but systems biology describes the overall cell function through quantitative description of the interaction between all the individual components in the cell, such as gene transcription, translation, protein–protein interaction,

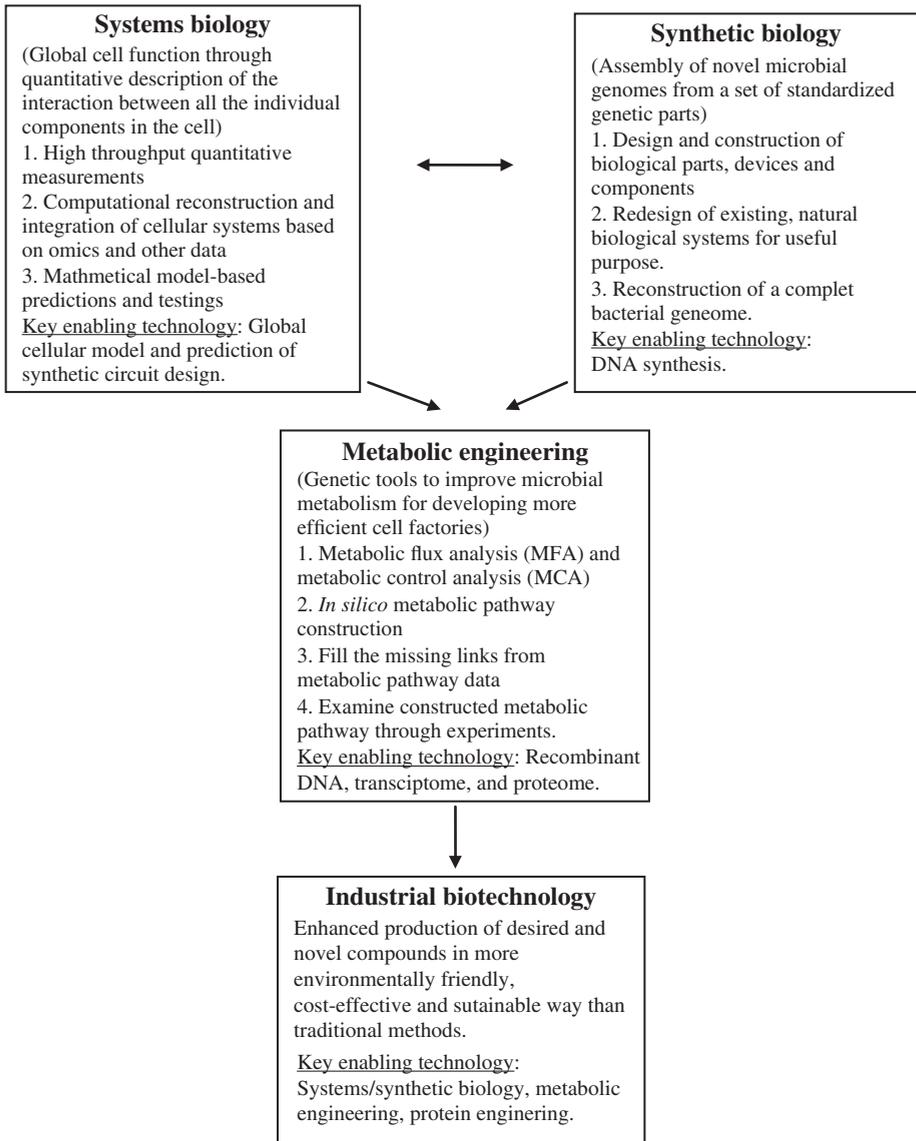


Figure 1.8 Contribution and overlap of systems/synthetic biology to metabolic engineering for industrial biotechnology.

enzyme catalysis of biochemical reactions, and receptor–metabolite interaction. That is the global-scale study of biological processes at the cell or organism level in terms of their molecular components and their interactions. Systems biology does not investigate individual cellular components at a time, but it is about the behavior and relationships of all of the elements in a particular biological system while it is functioning. In fact, the goal of synthetic biology is to make the engineering of biology faster and more predictable,

and to harness the power of biology for the common good. Systems biology focuses on the interaction of myriad components and how these give rise to the dynamic and complex behavior of biological systems. More precisely, synthetic biology is to gain an increasingly precise control over cellular processes, considering ground elements – that is, custom DNA sequences, custom proteins produced by genetic code expansion, standard biological parts, and basic synthetic circuits – but also higher-order elements, composite synthetic circuits, and engineered metabolic pathways. The degree and the tools with which synthetic biology intends and controls cellular processes are what distinguish it from any other field of the biological sciences. Even a simple biocatalyst like *E. coli* is a complex system of an estimated 4603 genes, 2077 reactions, and 1039 unique metabolites, and while the engineering steps are relatively straightforward, it is still difficult to quickly and reliably engineer a biocatalyst to perform desired behaviors. Systems biology, the standardization of biological systems, and metabolic evolution are all vital to compensate for this disconnect between the expected and actual biocatalyst behaviors. Applications of synthetic biology have already been successful in the (i) production of antimalarial drug precursors, *artemisinic acid* in *E. coli* and yeast, (ii) creation of synthetic oscillators and light sensors, (iii) transplantation of synthetic genomes and chromosomes, engineering of cells able to break down toxins, detection of explosives by plants, creation of bacteria that produce toxins in response to pathogens, and (iv) creation of cell factories tailor-made for efficient production of fuels and chemicals. One successful achievement by synthetic biology is the microbial production of the antimalarial drug *artemisinin*. Artemisinin, a sesquiterpene lactone endoperoxide extracted from *Artemisia annua* L (a sweet wormwood) is highly effective against the multi drug-resistant malaria parasite *Plasmodium falciparum*, but it is in short supply. Most people suffering from malaria are unable to get it due to its high costs. It was possible to modify *S. cerevisiae*, which can produce high titers (up to 100 mg/L) of artemisinic acid using an engineered mevalonate pathway, amorphaadiene synthase, and a novel cytochrome P450 monooxygenase (CYP71AV1) from *A. annua* that performs a three-step oxidation of amorpha-4,11-diene to artemisinic acid (<http://newscenter.berkeley.edu/2013/04/11/launch-of-antimalarial-drug-a-triumph-for-uc-berkeley-synthetic-biology/>). The synthesized artemisinic acid is transported out and retained on the outside of the engineered yeast. Although the engineered yeast is already capable of producing artemisinic acid at a significantly higher specific productivity than *A. annua*, cost-effective optimization and industrial scale-up will be required to produce artemisinic acid production to significantly lower their current prices. However, challenges in synthetic biology are also many because biology is complex and synthesis capabilities far exceed design capabilities. Potential benefits are enormous in the fields of medicine, food, and energy, but the safety and ethical issues are being revisited. Similar to the genetically modified organism (GMO), potential risks include unintended harmful consequences for human health or the environment or deliberate misuse for hostile purposes. ME is basically the use of directed genetic modification to improve the properties of a given cell, for example, to improve the yield or productivity, expanded substrate range, and production of novel products. ME is to improve cellular activities by manipulations of enzymatic, transport, and regulatory functions of the cell with the use of rDNA technology. In fact, ME studies are also considered to be part of synthetic biology. Recent developments in synthetic biology promise to expand the ME toolbox further by creating novel biological components for pathway design. The holistic understanding of metabolic flux, promoter activity, preferential codons, and scaffold proteins are the basics for synthetic ME. These integrated approaches promise to do more than simply improve product yields by recombination DNA and other molecular biological tools; they can expand the array of products

that are tractable to produce biologically. That is why ME has recently been upgraded to systems level (so-called systems ME) by the integrated use of global technologies of systems biology, fine design capabilities of synthetic biology, and rational–random mutagenesis (RM) through evolutionary engineering.

By metabolic flux analysis (MFA), the intracellular metabolic fluxes can be quantified by the measurement of extracellular metabolite concentrations in combination with the stoichiometry of intracellular reactions. MFA can be applied to evaluate the intracellular metabolic conditions and to identify key metabolic pathways or metabolites. *Metabolic control analysis (MCA)* is a statistical modeling technique used to understand how metabolic fluxes are controlled by certain enzyme activities and metabolic concentrations. Those responses of small changes to metabolic flux distribution can be predicted by MCA. Tools of ME require: (i) molecular biological tools such as *genomic, proteomics, transcriptomics, interactomics, bioinformatics*, and so on; and (ii) metabolic pathway analysis tools such as identification of the metabolic network structure (or pathway topology), and quantification of the fluxes through the branches of the metabolic network, and identification of the control structures within the metabolic network. The metabolic flux is an essential concept in the practice of metabolic engineering. Because gene expression levels and concentrations of proteins and metabolites in the cells provide clues to the status of metabolic networks, they have inherent limitations in describing the cellular phenotype. In *in silico* construction of a strain's metabolic pathway, (i) one must obtain the complete sequence of the strain, search *open reading frames (ORFs)* from sequences (using ORF finder or ORF data from Genbank), (ii) identify the functions of ORFs based on literature information (e.g., *MetaCyc database*), (iii) search enzymatic reactions using metabolic pathways (*KEGG* or *Swiss-Prot*), (iv) construct metabolic pathway from enzymatic reaction list, (v) fill the missing links from reference metabolic pathways, and (vi) compare the constructed metabolic pathway through real experiments. Knowledge acquired from this research will benefit the society in a number of ways, including the ability to modify biological pathways to produce biological substitutes for less desirable chemical processes. This technology will allow greater agricultural production, permitting more efficient and safer energy production, and provide better understanding of the metabolic basis for some medical conditions that could assist in the development of new cures. The continued development of tools in synthetic biology is a way to reduce the cost and time required to engineer biological systems for the production of pharmaceutical ingredients, fine chemicals, biofuels, and so on. However, in the classical method of ME, identifying a rate-determining step in a pathway and alleviating the bottleneck by enzyme over-expression has enjoyed only limited practical success. Thus, an alternative method for cellular perturbations to identify targets, termed *inverse metabolic engineering (IME)*, takes advantage of recent advances in high-throughput screening and genome sequencing. Although this IME approach is potentially very powerful, elucidating the genetic basis of a relevant phenotype can be difficult. This is especially true in the context of modern evolutionary techniques such as genome shuffling and directed enzyme evolution among others. In other words, the classical approach of ME requires detailed knowledge of the enzyme kinetics, the system network, and intermediate pools involved, and on such bases, a genetic manipulation is proposed for some presumed benefits.

In contrast, the concept of IME inverse metabolic is first to identify the desired phenotype, then to determine environmental or genetic conditions that confer this phenotype, and finally to alter the phenotype of the selected host by genetic manipulation. The process of IME consists of: (i) construction or identification of a certain phenotype, (ii) determination of the factors conferring that phenotype, and (iii) engineering the factors into another

strain or organism. However, the use of functional genomics on IME is still in its infancy, and thus the numbers of published data are still small.

Summary

Strain improvement is an essential part of process development for biotech products as a means of reducing costs by increasing productivity and yield by redirecting multiple-step metabolic pathways (catabolism and anabolism). ME aims to control this complexity in order to establish sustainable and economically viable production routes for valuable chemicals. Recent advances in systems-level data generation and modeling of cellular metabolism and regulation together with tremendous progress in synthetic biology has provided the tools to put biotechnologists on the fast track for implementing novel production processes.

The rapid advance of ME has been in part due to notable advances in fields adjacent to ME such as rapid DNA-sequencing techniques, extensive databases of gene expressions, metabolic reactions, and enzyme structures, new genetic tools enable more precise control over metabolic pathways, new analytical tools enable the metabolic engineer to track RNA, protein, and metabolites in a cell to identify pathway bottlenecks, and detailed models of biology aid in the design of enzymes and metabolic pathways (systems biology).

Yet even with these substantial developments, metabolic engineers must weigh many trade-offs in the development of microbial catalysts. In particular, there are many feedback loops in the control structure; that's why it is difficult to predict the overall effects of a specific genetic modification. It is thus necessary to go through the cycle of ME and inverse ME several times when intracellular fluxes needed to be redirected. Even with these many challenges, ME has been successful for many applications, and with continued developments, more applications will be possible. Recently, ME has been upgraded to systems level, called systems ME by the integrated use of global technologies of systems biology, fine design capabilities of synthetic biology, and rational–RM through evolutionary engineering.

1.7 Bioengineering and scale-up process

As the food science major does not have sufficient background on bioengineering, a detailed description has been added in this subject. *Biotechnological engineering or Bioengineering* is a branch of engineering that focuses on biotechnologies and biological science. It includes different disciplines such as biochemical engineering, biomedical engineering, bioprocess engineering, biosystem engineering, and so on. The definition of a *bioengineering* is in general an integrated approach of fundamental biological sciences and traditional engineering principles. Bioengineers are often involved to *scale up bioprocesses* from the *laboratory scale* to the *manufacturing scale*. Moreover, as with most engineers, they often deal with management, economic, and legal issues. Since *patents* and *safety regulations* are very important issues for biotech enterprises, bioengineers are often required to have knowledge related to these issues.

Processing of biological materials such as *cells*, *enzymes*, *metabolites*, or *antibodies* are the central domains of bioengineering. Success in bioengineering requires integrated knowledge of governing biological properties by process-oriented microbiologists, and the methodology and strategy of chemical engineers. In terms of annual world-wide

sales, biotechnology-derived products can be divided into the three categories. First, fine chemicals as low volume products and as bulk chemical products usually fall within the 100 kg per year to 100 tons per year range. This category broadly includes bioproduction of high-value molecules such as *vaccines*, *rDNA products*, *5'-nucleotides*, some of the *amino acids*, *enzymes* (for medical applications), *monoclonal antibodies*, and bioconversion of high-value starting materials such as antibiotics, and steroids, and so on. A significant fraction of the production costs of these products are involved in purification and testing of the product to meet the demand of quality and safety specifications. Intermediate volume chemicals are usually chemicals or ingredients that are produced microbiologically in the range of 100 and 20,000 tons per year. Such products have less vigorous quality and safety specifications than do fine chemicals. Some examples are *glutamic acid (monosodium glutamate (MSG))*, which is used as a flavor enhancer, *antibiotics* used for protecting agricultural crops, *food and industrial enzymes*, *organic acids (citric, lactic, gluconic acids)*, *solvents (acetone, butanol)*, many *fermented beverages*, and *food products*.

Finally, the bulk product sector consists of products that are usually produced in continuous reactors, their number exceed 20,000 tons per year. These products are marketed on the basis of commodities and overall product performance criteria rather than on the basis of rigid quality specifications. The microbial products that fall within this category are *SCP*, *gasohol (ethanol)*, *biogas (methane)*, and *biopolymers* for enhanced oil recovery.

The primary goal of bioengineering practice is then to create processing systems that economically transform raw materials into marketable products using *fermentation* and *downstream (or product recovery)* processing. However, the recovery process that involves extraction, purification, drying, and so on, of biological products differs from chemical recovery in that these materials are much more labile than chemicals. Many of the techniques used in the biological products are still similar to those of chemical processes (e.g., separation, distillation, heating, cooling, drying). There is, however, increasing use of non-denaturing methods such as various chromatography and electrophoresis techniques for biological materials. In the fermentation process, engineering is only an aid in the development and regulation of biological processes such as regulation of microbial metabolism by optimizing culture media, oxygen requirements under sterile conditions, genetic manipulation, and so on. The economic evaluation of industrial processes and process routes is also an essential activity of the engineers. The gross profit obtained as a result of operating a particular plant is the difference between the net income from the annual sales, after distribution, promotional and sale costs as well as the annual manufacturing costs have been deducted.

To improve its profitability by process integration and optimization, it is essential to understand the technological factors that affect the overall economics of manufacture significantly. In commercial processes, the *microbial factors* with the greatest impact on the process are (i) yield coefficients for the product(s), (ii) growth rates and/or product production rates, (iii) affinity of the culture for carbon energy substrates, (iv) stability and fastidiousness of the culture. The *process engineering factors* with the greatest impact on the process are (i) feedstock conversion efficiencies, (ii) productivity, and (iii) product concentration. Both the microbial and the process engineering factors are closely related in processes that are technologically and commercially successful. When evaluating any new production process, it is then essential to understand the technical factors that affect the overall economics of manufacture significantly. To improve profitability by process integration and optimization, it is essential to understand the technological factors that affect the overall economics of manufacture significantly. In commercial processes, the microbial factors having the greatest impact on process are yield coefficients for the product(s), growth rates and/or product production rates, affinity of the culture for carbon

energy substrates, and stability of the culture. The process engineering factors having the greatest impact on the process are feedstock conversion efficiency, productivity, and product concentration.

1.7.1 Microbial and process engineering factors affecting performance and economics

The general fermentation process is largely divided into a fermentation section and a product recovery section as shown Figure 1.9. The upstream section receives and stores raw materials for eventual preparation of media; the downstream part is the site of final product preparation, packaging, and shipping. A full-process design must include for

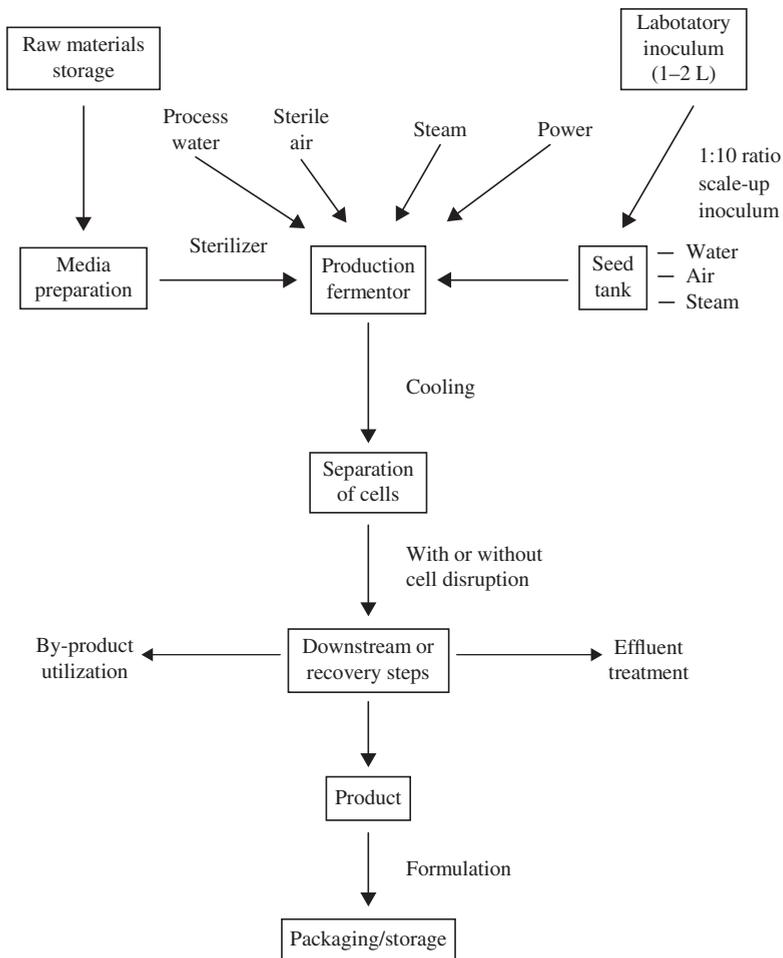


Figure 1.9 The general process scheme for upstream and downstream fermentation.

each section operations, utilities (water, gas, electricity, etc.), and effluent or by-product disposal. Since bulk chemicals from fermentation involve large-scale operations, and cell sludge or waste disposal is responsible for a major fraction of plant operating costs, a market for by-products must be found. Most modern fermentation cultures have been pure monocultures isolated from natural sources under aseptic operation. However, the cultures have been subjected to strain improvements by manipulating growth conditions, encouraging mutations, or using rDNA techniques. The concepts of microbial growth kinetics, yield coefficients, and batch or continuous cultures have been discussed. However, the overall suitability of a selected culture for a commercial process, which is a critical factor for process scale-up, has not been covered.

Heat and mass transfer in fermentors/bioreactors are often achieved by an intense mechanical agitation to disperse air and to promote adequate mixing. This shearing action of rotating impellers tends to damage the cells, particularly those of filamentous molds, actinomyces, and bacteria with appendages (*flagella* and *fimbriae*). Microbial strains whose morphology is significantly affected by such culture conditions are obviously not suitable for commercial process application. It is thus important to select more robust microbes that are better suited to the harsh conditions encountered in intense processes. This option is restricted for animal and plant cell cultures.

Microbial strains that exhibit broad ranges of pH, temperature, and DO optima are certainly more advantageous for process application than those of fastidious strains. Robust microbes that can adapt to high levels of performance under rapidly changing physical and chemical environments are essential for good bioprocess economics.

Any microbial process can be divided into the production of live microorganisms and the production of the desired end product or the decomposition of feedstock by chemical transformation due to, for example, the actions of enzymes present in the cells. On the industrial scale, biotransformation by growing microbial cells is the most commonly used technology. The immobilized systems using either cells or enzymes are developing rapidly for some industrial-scale transformations. However, problems still exist with respect to maintaining longer shelf life of enzymes, preventing microbial contamination, generating cofactor, and overcoming the intrinsic diffusional resistance. Productivity and conversion efficiency are the key factors that affect process economics, but each in turn is controlled by the physical phenomena occurring in the fermentor through its mass and heat transfer capacities.

For a project that eventually achieves commercialization, life cycle stages include the product idea, preliminary evaluation of economics and markets, development of data needed for final design, final economic analysis, detailed engineering design, procurement of site and equipment, construction of buildings and process, process start-up and trial runs, and regular production operation. It is beyond the scope of this text to describe the elements of total product cost and typical values for estimation.

1.7.2 Fermentor and bioreactor systems

Basically, there is not much difference between fermentors and bioreactors. Regardless of device type and size, the basic fermentor consists of a closed vessel, fitted with an air inlet and an agitator, in which microbial or biochemical reactions are carried out for commercial ends under a controlled environment. Vessels used to grow microbial cells are often referred to as *fermentors*, while those in which plant and mammalian cells can be cultured are called *bioreactors*. Essentially, however, all such equipment features heterogeneous systems consisting of two phases, usually gas and liquid. We will use the term *bioreactors* to denote cells of all types in this text.

Table 1.9 Historically developed types and applications of bioreactors

Reactors	Applications
1. Anaerobic vats	Alcohol and yeast production
2. Surface koji (solid state) culture	Acetic acid, citric acid, fungal commercial enzymes
3. Stirred tank–submerged culture (e.g., mixed flow, continuous flow (CSTR), backmixing)	Many examples of cell-free enzymes (e.g., glucoamylase) Free cells (e.g., steroid) Immobilized beads Biomass
4. Single-cell protein (SCP) culture (e.g., continuous, large volume, tower, high density)	
5. Packed bed (e.g., immobilized system)	Glucose isomerization Penicillin hydrolysis Selective separation of racemic amino acids
6. Bubble column (e.g., tower, air lift)	Beer production Biomass Vinegar Plant metabolites
7. Fluidized bed	Continuous beer and cider production
8. Trickle bed	Wastewater treatment Vinegar manufacture

Bioreactor classification is usually based on whether the reaction occurs in a single-phase or a multiphase environment. Reactions in the first case are termed *homogeneous reactions*, while those occurring in multiple phases are called *heterogeneous reactions*. For biological reactions involving microbes and for most enzymatic reactions, however, the concepts of homogeneity and heterogeneity cannot be applied consistently.

With a few exceptions, such as the continuous flow of aerobic wastewater and sewage treatment, the making of vinegar, and microbial biomass production, most bioindustries still prefer to use the batch mode of operation for transformation and the semicontinuous mode of operation for microbial biomass production, for reasons of reliability and versatility. Table 1.9 shows how bioreactors have been developed historically and compares reactor types. The discussion of reactors has been restricted in this text because there are so many different types and also because newer systems are continually developing, especially for systems that offer more savings in energy. Alternative nonstirred reactors such as immobilized cells and enzymes are also developing rapidly and may replace fermentation processes in the future.

Packed-bed bioreactors *Packed-bed reactors* are operated in most cases with a continuous gas atmosphere. The nutrient solution is evenly distributed over the packing through a feed device. In packed-bed (or fixed-bed) reactors, columns packed with immobilized biocatalyst particles are currently used for glucose isomerization, selective penicillin hydrolysis, and selective reactive separation of racemic mixtures of amino acids. Many immobilized cell systems in packed-bed reactors have been examined, and a useful model is the *plug-flow reactor*, which is also one of the simplest ones. The flow is from the bottom to the top, affording gradients of concentration of substrate and product in which the product of unreacted substrate flows out of the reactor continuously. It is very difficult to provide effective aeration at a significant scale as well as to regulate the temperature and pH.

Bubble-column bioreactors All reactors in which the compressed gas at the bottom of the vessel rises through the liquid because of its buoyancy belong to the bubble-column group. In several recent designs, the so-called *air-lift reactors*, an external loop is used to provide fluid circulation; this arrangement permits high efficiency of heat exchange and enhances flow and mixing in the vessel. It has been stated that the energy demand for a loop reactor is about one-fifth that of a conventional stirred vessel. As shown in Figure 1.10a, the air is introduced at a high velocity by static gas distributors (perforated or sintered plates). The use of a draft tube enables the liquid to circulate with considerable turbulence. For efficient oxygen transfer, it is necessary for the reactor to have a much higher tower (height-to-diameter ratio of 10:1), than a conventional stirred-tank reactor. As the bubble ascends the column, however, the partial pressure of the oxygen within it decreases, and this decreases the rate of oxygen transfer to the liquid.

The other reactor design that is able to overcome the foregoing disadvantages is the *deep-shaft reactor* (Figure 1.10b). The air is introduced at above midheight of the reactor and this forces the liquid up and down. Both the air-lift and deep-shaft reactors can be operated continuously. Reactors of the bubble-column type have been used for the chemical industry; in biological systems, they have been used mainly for the production of beer, vinegar, and single-cell protein (or animal feed).

Fluidized-bed reactors *Fluidized-bed reactors* are generally similar to those of bubble-column geometry but are considerably more complex than the systems discussed thus far. Heterogeneous biocatalyst particles such as flocculated organisms, pellets of immobilized enzymes, or cells are suspended by drag forces exerted by the rising liquid. By maintaining a careful balance between operating conditions and cell characteristics, the biocatalyst is

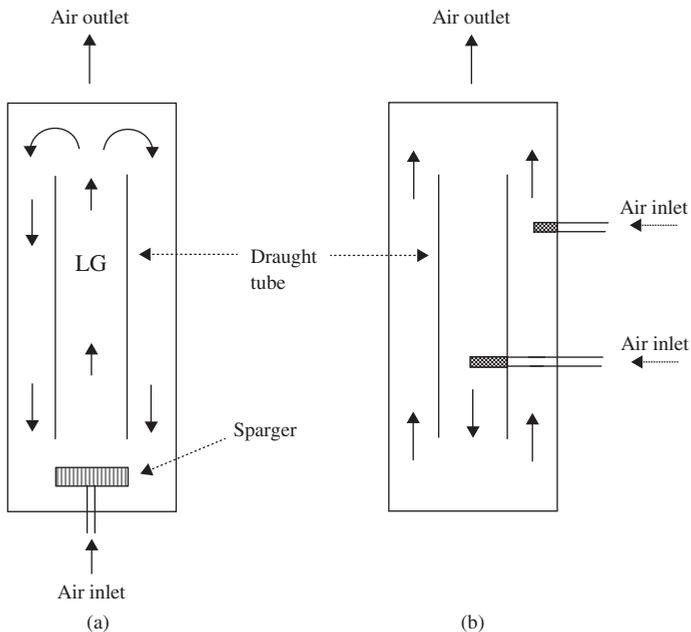


Figure 1.10 Two novel reactor designs: (a) air-lift (tower loop) and (b) deep-shaft reactors.

retained in the reactor, while the medium flows through it continuously. However, the height of the catalyst must be somewhat greater than that of the substrate solution; thus recycling of the fluid is not possible and scale-up is quite difficult.

Trickle-bed reactors *Trickle-bed reactors* are continuous gas-phase systems containing a packed-bed of heterogeneous catalyst, flowing gas and liquid phases. Providing good gas and liquid supply to biocatalysts is a major advantage of these reactors they being substantially influenced by the physical state of gas–liquid flow through the fixed bed and by the associated mass-transfer processes. The first application of this system is the trickling biological filter used for wastewater treatment. A similar system has been used for biological oxidation of ethanol to acetic acid (vinegar).

Reactors for immobilized enzymes or cells Among the newer bioreactors are immobilized enzyme or cell reactors. Immobilized biocatalysts are enzymes – cells and organelles, bound individually or in combination – that have been confined to allow their continuous reuse. Both physical and chemical techniques are used to immobilize enzymes or cells. Physical techniques tend to be less expensive and less reliable. Bioreactors for use with immobilized enzymes or cells must be constructed in such a way that the rate of movement between substrate and the biocatalyst is not rate-limiting.

Before considering methods to achieve enzyme or cell immobilization and some of the experiments conducted to date with immobilized systems, we will review some of the reasons for immobilization. When enzymes are present in solution, some enzymes will leave the reactors with the final product. This means that not only the new enzymes must be introduced to replace the lost ones, but also that enzymes must be removed from the product because they constitute undesirable impurities. Also, immobilized enzymes may retain their activity longer than those that are in solution. Most enzymes are labile under normal operating conditions, having only a very limited life. An immobilized enzyme may be fixed in position near other enzymes that are participating in a catalytic sequence, thereby increasing the catalyst efficiency for a multistep conversion.

The cost of isolating and purifying intracellular enzymes for commercial processes can so dampen profitability that their use becomes unattractive. However, advantages of isolated enzymes must be balanced against cost, depending on the nature of the conversion process. Isolated enzymes offer greater purity, which can yield higher conversion of products that are less contaminated by miscellaneous materials. Immobilizing whole cells should be considered when the extracted enzyme is unstable. Many industrial applications using continuous enzyme reactions are now carried out using immobilized microbial cells. Immobilized cells are limited in their diffusion through the cell membrane. The presence of many enzymes in the cells may also lead to side reactions, but these problems can be solved, and the enzyme systems within the microorganism can be efficiently utilized.

Reactions by immobilized cells are advantageous under the following circumstances:

- When enzymes are intracellular
- When enzymes extracted from cells are unstable during and after immobilization
- When the microbe contains no interfering enzymes (or the interfering enzymes can be readily inactivated)
- When the substrates and products are low-molecular-weight compounds

One important advantage of cell immobilization is that it gets around the washout problem of chemostat culture, providing much higher dilution rates and volumetric productivities than would otherwise be obtainable. Additional advantages are listed in Table 1.8.

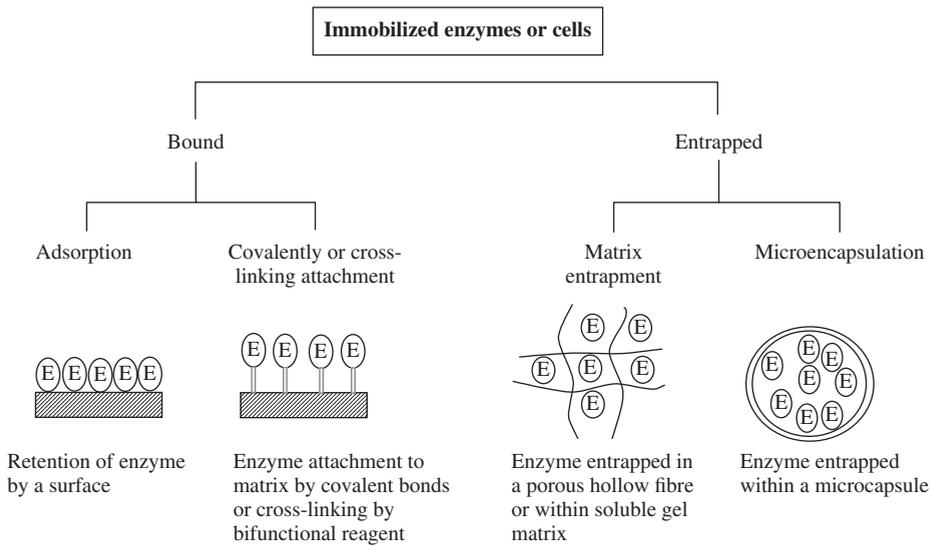


Figure 1.11 Two basic immobilization methods.

No ideal, general methods that are applicable to the immobilization of enzymes or cells of all types have been developed. Thus, it is necessary to choose suitable methods and conditions for immobilization of each system. Toxicity of immobilization reagents should also be considered in connection with the process of waste disposal. Resistance of cell wall and cell membrane to substrate and product transport would constitute a considerable shortcoming of cell immobilization.

New methods of coimmobilization of cells and enzymes are also available. The dried cells are resuspended in an aqueous solution of the enzyme and microbial cells, which are coentrapped by alginate gel formed with Ca^{2+} ions or enzyme molecules. They are covalently bound on alginates by means of carbodiimides or glutaraldehyde. Also, immobilized whole cells have been permeabilized by lytic enzymes, alcohols, or dimethyl sulfoxide, and some are now in commercial use. Figure 1.11 summarizes two basic types of immobilization methods. Basically, there are two types: those in which the catalyst is bound to a variety of carriers by adsorption or covalent attachment, and those in which the enzyme or cell is entrapped physically in a matrix or a membrane (Table 1.10).

Immobilization methods In adsorption processes, the enzyme is attached to surface-active materials such as alumina, activated carbon, clay, glass, cellulose and ion exchange resins, as shown in Table 1.11. Even for these so-called inert carriers, the binding mechanism is not simple and probably involves a combination of van der Waals (VDW) ionic and hydrogen bondings. Ion exchange resins have been shown to result in improved activity and stability characteristics. Elution of the enzyme still occurs, but it can be reduced by altering the pH, temperature, and ionic strength. The first commercially successful immobilized enzyme was prepared by adsorption, and this approach still offers advantages for large-scale industrial applications. Adhesion of cells to anion exchange resins has also been used to immobilize whole cells. One of the first attempts to immobilize cells involved *E. coli* and *Azotobacter agile*.

Table 1.10 Advantages of immobilized enzymes and cells

Enzymes	Whole cells
1. Reuse of the enzyme, which permits continuous operation	1. Reuse of the cell, which permits continuous operation
2. Increased stability	2. Unnecessary for enzyme extraction or purification
3. Enhancement of enzyme kinetic properties; greater control of catalytic process	3. Maintains intact enzyme activity and less sensitivity to changes in operating conditions
4. Enzyme-free product	4. Lower costs
5. Lower costs for expensive enzymes	5. Use of multienzyme system, which requires many enzymes and cofactor renewal
6. Use of multienzyme system	6. New medical/industrial uses
7. New medical/industrial uses	7. Reduces plant pollution problem because of continuous process (smaller volume)
8. Use of enzymes from non-GRAS* microorganisms	

*GRAS: generally regarded as safe for the purposes of the US Food and Drug Administration.

Table 1.11 Some adsorbents for the immobilization of enzymes

Process	Adsorbents
Physical adsorption	Alumina, activated carbon, silica gel, starch, clay, glass, cellulose, bentonite, collagen, titania (transition metal oxide)
Ionic binding	Cation exchangers (carboxymethyl cellulose, Amberlite, Dowex 50) Anion exchangers (DEAE-cellulose, DEAE-Sephadex, Amberlite IR45, Agarose)

The *covalent binding method* is one of the most commonly used techniques for enzyme immobilization through inter- and intramolecular reactions with bifunctional agents, resulting in high operational stabilities. Because of the toxicity of the coupling agents involved, however, this process is not widely used for cells. Any reactive component of the cell surface (e.g., the amino, carboxyl, thiol, hydroxyl, imidazole, or phenol groups of proteins) can be used for linking. To introduce the covalent linkage, chemical modifications of the carriers (inorganic and organic) have been used. Silica and ceramics are the major inorganic carriers. Among coupling agents, the most important are glutaraldehyde, diimido esters, toluene diisocyanate, diamines, dithiols, *p*-nitrobenzyl chloride, and succinic anhydride. Such coatings can be derivatized to aldehyde groups using glutaraldehyde, to acrylamine groups using *p*-nitrobenzyl chloride, or to carboxyl groups using succinic anhydride.

A series of enzymes such as lactate dehydrogenase, trypsin, chymotrypsin, asparaginase and lysozyme, and aldolase and glycogen phosphorylase have been modified with glutaraldehyde. In the case of organic carriers *Micrococcus* cells are immobilized on the carboxyl groups of agarose beads in a two-step process that avoids exposure of the cells

to carbodiimide. These immobilized preparations are generally difficult to handle because they are gummy or gels. They present multiple problems of high pressure drops in a packed-bed reactor and poor fluidizing properties in a fluidized-bed reactor. The method of directly entrapping enzymes or cells into polymer matrices such as polyacrylamide gel, alginate gel, carrageenan, and photo-cross-linking resin have been used most extensively. Entrapment strategies can be divided into two forms: (i) the enzyme is embedded into a polymer network and (ii) the enzyme in solution is retained by a membrane that is permeable to substrates and products. Entrapment is probably the most popular method of immobilization of microbial cells.

In another immobilization method known as microencapsulation, enzymes are entrapped in small capsules ranging from 5 to 300 μm in diameter. The enzyme is too large to move through the pores of the semipermeable membrane, but smaller substrate and reaction product molecules can move back and forth. Two membranes, cellulose nitrate and nylon, are often used, but the most promising delivery system is *liposome encapsulation*. Since liposomes differ from solid, semipermeable microcapsules in that enzyme action occurs only upon disruption of the carrier, liposomes are actually delivery devices rather than true immobilization systems. Liposomes are lipid vesicles that contain the enzyme in an aqueous environment surrounded by one or more concentric lipid bilayers. Other forms of physical entrapment processing include the use of ultrafiltration or hollow fibers to immobilize enzyme within the walls of membranes porous enough to allow the fluid to be forced through the interface, avoiding transport limitation by diffusion. Any reactions that require multienzymes or coenzymes can be immobilized in this way.

Immobilized reactors and applications Several types of immobilized enzyme reactor are known for industrial enzymes in use or under development (Tables 1.12 and 1.13). Basic construction types for immobilized reactors are presented in Figure 1.12.

In the batch-stirred reactor (Figure 1.12a), the total amount of enzyme is normally exposed to high substrate concentrations, resulting in high product concentrations. This type of reactor is thus not suitable for reactions that are subject to substrate or product inhibition.

The *continuous stirred-tank reactor (CSTR)* (see Figure 1.12b) is started up as a batch reactor. As soon as a certain conversion has been reached, the substrate is pumped continuously into the reactor and the product is simultaneously pumped out of the reactor. This type of reactor, called a chemostat in the cultivation of cells, is not affected by pH shifts. Stirred reactors have the disadvantages of product inhibition and damage due to shearing of the catalyst.

In another type of system, the catalyst is immobilized in a bed; examples include packed-bed, fluidized-bed, and cyclonic reactors. In a *packed-bed (plug-flow or fixed) reactor* (Figure 1.12c), the substrate is converted into the product during upflow passage through a column packed with immobilized enzymes. This reactor adapts well to large-scale operation because there is a high rate of substrate conversion per unit time. Gas formation may reduce the contact surface between substrate and enzyme, however, and regulation of temperature and pH is difficult. The fluidized-bed reactor (Figure 1.12d) is theoretically attractive in terms of mass transfer. Because of the plug-flow nature of fluid movement, the immobilized enzyme particles are kept in suspension. The flow rate cannot be varied, and scale-up is quite difficult.

In *membrane reactors* (Figure 1.12e), the soluble or fixed enzymes and substrate are introduced on one side of the ultrafilter membrane. By means of a pump, the product is forced through the fiber membrane, representing a series of simple physical barriers that

Table 1.12 Applications of immobilized enzyme reactors in use and under development

Enzymes	Products	Reactors
Aminoacylase	Amino acids	Stirred tank/packed bed
α -Amylase (bacterial)	Dextrinization	Stirred tank/fluid bed
Endo/exonucleases	Nucleotides	Stirred tank/packed bed
Ficin	Soluble proteins	Packed bed (with cofactors)
Glucosylase	Saccharification	Packed bed
Galactosidase	Raffinose hydrolysate	Stirred tank
Glucose isomerase	Fructose syrups	Packed bed/fluid bed
Glucose oxidase/catalase	Food preservation	Stirred tank
	Soft drinks	Packed bed
	Gluconic acid	Packed bed
Hydrogenases	Hydrogen by photosynthesis	Stirred tank
Invertase	Sucrose inversion	Packed bed
Lactase	Milk, whey, lactose	Tubular/packed bed
Lipases	Fatty acids	Fluid bed
Papain	Soluble proteins	Packed bed
Pectin esterase	Juice clarification	Fluid bed
Protease (microbial)	Soluble proteins	Packed bed/fluid bed/stirred tank
Rennet	Milk coagulation	Packet bed/fluid bed/open surface
Steroid esterase	Steroid modification	Stirred tank
Sulfhydryl oxidase	Flavor control in heat-treated milk	Stirred tank
Tannase	Instant tea	Packed bed
Trypsin	Soluble protein	Packed bed/stirred tank

Table 1.13 Half-lives of some immobilized enzymes

Enzyme	Support	Substrate	Temperature (°C)	$t^{1/2}$ (days)
Lactase	Porous silica or titania	5% Lactose	50	100
		Whole acid whey	50	8
		Deionized whey	40	400
		Deproteinized whey	60	8
Lactase	Zirconia-coated glass	Lactose	30	44
			50	3
		Whey ultrafiltrate	40	25
Glucose isomerase	Zirconia-coated glass	Sweet whey	40	54
		2.8 M glucose	50	24
Glucose isomerase	Zirconia-coated glass	1 M fructose	70	3
			50	240
			60	11
			75	1.0
			80	0.1
L-Amino acid oxidase	Porous glass	L-Leucine	37	43
Alkaline phosphatase	Porous glass	<i>p</i> -Nitrophenyl phosphate	23	55
Papain	Zirconia-coated glass	Casein	45	35
Glucosylase	Zirconia-coated glass	Starch	40	900
			45	645
			50	100
			60	13

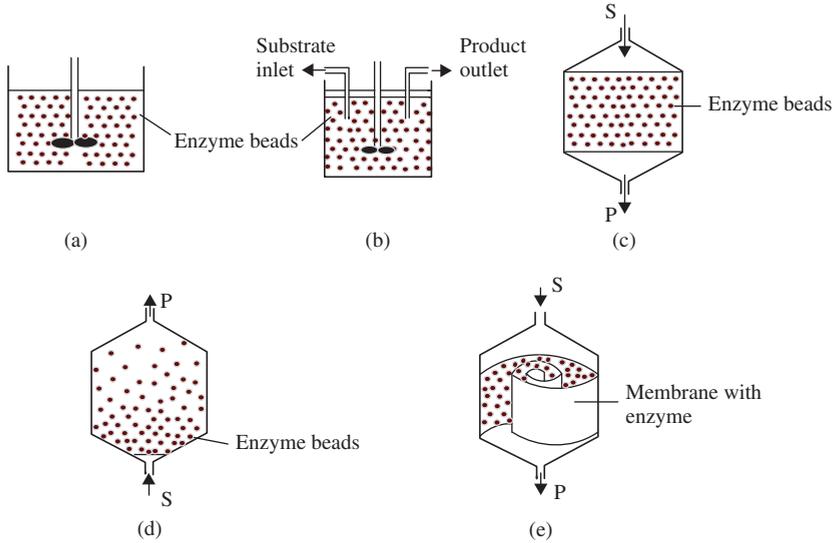


Figure 1.12 Basic immobilized enzyme or cell reactor types. (a) Batch-stirred reactor, (b) continuously fed and stirred tank reactor (CSTR), (c) packed bed or plug-flow reactor, (d) fluidized bed reactor, and (e) UF membrane/enzyme reactor.

retain macromolecules in the compartment under 0.5–5 atm pressure. Many of the commercial enzymes are depolymerases such as amylases and proteases. Membrane circulating bioreactors for depolymerases have thus many advantages and probably will be the reactors of choice for many applications. For high molecular weight substrate such as starch, a tubular reactor is recommended.

Various applications of *ultrafiltration* and *hollow fiber* systems such as the bioconversion of cheese whey into organic acids (lactate, propionate, etc.) have been well known. The main disadvantages of membrane systems are shear stress from the liquid flow, membrane adsorption of the enzyme, and fouling of the membrane, which is manifested by a progressive reduction in the permeation rate (i.e., the polarization phenomenon) and consequently limits overall reactor productivity. Many different geometries for immobilized cell reactors have also been explored, but the configurations are essentially the same as that of the enzyme reactors.

Some examples of applications of immobilized cells for the production of useful chemicals and foods are listed in Table 1.14. Here, the immobilized cell essentially serves as an immobilized enzyme catalyst, with the entire cell used to minimize treatment and processing costs involved in enzyme preparation. Immobilized enzymes have also made other important contributions to medical and analytical applications. *Immobilized enzyme electrodes* have now been constructed for many biologically important compounds (Table 1.15). An enzyme-based electrode consists of an electrochemical sensor (electrode) in contact with a semipermeable membrane of an immobilized enzyme. The enzyme acts on the substrate, and changes of substrate consumption, product formation, or cofactor concentration can be measured by means of spectrophotometer, polarimeter, and photometer. Enzyme thermistors (e.g., a palladium-coated semiconductor device) can also be used to measure ion species.

Table 1.14 Application and stability of various immobilized cells

Product	Microorganisms	Half-life (days)*	Scale
Aspartic acid	<i>E. coli</i>	120 [†] 680 [‡]	Industrial (1978) –
Malic acid	<i>Brevibacterium flavum</i>	52.5	Commercial (1977)
Citric acid	<i>Aspergillus niger</i>	–	–
Glucose	<i>Actinomyces missouriensis</i>	45 [†]	–
Fructose	–	280 [‡]	–
Ethanol	<i>Kluyveromyces maxilans</i>	15	Pilot scale
	<i>Saccharomyces cerevisiae</i>	10	Pilot scale
Beer	<i>Saccharomyces cerevisiae</i>	–	Commercial (Japan)
Lactic acid (from whey)	<i>Lactobacillus helveticus</i>	–	–
Propionic acid (from whey)	<i>Propionibacterium shermanii</i>	–	–
Penicillin	<i>Penicillium chrysogenum</i>	–	–
6-Amino-penicillanic acid	<i>E. coli</i>	42	–

*The enzyme activity involved in bioconversion is half its original value at optimum conditions (pH and temperature).

[†]Polyacrylamide.

[‡]Carrageenan.

Table 1.15 Examples of enzyme electrodes

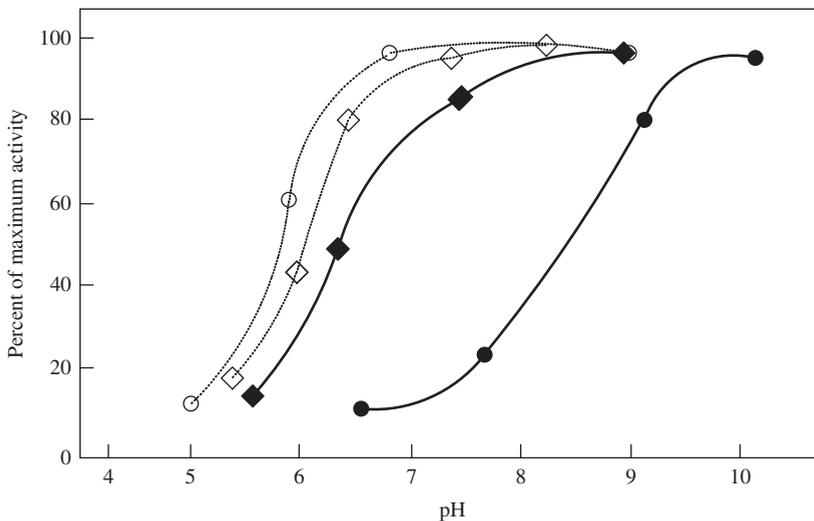
Ion species	pH range for direct measurement	Concentration range (mM)
Ammonia	11–13	10^{-3} to 10^{-9}
Calcium	6–8	10^{-3} to 10^{-8}
Carbon dioxide	5	10^{-5} to 10^{-7}
Chloride	2–11	10^{-2} to 8×10^{-9}
Magnesium	–	10^{-7}
Nitrate	3–10	10^{-2} to 6×10^{-7}
Nitrate	0–2	10^{-5} to 5×10^{-10}
Potassium	3–10	10^{-3} to 10^{-9}
Sodium	9–10	10^{-3} to 10^{-9}
Phosphate	–	5×10^{-6} (min)
Sulfate	–	5×10^{-6} (min)
Metabolite	Immobilized enzyme(s)	Concentration range (mM)
Cellobiose	β -Glucosidase + glucose oxidase + catalase	0.05–5
Creatinine	Creatinine iminohydrolase	0.01–10
Ethanol	Alcohol oxidase + catalase	0.01–2
Glucose	Glucose oxidase + catalase	0.002–0.8
Lactate	Lactate 2-monooxygenase	0.005–2
Oxalic acid	Oxalate oxidase	0.005–0.5
Penicillin	β -Lactamase	0.01–500
Sucrose	Invertase	0.05–100
Triglycerides	Lipoprotein lipase	0.1–5
Urea	Urease	0.01–500

Table 1.16 Comparison of K_m and activation energy (kcal/g-mol) values of some soluble and immobilized enzymes

Enzyme	K_m (mM)*		Activation energy (kcal/g-mol) [†]	
	Soluble	Immobilized	Soluble	Immobilized
Glucoamylase	1.22	0.30	16.3	13.8
Alkaline phosphatase	0.10	2.90	–	–
Glucose oxidase	7.70	6.80	6.6	9.0
Papain	–	–	13.8	11.0
Lactase*	13.1	22.1	10.5	11.3

*Values from *Aspergillus niger* source.[†]Values from *Kluyveromyces maxilans*.

The same type of enzyme deactivation and reaction parameter changes that occur in soluble enzyme are likely to influence immobilized enzymes. Soluble and immobilized enzymes often show the differences in kinetic constants that can be attributed to diffusion influences rather than to changes in intrinsic kinetics (Table 1.16). Stability and activity optima of immobilized enzymes are also rarely the same as soluble enzymes. In engineering, *optimal* has an economic implication that has rarely been applied to immobilized enzymes. Whenever a charged substrate reacts with an enzyme attached to a charged surface, the pH *optimum* for immobilized enzyme, for example, trypsin, was shifted down 3 full pH units from that of the soluble enzyme. High ionic strength tends to diminish these charge-interaction effects and brings the curve together (Figure 1.13). Charge-interaction effects can thus be made to work for the biotechnologist if they are

**Figure 1.13** pH activity curves for trypsin (dotted) and trypsin covalently bound to ethylene-maleic acid copolymer (solid) at ionic strength of 0.06 (circles) and 1.0 (squares). Benzoylarginine ethyl ester is the substrate.

properly understood and anticipated. Further discussions on immobilized biosensors are also found in Section 1.11.

1.7.3 Mass transfer concept

There are many situations in bioprocessing where concentrations of compounds are not uniform; we rely on the mechanisms of mass transfer to transport material from regions of high concentration to regions where the concentration is low. An example is the supply of oxygen in fermenters for aerobic culture. Concentration of oxygen at the surface of air bubbles is high compared with the rest of the fluid; this concentration gradient promotes oxygen transfer from the bubbles into the medium. Another example of mass transfer is extraction of penicillin from fermentation liquor using organic solvents such as butyl acetate. When solvent is added to the broth, the relatively low concentration of penicillin in the organic phase causes mass transfer of penicillin into the solvent. Solvent extraction is an efficient downstream-processing technique as it selectively removes the desired product from the rest of the fermentation fluid.

Mass transfer is the most critical factors in the operation of a large-scale bioreactor. These are caused by *bulk flow (convection)* as well as *conduction (diffusion)* processes. For the ultimate supply of nutrients or other reactants to cells or cell products such as enzymes, diffusion processes dominate. Fick's law of diffusion states that the mass flux is proportional to the concentration gradient, that is,

$$\begin{aligned} JA \text{ (moles/cm}^2\text{/s)} &= -D_A \frac{dCA}{dX} \\ &= (\text{cm}^2\text{/s})(\text{moles/cm}^3\text{/cm}) \end{aligned}$$

where the diffusivity D_A depends on temperature by the Arrhenius equation ($D_A = D_{A_0} e^{-E/RT}$). Diffusivities for typical biological reactants range from about 10^{-4} cm²/s (small molecules) to 10^{-8} cm²/s (macromolecules) in aqueous solution.

Three mass-transfer situations that occur in bioprocessing are liquid–solid mass transfer, liquid–liquid mass transfer between immiscible solvents and gas–liquid mass transfer. The overall rate of microbial growth or product formation is governed by one of many mass transfer or reaction rates, including transfer of reactant from bulk fluid to surface of microorganism, transfer across the cell membrane, transfer within the cell or organelle, and reaction within the cell or organelle. In analyzing these systems involving mass transfer, recall that events in series are governed by the slowest step, while the overall rate of processes that run in parallel are governed by the fastest rate step. Since many of the systems of interest to biologists and biochemical engineers consist of processes occurring in series, the concept of the rate-limiting step becomes particularly important.

When oxygen is required as a microbial gaseous substrate, it is frequently a limiting factor in fermentation. This is because of the very low solubility in water of oxygen as compared with other substrates and metabolites. The solubility of oxygen is only approximately 0.01 g/L (Table 1.17). Moreover, the influence of the culture medium ingredients causes the maximal oxygen content to be lower than it would be in pure water. A rapidly respiring culture will use all the oxygen available in solution within a few seconds, and *oxygen transfer* therefore has been a key problem of biochemical engineers concerned with bioreactor design. The oxygen requirement of an aerobic culture is controlled by the cell density in the reactor, the cell growth rate, and the approximate yield coefficient. The yield coefficient Y_{ATP} is the mass of cells formed per mole of ATP consumed for biosynthesis.

Table 1.17 Solubilities of substrates and metabolites in water at 25 °C

Substance	Solubility (g/L)
Alanine	147.0
L-Asparagine	24.6
Glucose	590.0
Glycine	21.7
Lactose	170.0
L-Leucine	22.4
Sucrose	909.0
Urea	620.0
DL-Valine	66.8
Oxygen	0.01

Table 1.18 Effect of substrate and cell yield on oxygen requirement and heat production

Microorganisms	Substrates	Cell yield, Y_s (g cell/g substrate)	Oxygen required, Y_{O_2} (g cell/g O_2)	Heat released, Y (g cell/kcal)
Bacteria	<i>n</i> -Alkanes	1.0	0.58	0.13
Yeasts	Carbohydrates	0.5	1.49	0.26
Yeasts	<i>n</i> -Alkanes	1.0	0.51	0.13

The amount of oxygen required is strongly influenced by how highly reduced the carbon substrate source is. From Table 1.18 it is seen that up to 200 g of oxygen may be required for yeast fermentation on *n*-alkane to produce 100 g of cells. Production of 100 g of cells per liter over the course of a fermentation might thus result in the replacement of the oxygen at the rate of 200 g/L per 0.01 g/L or 20,000 times. Obviously predicting oxygen transfer rates can be very important. Hence, aerobic microbial processes frequently operate under conditions of oxygen limitation; thus, there are reductions in productivity, and the costs incurred for oxygen transfer comprise a significant fraction of total operating expenditure.

The solubility of a gas in a liquid has a limited and definite value, which depends on the nature of both the gas and the liquid and on the temperature and pressure of the system. When other factors remain constant, an increase in temperature usually results in a decrease in solubility, while an increase in pressure (or partial pressure) results in an increase in solubility.

The quantitative relationship between solubility and pressure is known as Henry's law. Henry's law describes the solubility of O_2 in nutrient solution in relation to the O_2 partial pressure in the gas phase,

$$CO_2 = \frac{P_{O_2}}{H_{O_2}}$$

where CO_2 is the DO concentration, P_{O_2} is the partial pressure of oxygen in air and H_{O_2} is the Henry's law constant for the system at the appropriate temperature. As the oxygen concentration increases in the gas phase, the oxygen proportion of the nutrient solution apparently increases. The general nature of the mass transfer problem of primary concern

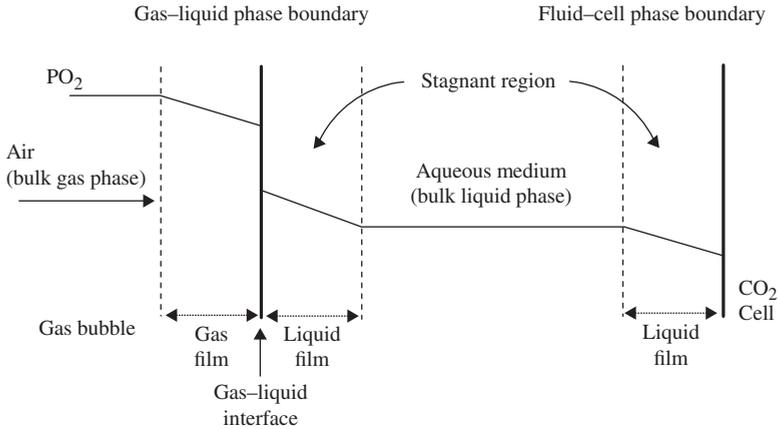


Figure 1.14 Resistance to oxygen transfer from the air bubble to a microbial cell based on the Lewis-Whitman model.

is frequently considered in the two-film model of Lewis and Whitman. A sparingly soluble gas, usually oxygen, is transferred from a source, rising through bubbles into a liquid phase containing cells. As shown in Figure 1.14, the oxygen must pass through several independent and different combinations of partial resistances: (i) resistance within the gas film to the phase boundary, (ii) penetration of the phase boundary between gas bubble and liquid, (iii) transfer from the phase boundary to the liquid, (iv) movement within the nutrient solute, and (v) transfer to the surface of the cell.

The *Lewis and Whitman model* assumes that at a gas-liquid interface, both the gas and the liquid that comprise the thin film immediately adjacent to the interface are essentially stagnant, while both the bulk gas and bulk liquid phases are in turbulent motion. Absorption results from steady state molecular diffusion processes in the two stagnant films. For fermentations carried out with single-celled microbes, the resistance in the phase boundary between gas bubble and liquid is the most important factor controlling the rate of transfer.

For most aerobic bioreactors, oxygen is transferred from air bubbles dispersed throughout the liquid medium in which the microbes are either growing or respiring. It becomes necessary to assess the rate of oxygen transfer on a unit volume basis. Oxygen transfer rates are generally predicted by

$$N_A = K_L a (C^* - C_L)$$

where N_A = volumetric O_2 transfer rate (mM O_2 /L/h)
 K_L = mass transfer coefficient at the phase boundary
 a = specific gas-liquid interfacial area (specific exchange surface)
 $K_L a$ = volumetric oxygen transfer coefficient (h^{-1})
 C^* = equilibrium oxygen, concentration
 C_L = DO concentration (mM/L)

The dispersion of gas bubbles in liquids is a complex process. Predicting oxygen transfer generally reduces to finding $K_L a$. This value is dependent on many variable factors (e.g., diameter, capacity, power, aeration system, aeration rate of the bioreactors) and on the density, viscosity, and composition of the nutrient, as well as the structure of the microbes,

the antifoam agent used, and the temperature. Obviously, to enhance oxygen transfer by increasing a , it is necessary either to increase gas hold-up, which is known as volume gas per volume reactor, or to reduce mean bubble diameter. The presence of dissolved inorganic and organic matter in aqueous systems markedly affects bubble size, either by influencing the coalescence characteristics or by changing the interfacial tension.

Surface-active agents such as *antiform compounds* reduce the value of $K_L a$ and cease the renewal of the bubble surface. Microorganisms themselves have a significant effect on the oxygen transfer by acting as a barrier. DO concentrations drop rapidly in the slime layer, approaching zero within 100 μm of the air–liquid interface; in other words, the film becomes anaerobic at those depths. It would certainly be useful to predict the conditions under which this will occur – that is, the conditions that will prevent the diffusion of oxygen into a region consuming oxygen (e.g., the slime layer) from keeping pace with the oxygen consumption rate. Since these processes occur in series, they are governed by the slowest step. In the design of aerobic bioreactors, we frequently use correlations of data more or less approximating the situation of interest to establish whether the slowest process step is the oxygen transfer rate or the rate of cellular utilization of oxygen (or other limiting substrate).

The maximum possible oxygen utilization rate is $X_{\mu_{\max}}/Y_{\text{O}_2}$, where X is cell density and Y_{O_2} is the ratio of moles of cell carbon formed per mole of oxygen consumed. We assume that all oxygen entering the bulk solution is rapidly consumed ($Cl = 0$) in this equation. If $K_L a Cl$ is much larger than $X_{\mu_{\max}}/Y_{\text{O}_2}$, the main resistance to increased oxygen consumption is microbial metabolism and the reaction is biochemically limited. Conversely, the reverse seems to be true in the mass transfer–limited mode. With unicellular bacteria, the O_2 absorption rate is constant during log growth until another substrate becomes limiting. However, in mycelial (streptomycetal and fungal) fermentations, the O_2 absorption rate decreases when O_2 becomes limiting as a result of increases of mycelium volume and viscosity.

Critical oxygen concentration ($\text{CO}_{2\text{cr}}$) is the value of the specific oxygen absorption rate that permits respiration without hindrance. The critical oxygen values for microorganisms lie in the range of 0.003–0.05 mmol/L. For the higher critical oxygen values (e.g., 0.02 mmol/L for *Penicillium* molds), oxygen mass transfer is evidently extremely important. The more important factors that can influence the total microbial oxygen demand (X_{μ}/Y_{O_2}) are cell species, culture growth phase, carbon nutrients, pH, substrate utilization, and biomass yield.

We have mentioned that oxygen utilization for growth is typically coupled directly to the amount of carbon source substrate consumed. Furthermore, more reduced substrates such as paraffins and methane require greater oxygen uptake by the cell than substrates such as glucose, which have approximately the same carbon oxidation state as the cell. There are four acceptable methods of determining oxygen transfer rate: the dynamic gassing-out method, the sulfite method, direct measurement of the volumetric O_2 transfer rate, and calculation from measurements of microbial growth.

1.7.4 Heat transfer concept

Energy balances allow us to determine the heating and cooling requirements of fermenters and enzyme reactors. Once the rate of heat transfer for a particular purpose is known, the surface area required to achieve this rate can be calculated using design equations.

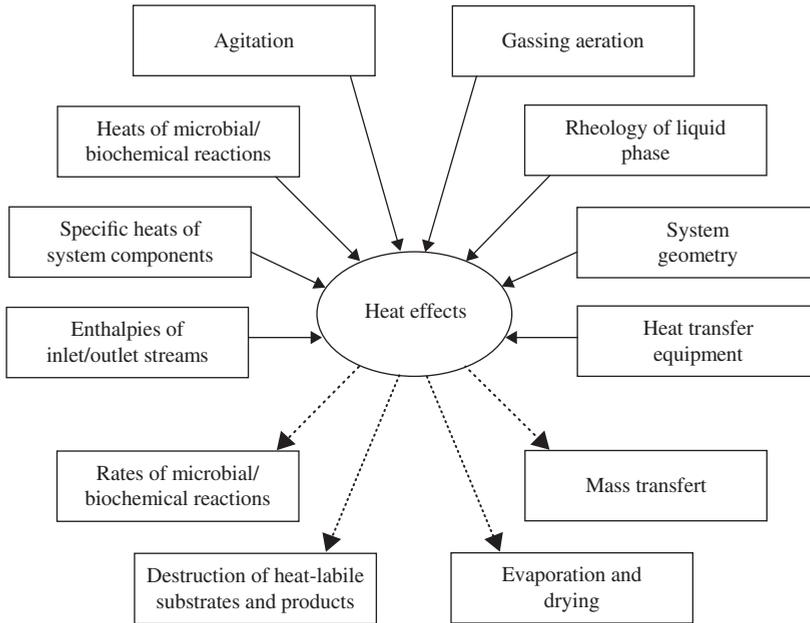


Figure 1.15 Factors contributing to and phenomena affected by heat effects in biological reactors. *Source:* Redrawn from Atkinson and Mavittura 1991.

Estimating the heat-transfer surface area is a central objective in design as this parameter determines the size of heat-exchange equipment.

The principles governing heat transfer are outlined with applications in the bioprocess design. Heat transfer is required to (i) sterilize a liquid reactor feed in a batch or continuous flow vessel (the temperature desired must be high enough to kill essentially all microorganisms in the total holding time), (ii) add heat to promote a desired reaction (as in an anaerobic sewage sludge digester operating between 55 and 60 °C), (iii) remove excess heat generated by most microbial fermentation processes, and (iv) concentrate microbial products by drying. The first three requirements relate to cell viability and metabolism, and we will emphasize (i) and (ii). The fourth is a unit operation, drying, which is covered on engineering unit operations.

Figure 1.15 displays some important factors contributing to heat transfer requirements and phenomena affected by heat production in biological systems.

These heat effects are accounted for in an energy balance equation. In a constant pressure system with negligible changes in potential and kinetic energies, the energy balance can be expressed in terms of changes in *enthalpy* (ΔH) (i.e., the heats of chemical transformation) or phase transformation (e.g., evaporation, condensation), the sensible heat flow in mass streams, and the heat transfer to or from second fluids acting as heating or cooling devices, as follows:

$$Q_{\text{met}} + Q_{\text{ag}} + Q_{\text{gas}} = Q_{\text{acc}} + Q_{\text{exch}} + Q_{\text{evap}} + Q_{\text{sen}}$$

where

- Q_{met} = heat generation rate from cell growth and maintenance
- Q_{ag} = heat generation rate due to mechanical agitation
- Q_{gas} = heat generation rate from aeration power input
- Q_{acc} = heat accumulation rate per unit volume
- Q_{exch} = heat transfer rate to the surroundings or to heat exchanger coolant
- Q_{evap} = heat loss rate by evaporation
- Q_{sen} = sensible enthalpy gain rate of streams (exit–entrance)

The rate of heat production due to agitation and the metabolic activity of the microorganisms must be balanced by the heat loss resulting from evaporation and radiation plus heat removal by the cooling system (the jacket of the fermentor or cooling coils). Such a balance can be used to calculate Q_{met} from measurements of Q_{acc} through monitoring the initial transient temperature rise of a nearly isolated fermentor. Viscous heating and metabolic heat during microbial growth are clearly the dominant factors that can lead to heat accumulation. This heat accumulation can easily lead to problems for the fermentation. Not all fermentations are exothermic, but sometimes ΔH is endothermic, which simplifies fermentation control because the heat-producing effect of agitation tends to be balanced by the heat of fermentation for endothermic systems. Not only may agitation lead to heat accumulation, but sensitive microorganisms (either filamentous or flocculating microbes) may be damaged by the impeller. Impeller tip speed ND (rpm \times impeller diameter) seems to be the key factor, where N is the rotational speed of the impeller and D is the impeller diameter.

Heat production is strongly related to the degree of reduction of the substrate. In general, hydrocarbons produce more heat than partially oxidized substrates ($Y_{\Delta}(\text{CH}_4) < Y_{\Delta}(\text{CH}_3\text{OH}) < Y_{\Delta}(n\text{-alkanes}) < Y_{\Delta}(\text{glucose})$). The yield coefficient Y_{Δ} is given in grams of cells per kilocalorie of energy released, Y_s indicates the ratio of grams of cells per gram of substrate utilized. At a yield coefficient of 0.5, four times as much heat is produced by cells growing on methane as by cells produced on methanol, a less highly reduced substrate (Figure 1.16). Y_{Δ} values also increase linearly in relation to μ , the specific growth rate of the cultures. Because of the “combustion” aspect of microbial metabolism in aerobic fermentations, the heat evolution rate correlates very well with the oxygen consumption rate for a number of microorganisms (Table 1.19). This is one important coupling between mass and heat transfer in biological systems.

Heat transfer problems that are insignificant during laboratory work may become very significant upon scale-up because heat production, which is proportional to volume of the fermentation fluid, increases more rapidly than the surface area available for jacketed heat transfer. The exchange surface available for removal of heat from the unit volume of culture decreases with scale, and this sets a maximum limit to the fermentor size. However, fermentors with internal cooling can be sized appropriately using such surface-to-volume considerations.

The heat production by power input also varies somewhat with the scale of the fermentation. Limitations on the ratio of surface area to volume can be reduced or eliminated by use of external heat exchangers. This strategy is aided by the batchwise operation of many fermentations, which mean that their heat production rates follow the batch mode. An example is provided by ethanol fermentation, in which heat release peaks at about 8 h into the fermentation and is largely complete within about 24 h. Why build an exchanger to handle the peak? Why not have two or more fermentors share a common external heat exchanger instead? If two large ethanol fermentors have a common external heat exchanger, savings in operating and capital costs should be substantial. The single

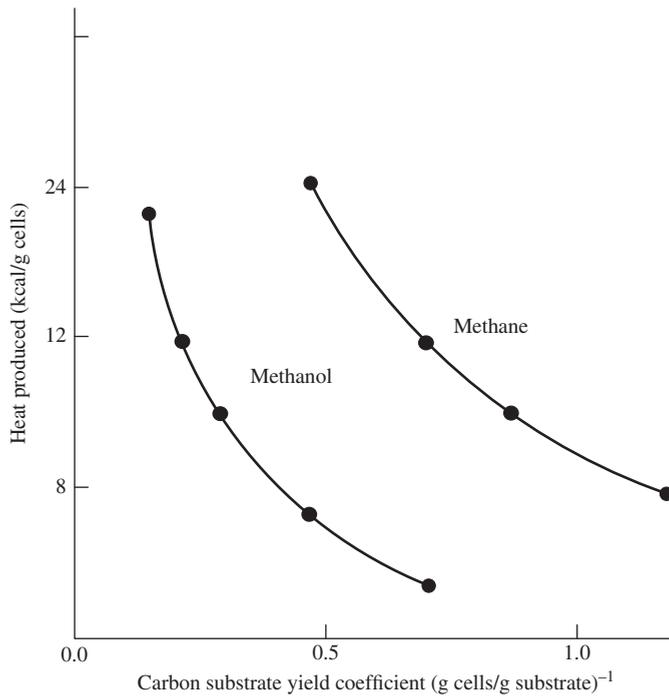


Figure 1.16 Correlation of heat production Y_{Δ} and biomass yield Y_s on different substrates.

Table 1.19 Heat production during the continuous culture of microorganisms using different substrates

Substrate	Y_s (g cell/g substrates)	Y_{O_2} (g cell/g O_2 consumed)	Y (g cell/kcal)
Acetate	0.36	0.70	0.21
Glucose	0.51	1.47	0.42
Maleate	0.34	1.02	0.30
Ethanol	0.68	0.61	0.18
Methanol	0.40	0.44	0.12
Isopropyl alcohol	0.43	0.23	0.074
<i>n</i> -Alkanes (C12–C18)	1.00	0.50	0.16
Methane	0.62	0.20	0.061

Source: Bailey, JE and Ollis, DF 1986. Biochemical Engineering Fundamentals (2nd Ed.). Reproduced with permission of McGraw Hill.

Table 1.20 Estimated operating costs for biomass production from different substrates

Substrate	Cost (\$/lb cells)			Total (\$/lb cells)
	Substrate	Oxygen transfer	Heat removal	
Acetate	0.167	0.0062	0.011	0.184
Alkanes	0.040	0.0097	0.014	0.054
Ethanol	0.088	0.0075	0.013	0.109
Glucose equivalent (molasses)	0.039	0.0023	0.0054	0.047
Maleate (as waste)	0.000	0.0046	0.0075	0.012
Methane	0.016	0.033	0.037	0.086
Methanol	0.050	0.012	0.019	0.081
Isopropyl alcohol	0.116	0.027	0.031	0.174

Source: Bailey, JE and Ollis, DF 1986. *Biochemical Engineering Fundamentals* (2nd Ed.). Reproduced with permission of McGraw Hill.

exchanger cannot service both fermentors at the same time, however. Thus, at about 24 h the flow is switched and the temperature in the first fermentor is allowed to rise slightly over the remainder of the fermentation. From the attention that mass transfer (especially oxygen transfer) and heat transfer have received in the literature, it is easy to assume that such processes must be economically dominant. More often than not, however, these costs will be important but not dominant. Substrate costs tend to dominate all other fermentation costs (Table 1.20).

1.7.5 Mass and heat transfer practice

Mass transfer and heat transfer equations are beyond the subject in this short chapter, and thus several books and references cited are suggested to read. The mass transfer theory finds applications in biotechnology in many product recovery areas. For the production of value-added products, it is impossible for the central production step to exist in isolation. The concentration of the desired product in the fermentation broths will be relatively low. Fermentation broths are complex aqueous mixtures of cells, soluble extracellular or intracellular products, and unconverted substrate. The recovery and purification of the product from the numerous undesirable contaminants which may be quite similar in size, charge, and molecular structure, may be considered to be one of the most critical aspects of industrial fermentation process. The principles referred to this section are applicable to the recovery of microbial, plant, or animal products. Product recovery is a largely undeveloped field for many of the more sensitive biological materials, especially for rDNA products. Eukaryotic gene products in prokaryotic organisms are often formed in the forms of highly insoluble inclusion bodies (IBs), frequently in inactive forms held together by disulfide bridges. Such IBs must be released, purified, and eventually converted into active proteins.

In most large-scale processes, the desired product is a metabolite that is present either intracellularly or extracellularly. *Intracellular metabolites* include nucleic acids, vitamins, enzymes, and certain antibiotics, while *extracellular metabolites* are amino acids, organic acids, alcohols, some enzymes, and most antibiotics. In either case, the product must be recovered from a solution in which a substantial amount of undesirable compounds are present. A typical unit operations approach to bioseparations can be conveniently divided into the following groups (Figures 1.17 and 1.18): cell separation/disruption, primary isolation, purification, and finishing operations such as drying, storage, and analysis. Unique

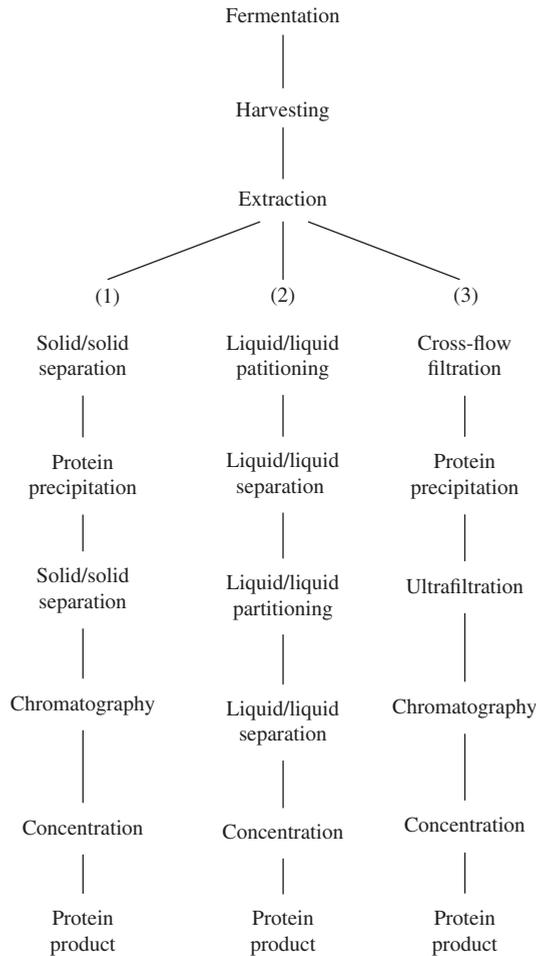


Figure 1.17 General schemes of protein recovery operation through sequences for solid–solid separation, liquid–liquid partitioning, and cross-flow filtration.

characteristics of biological fluids related to separation problems are (i) slimy gelatinous, viscous, non-Newtonian flows, caused often by the biopolymers secreted, (ii) poor filterability, poor sedimentation, and high water retention, and (iii) poor thermal stability and/or poor chemical stability as well as unfavorable forming and emulsification tendencies, hence poor mass and heat transfer properties. Fluids for which the viscosity is not a function of the rate of shear are referred to as *Newtonian fluids*, while many liquids found in bioprocesses do not exhibit this behavior and are referred to as *non-Newtonian fluids*.

Particles and molecules may be separated from a solution based on their differences in size, density, solubility, and diffusivity, which are the physicochemical properties. The size of a bacterial cell ranges from about 0.2–5 μm , and the specific gravity is very small, which makes separation extremely difficult. Separation methods include flocculation (or flotation), filtration, ultrafiltration and centrifugation (or ultracentrifugation). If an intracellular

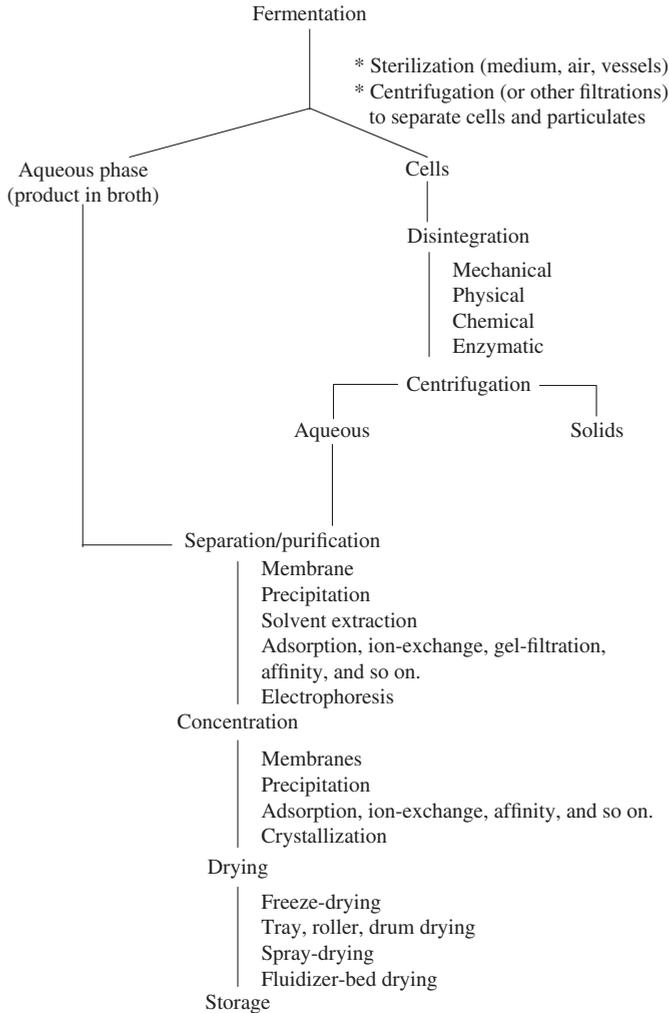


Figure 1.18 A process flow sheet for product recovery.

metabolite is to be isolated, it must be liberated from the cells by disintegration or direct extraction techniques before centrifugation. *Flocculation* is used to produce large aggregates, which settle more readily. Flocculating agents such as inorganic salts, organic poly-electrolytes or mineral hydrocolloids are often added. The flocculation of cells depends on many different factors, such as temperature, ionic strength, age of cells, surface force, and type of organism. Flotation is most readily accomplished by introducing gas bubbles into the liquid, whereupon the cells become adsorbed and rise to the layer that forms at the top, which can be removed.

Preliminary isolation can take the form of solvent extraction, sorption, precipitation or ultrafiltration. During primary separation, the concentration of the desired product increases significantly from that of the crude preparations. *Purification* usually includes

a series of adsorption/desorption, or chromatography columns for impurity removal as well as further product concentration. In finishing operations, the final volume is further reduced by centrifugation and subsequent drying of the crystallized product. Drying should not be so harsh that it damages the product, and storage conditions especially must take into account the special properties of biological matters before packaging. Analysis is also necessary not only at the end but also at various points along the way for purity requirements and bioassay.

Cell separation In the first step in process recovery, separation of cells (and solid particles) from a large volume of the culture broth is normally carried out by filtration or centrifugation. The principal separation methods based on physicochemical properties of particles and molecules are shown in Figure 1.18. Small fermentation batches can be filtered through a *plate-and-frame filter* (i.e., filter press) in which stacked flat, porous plates are used as supports for a filter (either cloth or membrane). Large samples rely on continuous filters mounted on the *rotary drum vacuum filters*, which in some cases require precoating (e.g., with diatomaceous earth) and a string or knife blade to scrape the cake from the drum. Two types of membrane filtration process – static and cross-flow – are commonly used for the convective transport of dissolved particles to the membrane surface. Under the influence of hydrostatic pressure, certain particles pass through the membrane, while other particles are retained at the membrane. The cross-flow method can reduce the tendency to clog, and there is a hundredfold increase in filtration rate compared with static flow. Three major types of filtration process are known, depending on the sizes of the particles being filtered; these are reverse osmosis, ultrafiltration and microfiltration. *Reverse osmosis* normally separates particles in the range of 0.0001–0.0001 μm or molecular weight cutoff less than 1000, while *ultrafiltration* is used for particles of 0.001–0.1 μm or molecular weight cutoff greater than 1000, and *microfiltration* serves for particles of 0.02–10 μm (Table 1.21). Ultrafiltration thus can separate compounds with molecular weights from 103 to 106 Da. However, both size (molecular weight) and shape are important, particularly for branched polymers such as polysaccharides (Figure 1.19).

Molecular diameter is also a key variable for ultrafiltration. Ultrafiltration is claimed to offer economic advantages over vacuum filtration due to the clarification it provides to the

Table 1.21 Flow characteristics of three types of pressure-driven membrane process: reverse osmosis (RO), ultrafiltration (UF), and microfiltration (MF)

Characteristics	Process RO	UF	MF
Separation range (μm)	0.0001–0.001	0.001–0.1	0.02–10
MW cutoff range (Da)	$<10^3$	$>10^3$ to 10^6	$>10^5$
Retentate	Salts, macromolecules, microorganisms	Macromolecules, microorganisms	Microorganisms
Pressure (psi)	250–1000	10–100	10–100
Membranes	–	–	Cellulose esters, polyvinyl fluorides, polycarbonate, polysulfones, cellulose
Modules	–	–	Cassettes, spiral-wound bundles of tubes (1–2 cm diameter), or capillary bundles

Separation principle

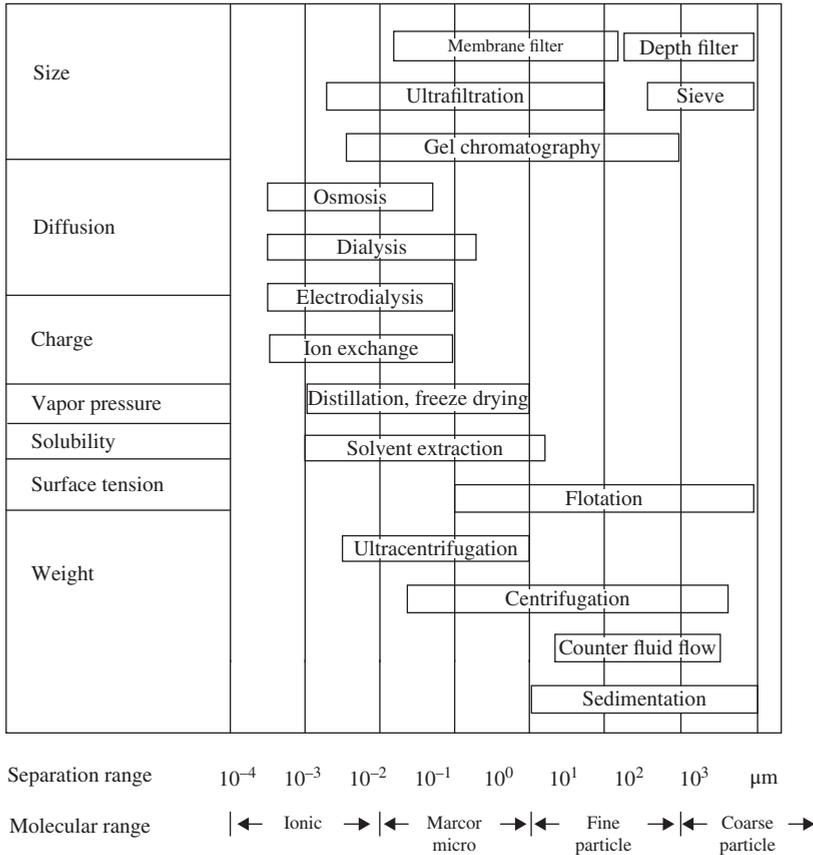


Figure 1.19 Separation methods based on physicochemical properties of particles and molecules. *Source:* Adapted from B. Atkinson and F. Mavitura 1991. *Biochemical and Biotechnology Handbook*, Macmillan.

fermentation broth. However, numerous parameters must be considered when selecting a filtration system. Among these are pore size and particle selectivity, cross-flow rate, cost and life span of the filter, and sterilizability of the system.

Centrifugation is used not only for achieving (fluid–particle) separation but also for enabling liquid–liquid and liquid–liquid–particle separations. For example, liquid–liquid separation is used in the manufacture of antibiotics (e.g., penicillin) by extracting the solvent from the aqueous phase by means of a two-stage continuous countercurrent extractor. The filter-and-sieve and solid-bowl centrifuges are of two distinct types. In the *filter and sieve centrifuge*, separation occurs as the particles are forced against a filter material. These centrifuges are used in great numbers for the separation of bioproducts. In the *bowl (or baffle) centrifuge* the separation occurs because of the specific gravities of the phases present, and the products are collected at the drum wall.

A wide variety of centrifuges are on the market for large-scale operations: separators (plate or nozzle) and decanters are examples. Separators have been used traditionally

for the production of baker's yeast and the clarification of the most diverse fermentation broths. A large-capacity (300 m³/h) nozzle separator is best used for the continuous separation of bacteria. In *decanter centrifuges (scroll type)*, sedimentation of the solid particles takes place on the rotating drum wall. Decanter centrifuges are used for the further dewatering of large single-cell proteins (yeast, algae) obtained in separators. In contrast to continuous centrifuges of other forms, *tubular bowl centrifuges* permit very high centrifugal force (g), averaging from 15,000 to 60,000 g. They thus permit the separation of very small particles and protein precipitates but use the discontinuous mode of operation, with low sludge hold-up capacity.

Cell disintegration Cells are usually disrupted after dilution of buffer to approximately 10% solids by one of the following types of methods: mechanical (milling), physical (ultrasonic, high-pressure homogenizer, freezing/thawing, drying), chemical (detergents, osmotic shock, acids, solvents, or antibiotics), or enzymatic lysis (lysozyme and other cell wall-digesting enzymes, autolysis, phages). Other means, including leaky mutants and genetically induced secretion, are also used in the production of rDNA products. The selection of a method depends principally on the nature of the cells and the cost of the method. Gram-positive bacteria and yeasts are more resistant to breakage than Gram-negative bacteria. Cell walls become more rigid, hence are better able to resist breakage, after the logarithmic stage of growth.

A *ball mill* is filled with the microorganisms together with small glass beads and subjected to high speed mixing; the action of the glass beads brings about the disintegration of the cells. One can expect a maximal breakage of 80–90% of the cells under optimum conditions. Another popular method on the industrial scale is the use of a *high-pressure homogenizer*. Cell disintegration takes place upon the application of high hydrostatic pressure followed by immediate pressure release as the cell suspension passes through a valve. To achieve good breakage (≈90%), two or three passes of the material through the homogenizer must be carried out. *Ultrasonic disintegration* is widely used in the laboratory, but it is not suitable for large-scale processes because of the high cost. Various drying methods have been used for the disintegration of cells. The mode of action is mainly due to a change in the structure of the cell wall during drying. Similarly, freezing and thawing, or heat-shock techniques can be used for the release of metabolites.

Autolysis is often used for the preparation of autolyzed yeast-flavoring ingredients. Endogenous autolytic enzymes of the yeast cells are responsible for lysis of the cell wall. Another similar technique, *plasmolysis*, is salt-induced autolysis obtained by adding a high concentration of NaCl. Osmotic shock is usually suitable for very sensitive cells such as animal cells. Detergents such as sodium dodecyl sulfate (SDS) or Triton X-100 are very effective for liberating intracellular molecules. On a small laboratory scale, investigators often rely on the enzymatic lysis of microorganisms using lysozyme or other cell wall-degrading enzymes and phage lytic enzymes.

Preliminary isolation (concentration)

Extraction Liquid extraction is based on the removal of the desired component from one of two immiscible liquids into the other, from which the components presumably may be recovered more easily. Many products can be concentrated and purified by use of a two-phase system (aqueous–organic or aqueous–aqueous). Aqueous–organic phases (amyl or butyl acetate) have been used for antibiotic recovery, but a novel aqueous–aqueous system has also been used for the purification of labile enzymes or other proteins. In solvent extraction, a two-phase system can be set up, using an organic

solvent that is immiscible with the aqueous broth. An extraction solvent should be selective (i.e., it should not dissolve unwanted components) and should have a high capacity for the solute; it should be low in volatility, relatively low in viscosity, noncorrosive, and relatively inexpensive. Selection of an appropriate extraction solvent can be particularly difficult for biological systems, which tend to be sensitive to many of the common solvents. The efficiency of the separation may be described by the distribution coefficient K as follows:

$$K = \frac{y_a}{x_a} \quad \text{at equilibrium}$$

where y_a is the mole fraction of the desired component (solute) in the extract and x_a is its mole fraction in the raffinate.

A variety of contacting patterns are possible in liquid–liquid extraction: countercurrent, fractional extraction, and cross-current extraction are examples. As a rule, the countercurrent technique offers higher driving forces, which impel the two fluids countercurrent to each other. If the solute to be removed is a weak acid or base, the distribution ratios may be markedly improved by adjusting the pH of the aqueous phase. For the purification of a typical penicillin, acidification of the broth to a pH in the range 2.0–3.0 causes protonation of the components, rendering the maximum selectivity for penicillin extraction by organic solvents. Organic solvents cannot be used for the purification of enzymes in the natural state, but aqueous two-phase systems can be readily scaled up for the recovery of the product.

Separated aqueous phases are produced by the dissolution of two incompatible polymers, such as PEG and dextran. The resulting phases are more than 75% water but rich in either PEG or dextran. The proteins are also dissolved in potassium phosphate buffer and precipitated by PEG. Cells remain in one of the phases, and the enzyme is transferred to the other phase without any loss of activity. Dextran, even its crudest form, dominates the medium makeup costs and thus must be reused. Some examples of enzymes that are purified by aqueous two-phase systems are shown in Table 1.22. However, this method is still expensive in terms of the PEG cost.

Another interesting variant of the foregoing technique, *supercritical extraction* (in which CO_2 plays a role), is used in the extraction of caffeine from coffee beans, hops for brewing, and pigments and flavor ingredients from biological materials.

Precipitation Precipitation may be brought about in many ways, such as by adding salt (e.g., ammonium sulfate or sodium sulfate) in high concentrations for salting out and adjustment of pH to the isoelectric point. Other possible additives include miscible organic solvents (ethanol, methanol, acetic acid, acetonitrile), nonionic polymers, polyelectrolytes, and polyvalent metal ions (to form a reversible protein precipitate). In concentrated salt solutions, the decrease in the solubility of proteins is a function of the ionic strength of the solution. If the desired enzyme is thermostable, contaminant host proteins may be selectively precipitated by heating at high temperatures, while the desirable thermostable enzyme remains in solution.

Biopolymers such as xanthan gum and alginate are recovered by adding divalent cation (Ca^{2+}) to form a gel precipitate. This method is thus used to form calcium alginate gels for cell immobilization. The gum polysaccharide is also directly precipitated by adding methanol or isopropyl alcohol.

Adsorption Ion exchange or adsorption with special polymer resins may be considered for the isolation of hydrophilic metabolites. Ion exchange materials are merely dissociable ion

Table 1.22 Two-phase aqueous extractions of enzymes from microbial cells*

Enzyme	Organism	Biomass concentration (%)	Phase system	Partition coefficient	Yield (%)	Purification factor
Isoleucyl-tRNA synthetase	<i>Escherichia coli</i>	20	PEG/salt	3.6	93	2.3
Fumarase		25	PEG/salt	3.2	93	3.4
Aspartase		25	PEG/salt	5.7	96	6.6
Penicillin acylase		20	PEG/salt	2.5	90	8.2
α -Glucosidase	<i>Saccharomyces cerevisiae</i>	30	PEG/salt	2.5	95	3.2
Glucose-6-phosphate dehydrogenase		30	PEG/salt	4.1	91	1.8
Alcohol dehydrogenase		30	PEG/salt	8.2	96	2.5
Hexokinase		30	PEG/salt	–	92	1.6
Glucose isomerase	<i>Streptomyces</i> sp.	20	PEG/salt	3.0	86	2.5
Pullulanase	<i>Klebsiella pneumoniae</i>	25	PEG/dextran	3.0	91	2
Phosphorylase		16	PEG/dextran	1.4	85	1
Leucine dehydrogenase	<i>Bacillus sphaericus</i>	20	PEG/crude dextran	9.5	98	2.4
Diacetate dehydrogenase	<i>Lactobacillus</i> sp.	20	PEG/salt	4.8	95	1.5
L-2-Hydroxyisocaproate dehydrogenase	<i>Lactobacillus confusus</i>	20	PEG/salt	10	94	16
D-2-Hydroxyisocaproate dehydrogenase	<i>Lactobacillus casei</i>	20	PEG/salt	11	95	4.9

*Except for pullulanase recovery, cells were disrupted by high-pressure homogenization or wet milling.

Source: Kula MR, et al. Purification of enzymes by liquid-liquid extraction. *Advances in Biochemical Engineering* 24, 773–778, 1982. With kind permission from Springer Science and Business Media.

pairs in which one type of charge is immobile. The separation of an antibiotic directly from the culture solution without prefiltration is an example. The total culture broth is treated with an anion exchanger in stirred columns connected in series. The use of ion exchangers for the separation of bacterial mixtures has also resulted in the development of adsorber resins without functional groups. The binding of substances to these resins takes place non-stoichiometrically by adsorption. In contrast to ion exchangers, the capacity of adsorber resins increases in the presence of salts. Whole broth processing is well known for the production of antibiotics (cycloheximide) using Amberlite (XAD) or streptomycin and novobiocin using fixed-bed ion exchange adsorbers at the pilot and process levels. This process was developed to eliminate a costly mycelial filtration, which resulted in loss of antibiotics in filter cake materials.

Purification A number of different processes are used for further purification of biologically sensitive products such as pharmaceuticals, diagnostic reagents, and research-grade enzymes. To isolate the desired compound, it will be necessary to carry out one or more chromatography steps, in sequence. The step using chromatography is the most expensive stage in product recovery.

The purification of the products by chromatography is based on the high selectivity of this technique. Separation occurs in a column, where a stationary phase (generally resin) is used to adsorb the product, which is then eluted with a mobile (liquid) phase. In chromatography, the partition coefficient α is a useful parameter. Thus in the relation

$$\alpha = \frac{q}{p_t}$$

where q is the concentration of matrix-bound protein and p_t is the concentration of protein in free solution plus the concentration of matrix-bound protein; α is affected by many factors.

The partitioning of a solute between a solid matrix packed in a column and the solvent is one of the most versatile methods for the fractionation of biological materials. The partitioning can be based on adsorption (called adsorption chromatography), molecular size (gel filtration chromatography), ionic charge (ion-exchange chromatography), selective adsorption using biospecific effectors (affinity chromatography) or molecular recognition (immunosorbent chromatography). By means of isoelectric focusing, proteins can also be purified by the use of pH gradients in association with ion exchange gels. In this process, called chromatofocusing, the proteins are separated according to their isoelectric points. The method is very good, although only small volumes can be handled.

Major advantages of chromatographic methods are characteristically high selectivity in separation and the mild reaction conditions. Table 1.23 shows various types of chromatography, which are distinguished according to the nature of the binding forces, the principle of separation, and the support materials.

In *adsorption chromatography*, separation is based on hydrophilic or hydrophobic interactions between the support materials and the biological compounds. Elution and fractionation are accomplished by higher or lower solvent systems or ionic strength. Support materials are inorganic substances such as silicates, hydroxyapatite, and alumina, or organic materials such as activated carbon and synthetic polymers (e.g., dextrans). Adsorber resins can also be used for the purification of proteins. Waters Associates and other companies make large-scale preparative systems that can handle up to 25 g or kilogram quantities of specific compounds.

In *ion-exchange chromatography*, the protein passes through a fixed bed containing ion exchange resin. An ion exchange resin is made up of an insoluble matrix to which charged groups have been covalently attached. One common cation exchange resin, carboxymethyl cellulose, often used for protein purification, is obtained by linking negatively charged carboxymethyl groups to a cellulose backbone. Cationic (positively charged) proteins will bind to this resin by electrostatic forces. After protein has been adsorbed to the resin, the column is eluted with buffers of defined ionic strength at fixed or altered pH. Such charges in the carrier solution cause weakly bound proteins to detach from the resin first; more tightly bound molecules are induced to detach from the resin by increasing the salt gradient (ionic strength) of the eluting solution.

Ion-exchange chromatography is also used on the technical scale for the purification of antibiotics, enzymes and other proteins. In industrial applications of ion exchange, a large resin particle size is normally selected for high flow rates and for minimum pressure buildup. Finer mesh resins give higher resolution, but the increased pressure drop requires lower flow rates. Fast protein liquid chromatography (FPLC), developed by Pharmacia, permits outstanding separations of protein molecules on preparative or technical-scale columns with a volume of 300 L or more. Most typical ion-exchange resins are strongly acidic cations, weakly acidic cations, strongly basic anions, and weakly basic

Table 1.23 Different methods and characteristics of chromatographic processes

Process types	Binding nature	Separation principle	Support materials
Adsorption chromatography	Surface binding	Surface affinity	Silicates, hydroxyapatite, alumina, cross-linked dextrans, activated carbon
Ion-exchange chromatography	Ion binding	Charge	Cation exchangers (Dowex, Amberite, carboxymethyl cellulose, etc.) Anion exchangers (DEAE-cellulose, Sephadex, Sepharose, Mono Q series)
Gel filtration chromatography	Pore size	Molecular size, molecular shape	Biogel type, P and A, Sephadex and Sepharyl series
Affinity chromatography (or immunosorbent chromatography)	Biospecific adsorption/desorption	Molecular structure	Biospecific effectors (enzyme inhibitors, monoclonal antibodies, metal chelates, protamin A, lecithins, etc.). Attached Sepharose or Sephadex series
Hydrophobic chromatography	Hydrophobic interaction	Molecular structure	Cyanogen bromide activated agarose, and so on.
Covalent chromatography	Covalent binding	Functional group (thiodisulfide exchange).	Agarose pyridine-2-yl disulfide, Sepharose-(glutathione-pyridine-2-yl disulfide) conjugate
Chromatofocusing chromatography	Net negative charge	Isoelectric point	Mono P

anions. These resins remove ions from solutions to form insoluble resin salts. Positively charged molecules are not bound by an anion exchange resin, whereas negatively charged molecules are retained.

Gel filtration chromatography is a separation technique based on the distribution of molecules between the external and internal solvents in gel particles with pores of a characteristic size. Molecules larger than this pore size cannot diffuse into the gel and pass directly through the column, while smaller molecules penetrate the gel, and their mobility is thus retarded from the solvent front. Relative molecular weight can be calculated from standard curves of known proteins. Gel filtration is used mainly to remove salts and to separate low molecular weight impurities at the industrial scale. On a small scale, however, gel filtration is used to fractionate and purify protein molecules.

Affinity chromatography uses a biospecific ligand that has been fixed covalently to an inert carrier and packed into a column to purify a molecule by highly selective adsorption and desorption. High molecular weight compounds such as enzyme inhibitors (e.g., Kunitz inhibitor for trypsin) and low molecular weight compounds such as substrate analogs (e.g., N^6 -(6-aminohexyl) adenosine for dehydrogenases) can be used as group-specific adsorbents for the purification of biological compounds. Other examples

of very specific pair interactions include antigen–antibody, protein A, metal chelates, lecithins, and antibiotic bacitracin, which is a useful ligand in the purification of serine-, cysteine-, and metalloproteases from a crude mixture. The most expensive ones are protein A affinity resins, but because of the strong affinity for certain antibodies, high-yield protein A affinity, despite its cost, handles the largest, least-refined process stream (after clarification removes debris) that can deliver 99% purity in a single step. With the availability of monoclonal antibody, which is a single type of antibody produced from animal cell culture, the immunosorbent column separation form of affinity chromatography now makes it possible to purify a particular protein or other molecule with a very high specific binding constant. An example of a commercial application can be found in the purification of human leukocyte recombinant interferon produced in *E. coli*. As a result of highly specific binding, the affinity column can accept a continuous feed input until saturation is obtained. The antibody needed to make the immunosorbent column is a dominant cost and is feasible only for high value-added products.

Hydrophobic chromatography is based on an interaction between a hydrophobic matrix and corresponding hydrophilic regions in proteins and polypeptides. In aqueous solutions, protein binds to alkyl or aryl residues of the substituted agarose. Alkyl-substituted agarose is obtained from cyanogen bromide (CNBR)-activated agarose by treatment with primary amines with different chain lengths. The proteins adsorbed at a low ionic strength can be desorbed selectively with the buffer having increasing ionic strength. Since the separating power depends on the tertiary structure of the proteins, a special hydrophobic carrier must be developed or optimized, involving a high expenditure on development. The combination of hydrophobic chromatography with other chromatographies permits the production of biological compounds of high purity especially for use in the pharmaceuticals division or for diagnostic purposes.

Covalent chromatography is based on the interaction of specific groups. For example, the purification of urease is achieved by reciprocal thiol–disulfide exchange on an agarose pyridin-2-yl disulfide as water-insoluble effector. The enzyme is bound to a support through a covalent bond in the form of disulfide bridge and is eluted by low molecular weight sulfhydryl compounds such as dithiothreitol.

Chromatofocusing is a technique used for separating biomolecules according to their isoelectric points. By means of isoelectric focusing, proteins can be purified by using pH gradients in high resolution ion exchange on monobeads (e.g., Mono P, Pharmacia). Mono P is substituted with various tertiary and quaternary amines. The mechanism is based on the buffering action of the charged groups on the Mono P. A molecule has a net negative charge at a pH above its isoelectric point (pI), and any component with a net negative charge is retained on the column when the sample is applied. A polybuffer is used to titrate the column and the sample components. Titration of the Mono P results in the generation of a linear, descending pH gradient and yields focused zones of molecules with different isoelectric points. The proteins are separated close to their pI by a pH gradient. The resolving power of this technique is the most effective when sample components have a negative charge.

Recent bioprocessing separation uses disposable *membrane adsorbers* and monoliths, which function as a specialized form of tangential-flow filtration. These maintain high resolution and capacity regardless of flow rate and molecular size for shear-sensitive products such as DNA plasmids, live viruses and labile proteins. Also *expanded-bed adsorption and multimodal* (mixed mode) chromatography is used in a number of market-approved processes. Further downstream processes in bioindustry on crystallization and chromatography are discussed in Section 1.8.2.

Drying To eliminate water or solvent from a liquid material, heat must be applied to the material to be dried. However, for biological compounds it is essential to dry the final product without thermal damage. Heat transfer can be achieved either by direct contact, by convection, or by radiation. Some common dryers are commercially available for carrying out the drying with convection dryers (e.g., pulverizing, rotating, spray) and contact dryers (e.g., thin layer, chamber, drum). The freeze-drying (sublimation drying) process has gained more importance for pharmaceutical and biological products (live vaccines, serum, hormones, enzymes, vitamins). Storage operations are widely used in other process industries and therefore this process operation is not discussed in this chapter. In connection with the application of such operations dealing with biological compounds, however, two important requirements must always be stressed: first, the product should not be subjected to any harsh denaturation conditions by excessive overheating, chemical and biological contamination, or degradation; and second, the potential health hazards and dangers due to the explosive nature that is associated with dried proteinaceous dusts must be avoided.

Sterilization There are a very few sources of practical information on how to run large-scale equipment with a low probability of contamination. Thus in this section, we consider sterilization from a practical standpoint. The sterilization and prevention of contamination in fermentation is a good example of the heat transfer practice.

Sterilization is required for vessels, culture media, and air. Media sterilization is commonly achieved by moist heat in batch cycles. The design of sterilization procedures is based on the death kinetics of microorganisms. In the sterilization of vessels, however, cost-effective operation calls for the synchronous exposure to this cycle of the nutrient media, the vessel itself, the filters, and all connecting pieces and sampling devices. Typically, the *thermal death of microorganisms* is correlated by the first-order rate expression as shown in equation (1.1).

$$-\frac{dN}{dt} = -kN \quad (1.1)$$

where k is the first-order rate constant (min^{-1}), N is the number of viable organisms, and t is time of exposure to a given temperature. The decimal reduction time D is the time to reduce the microbial population by 90% or by 10-fold, $D = 2.303/k$. The rate constant k is actually dependent on temperature according to the Arrhenius equation, a form of which is shown in equation (1.2):

$$k = k_0 e^{-E/RT} \quad (1.2)$$

where E is the activation energy for thermal death of the microorganisms (kcal/g-mol), R is the gas constant (kcal/g-mol-K), T is the absolute temperature (K), and k_0 is an experimentally determined parameter (min^{-1}). E must be measured experimentally and represents the sensitivity of the microorganism to heat. All other things being equal, a large E indicates extreme sensitivity to heat, while a small E represents only slight sensitivity to heat and therefore a difficult sterilization. Typically, E ranges from 50 to 100 kcal/g-mol for vegetative cells and spores. Batch sterilization is designed on the basis of a certain percentage reduction in the number of viable cells, usually in natural logarithm or n cycles of death.

$$\ln \frac{N}{N_0} = kdt = k_0 e^{-E/RT} dt \quad (1.3)$$

In most cases, temperature is not constant; rather there is a heating up period, a constant temperature holding period (usually at 121 °C), and a cooling down period.

The sterilization level can be calculated from $k_0 e^{-E/RT}$ if an expression relating temperature to time is available.

Sterilization can be carried out by indirect heating of the medium by means of an internal or external heating steam coil, or by blowing direct steam into the medium. With direct injection of steam, the time of heating up is relatively short, but condensate accumulates within the fermentor and will increase the volume of the liquid. In indirect heating, the heating up time becomes considerably longer.

The drawbacks of the *batch sterilization* process are the extensive heating (2–3 h) and cooling (1 h) periods, while the holding period usually lasts for 30–60 min for the actual killing process at a temperature of 121 °C in a 3000 L fermentor. If the hot water obtained during cooling cannot be recovered, batch sterilization becomes very costly. Another disadvantage of batch sterilization is the extent of thermal damage to desirable components. Vitamins are destroyed, and the quality of the culture medium deteriorates as a result of undesirable browning reactions. For energy-saving reasons and to avoid undesirable reactions, *continuous sterilization* is always to be preferred to a batchwise process. Not only are savings of the order of 70–80% achieved when steam and cooling water are used, but the use of a higher temperature (135–140 °C) for a short time (5–8 min) contributes to gentle treatment of the labile components in the medium. For continuous medium sterilization, two distinct types of system exist – those employing direct steam injection into the nutrient medium, which necessitates extraclean steam, and those employing indirect heating by means of heat exchangers. There are some drawbacks to continuous sterilization – direct steam heating can add excess water to the medium; microorganisms may not spend identical amounts of time in the sterilizer; and the heat exchanger used for indirect heating or cooling can be fouled by suspended solids. In the fermentation industry, continuous sterilization is much less common than batch sterilization.

Filtration approaches may also be applied to media sterilization, but they are less reliable and not as well developed. Filtration approaches are, however, widely used in fermentation air sterilization, along with heating techniques. Air sterilization processes are required to remove microbes with dimensions as small as 0.5 µm from the vast volumes of air frequently required by aerobic processes. A fermentor having a working volume of 50 m³ with an aeration rate of 1 volume of air per volume of liquid per minute (vvm) will need 3000 m³ sterile air per hour. The critical importance of air sterilization on the industrial scale can be seen from these values. Among the methods available for sterilizing gases, such as filtration, gas injection (ozone), gas scrubbing, radiation (UV), and heat, only filtration and heat are used in industry. While depth filters such as glass wool filters are important historically, all filtration today associated with air sterilization relies on polymeric microporous membranes that use cartridge filters. Significant improvements in filter cartridge design have appeared within the last couple of years. Costs for air filtration depend on filter costs, pressure drop, power costs, and installation and removal costs. There is not yet an absolute filter for bacteriophage in industrial usage. Bacteriophage can cause total failure in the production of many bioingredients from microbial fermentation and of fermented foods with lactic starter cultures.

Recovery of recombinant DNA products Certain aspects of rDNA fermentation and protein recovery require careful examination not only during the initial cloning and expression, but also before process development is considered. The heterologous proteins expressed at high levels in recombinant *E. coli* often accumulate in the intracellular refractile bodies called IBs. These insoluble aggregates of highly cross-linked protein must be solubilized and renatured to obtain an active form. Methods of completely solubilizing the protein and refolding it in reducing conditions are now technically feasible but still expensive, unless

the product is high in value, like recombinant human insulin. A part of recombinant insulin and chymosin (rennin) in the IBs is present in so-called quasi-native form, in which the molecules are in the correct folded state but differ from the native form by the absence of cystine disulfide bond. Often the renaturation step used is oxidative sulfitolysis, or the dissolution of urea or guanine, a refolding and disulfide bond formation. Flow processes used in the production of recombinant chymosin from *E. coli* and *Kluyveromyces lactis* are shown in Figure 1.20.

The choice of microorganism, the available fermentation conditions, and the ability to select for secretion of the desired protein influence the choice of operations for recovery and purification of the product. Current examples of host microbes used for the production of recombinant chymosin are *E. coli* (Pfizer, USA to Chr Hansen), *K. lactis* (Gist-Brocades, Netherlands) and *Aspergillus niger* (Genencor, USA). *Aspergillus* could give the advantage of secreting high levels of enzyme. However, glycosylation, which is the addition of carbohydrate moieties to the protein, may require an extra step, namely deglycosylation.

Leader sequences from yeast proteins (pre-pro region of α -factor genes) were used in *K. lactis* to secrete prochymosin. The secretion of the desired protein should simplify

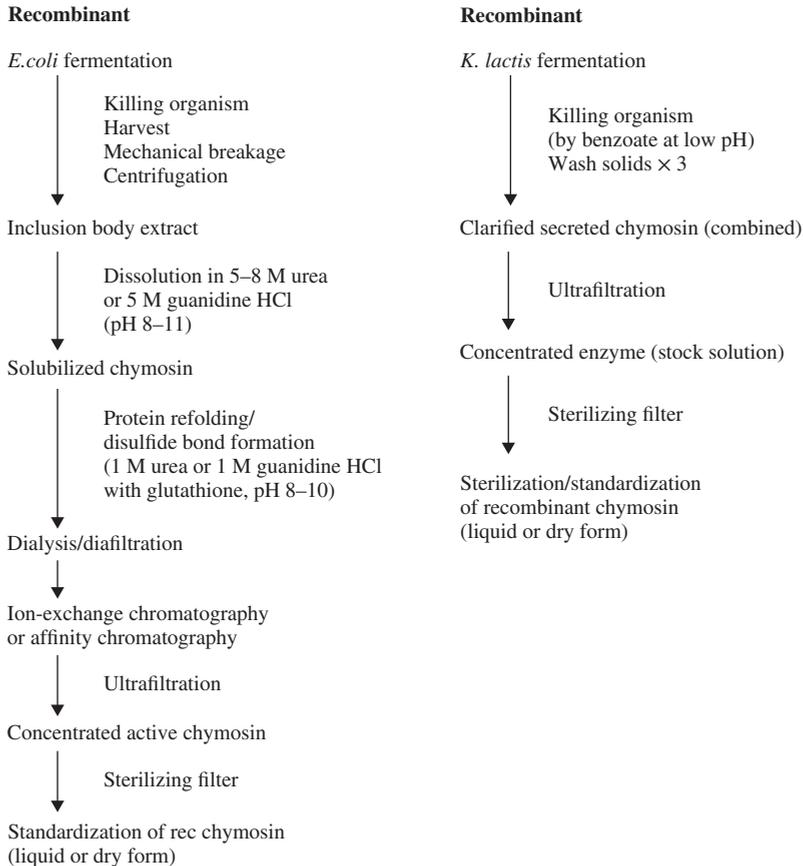


Figure 1.20 Flow processes in the production of recombinant chymosin.

downstream processing, but proteases are often excreted, which would cause severe product degradation. Prochymosin is inactive, and thus does not have proteolysis, and the prochymosin gene and a leader sequence were inserted under the control of the lactase promoter and terminator. To stabilize chymosin production, the expression vector (pUC19) was then integrated into the host chromosome at a site in the lactase promoter by recombination during DNA replication. The biochemical identity and functional properties of the recombinant chymosin were similar to those of the native chymosin. Then the production process was developed, using the existing lactase production protocol. Though a number of modifications were made to optimize the process, the general production scheme remained the same as for lactase production.

The release into the ambient atmosphere of genetically engineered microbes is not permitted in countries where such work is performed; therefore, the recombinant microbe must be inactivated before the fermentor is opened. Benzoic acid is used as a preservative for rennet products, and the low pH of the mixture needed to inactivate the cells also facilitates the autocatalytic conversion of prochymosin to chymosin. The specifications for avoiding microbial contamination of chymosin – namely, absence of any genetic information (DNA) that could be carried over, stability, and absence of β -lactamases, which inactivate the effects of antibiotics – have been met. Once the biochemical identity of recombinant chymosin had been established, the three main regulatory regimes were met, the process was carried out on a large scale, and the criteria for the demands of legislative procedures and cheese trials were verified. Furthermore, the large-scale protocol and equipment had to be suitable for use with recombinant strains. Also, the survival of the *K. lactis* strains was compared with the host strain in a number of soil and water samples. A pathogenicity study was performed with laboratory animals (e.g., mice), administering different doses by various routes, to see if the GM *K. lactis* caused any infection.

No special problems were encountered in the scale-up of the production process, since it was based on the commercial production of lactase. Besides the evidence of nontoxicity of the final product, as evidenced by acceptable levels of mutagenicity, acute oral toxicity, and allergenicity, as well as passing results from feeding trials, functional properties and cheese manufacturing trials had met. The product was then ready for commercial operation and for introduction in the market. When the genetically engineered microorganism was capable of producing chymosin on a large scale, a new source of rennet was available commercially. The recombinant microbial rennet was completely identical to calf chymosin. It is available from a virtually unlimited source, and it will eliminate the need to slaughter suckling calves. Although the absence of any health hazard to consumer had been proven, in most countries additional standards had to be met.

In addition to these legal issues, the dairy industry had to be persuaded to accept the recombinant product. Since the food industries are very conservative, this tends to be a difficult task. Once the benefits of recombinant chymosin on cheese quality and production costs are recognized, it will be a matter of time before the product is widely used. The safety issue on rDNA technology is dealt with in another chapter.

1.7.6 Scale-up and scale-down of fermentations

There are basically two ways to develop a new bioprocess: the *scale-up* and *scale-down* methods. Although the *scale-up* method is the most common one to develop a new process, drawbacks of the scale-up method exist in which the pilot plant dimension, primarily those of the reactor, may not be appropriate for the commercial process. Also, this does not include the development of a kinetic model, nor does it include simulation of the commercial-scale process. In the scale-down method, a simulation of the commercial

design exists prior to the design of the pilot plant. Simulation involves many parameters and the pilot plant is then designed to improve the estimates of the most important and least well-known parameters.

Scale-up is simply the conversion of a small-scale process developed at a laboratory to an industrial scale. The term “scale” refers to either the volume or the linear dimensions of a fermentor. It is seldom feasible to apply fermentation conditions that have worked in the laboratory directly on a commercial scale. This is because success in scale-up is evaluated on the basis of maximal yield in terms of the minimal operating cost and time. The information obtained in small units is not adequate for large-scale design, since the fluid dynamics, transport processes, and even the behavior of the cells may be changed considerably as a result of an intensification of turbulence when the size of the unit is increased. Ideally, the geometric enlargement of a reactor should be known. In the absence of this information, however, pilot plants must be constructed that subdivide the large step of this scale-up procedure between laboratory and production units to reduce the risk involved in the design of the production unit.

Most of the following information is obtained from small pilot plants; only a few results from industrial-scale plants have been published. Although many equations employing physical parameters have been derived and evaluated for use as scale-up criteria, there is no general formula because of the variation from one fermentation process to another. The most useful information concerning scale effects has been obtained using a series of geometrically similar fermentors. In reality, however, scale-up is not usually achieved with geometrically similar fermentors in laboratory, pilot plant, and industrial settings. The problem more often is do with scaling down from the production plant to carry out experiments on a laboratory scale under conditions of existing large tanks.

Scale-up is a highly interdisciplinary task, requiring the integrated use of concepts and methodologies of both biochemical engineering and microbial physiology (Figure 1.21). At the small laboratory stage, more microbiologists and biochemists are involved and data produced at this stage can be the high risks, but above the pilot plant stages, engineers are more involved and data produced at this stage can be more trusted for commercial production. This section is not intended to give an in-depth study of critical parameters; rather, it provides a general outline of scale-up procedures together with a description of the relevant techniques. The main parameters affected by scale are agitation and aeration, heat transfer, medium sterilization, and process monitoring and control. Since we have already discussed most aspects of fermentation parameters in mass and heat transfer practice, we discuss briefly the subject of agitation (or stirring) before turning to the common scale-up methods. Other biological factors (culture variability, medium components, water quality, selection of mutants, etc.) can bring different yields at different locations with similar equipment. In batchwise processing, the viscosity increases as the cell mass increases, and this is particularly important in mycelial fermentations used in antibiotics and enzymes.

Vigorous mechanical mixing of air–liquid dispersions is often necessary to obtain economic rates of biomass production, substrate consumption, or product formation. The agitation or stirring brings about homogenization of the culture and nutrients by bulk mixing, dispersion of air and immiscible liquids in the nutrient solution, and promotion of interface mass and heat transfer. All these functions require mechanical energy, which is supplied by the motion of impellers of various type and is eventually dissipated as heat. Various zones with different mixing intensity and fluid shear rate are often found in large fermentors. This nonuniform mixing leads to gas–liquid (O_2 , CO_2), and liquid–solid mass transfer problems. Stagnation zones also increase lag time in process-control sensors as a result of local concentration gradients. The optimum mixer design for gas–liquid mass transfer

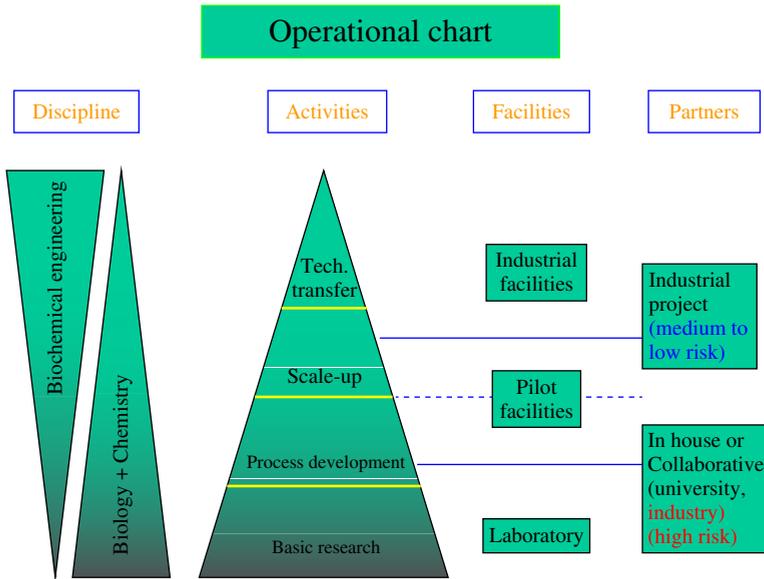


Figure 1.21 Scale-up requiring a highly interdisciplinary task, concepts and methodologies of biochemical engineers, microbiologists, and chemists.

could often turn out to be disastrous in terms of shear rate and blending of relatively fragile mycelial fermentations. In general, high-shear, low-flow impellers are inferior to low-shear, high-flow impellers. Large tanks often have high-shear rates and lower pumping capacity than small tanks (Figure 1.21).

The ability of impellers to transfer energy to a liquid is usually expressed in terms of the *power number* (*Newton's number*) N_p as follows:

$$N_p = \frac{\text{imposed force}}{\text{inertial force}}$$

$$= \frac{P_o}{N^3 D_i^5 \rho}$$

where P_o = stirring power (kW)
 N = stirring speed (s^{-1})
 D_i = stirrer diameter (cm)
 ρ = density of the medium (g/cm^3)

The power number has been correlated with the Reynolds number for several types of stirrer. The *Reynolds number* is a measure of the turbulence of the system and expresses the ratio of inertial to viscous forces in the liquid:

$$N_{Re} = \frac{ND_i^2 \rho}{\eta}$$

where N = stirrer speed (s^{-1})
 D_i = stirrer diameter (cm)
 ρ = density
 η = dynamic viscosity ($\text{g}/\text{cm} \cdot \text{s}$)

The Reynolds number describes the flow only at the periphery of the stirrer. The general relationship between N_p and N_{Re} has the form:

$$N_p = K(N_{Re})^{-m}$$

where K is a constant dependent on the container geometry and the shape of the stirrer, but not dependent on the reaction size, and $m = 1$.

With laminar flow, the power required for stirring is not dependent on the density, but is correlated with fermentation parameters. In the turbulent flow range of mixing speed ($N_{Re} > 10^4$), the power number is independent of the Reynolds number.

Scale-down to a lab scale bioreactor is often necessary to obtain more consistent process performance during the scale-up. Creation and qualification of scale-down models are essential for performing several critical activities that support process validation and commercial manufacturing. A key parameter that frequently limits a scale-down aerobic fermentation model is the volumetric oxygen mass transfer coefficient ($K_L a$), which is proportional to the rate of oxygen transfer from rising gas bubbles to the cells. This approach can be coupled with a small-scale experimental design to more accurately predict and optimize larger-scale fermentation conditions before the actual process transfer (growth and production temperature profile, feed rate, air/O₂ control scheme, induction cell density, etc.).

1.7.6.1 Scale-up methods A constant aeration and agitation regime can hardly be regarded as an optimal scale-up factor, since culture requirements and rheological properties of the fermentation broth differ substantially at different periods of the fermentation. A constant oxygen transfer rate, value $K_L a$ is the most commonly used method because $K_L a$ relates well with process results such as yield and titer of the products. Neither the fermentor type nor the volume was a crucial scale-up criterion, but there was good agreement between $K_L a$ values and yields using small (6–15 L) and larger (100–3000 L) fermentors. However, the $K_L a$ value cannot be used in highly viscous non-Newtonian fermentation solutions, which have been found in many antibiotic fermentations, or under conditions of high speed stirring. The region for effective scale-up is most likely to be before foaming, mechanical damage and product inhibition can occur. Another simple method is based on the power consumed per unit volume (P/V) in aerated bioreactors. The desired prerequisite for using P/V for scale-up is a geometric similarity that is rarely attained. For geometrically similar tanks,

$$P_g = K \left(\frac{P_o^2 N D_i^3}{Q^{0.56}} \right)^{0.45}$$

where N is the rotation speed of the impeller, D_i is the impeller diameter, and K is a function of broth characteristics. However, power usually measured by wattmeter is not a true measure of power transferred to liquid; also, power requirements change over the course of

Table 1.24 Type of data required from laboratory and pilot experiments for effective scale-up into production

Oxygen uptake data related to product formation
$K_L a$ (volumetric oxygen transfer coefficient, h ⁻¹)
Dissolved oxygen tension
Growth rate
Nutrient uptake data related to product formation
K_s (substrate specific constant)
Growth rate
Viability of environmental parameter rate constants
pH
Dissolved oxygen tension
Specific rate data
Temperature
Fluid characteristics during the course of fermentation
CO ₂ effects (including back pressure)
Definition of geometry for estimation of mixing behavior
Definition of pilot plant control system
Estimation of cooling requirements

a fermentation as viscosity and aeration requirements change. Other physical methods have been tried, but impeller tip velocity representing liquid shear, mixing time, and Reynolds number of stirrers are less useful physical parameter than others.

In executing scale-up and scale-down of biological reactors, one should pay primary attention to the microbial physiological response, disregarding the geometrical similarity between the original vessel and the replica. The $K_L a$ is well related to productivity and biological parameters. The main data required from laboratory and pilot plant experiments for effective scale-up into production are shown in Table 1.24. Generally, a 1:10 ratio is used in scaling fermentation vessels, as shown in Figure 1.22. There are several advantages associated with 1:10: (i) each vessel can be used to adequately seed the next larger scale vessel; (ii) within the pilot plant, a minimum of two log orders of scale-up can be evaluated for effects or trends on process performance and control; and (iii) a maximum of one log scale-up to production is possible, to minimize scale-up risks. Scale factors in translation of pilot plant data into production are also summarized in Table 1.25.

1.7.6.2 Bioinstrumentation and computer control

Bioinstrumentation Process monitoring and control have become essential elements in the optimization of fermentation processes that call for the performance of measurements during fermentation for data analysis and subsequent control of the process. The basis of understanding and controlling a biological process is the data obtained from the biosensors and instrumentation employed. Table 1.26 lists various physicochemical sensors that have been developed for monitoring fermentation parameters. The biological parameters, with the exception of the NADH₂ online measurement using a fluorescent method, must all be measured outside the fermentor. We will thus concentrate on instrumentation for online physical and chemical parameters for fermentation processes. A key limitation for complete bioprocess monitoring is the inability to measure accurately and quickly many variables online.

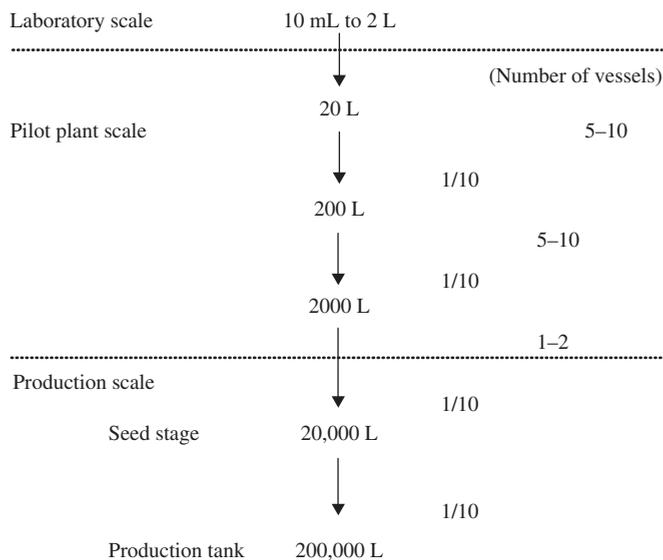


Figure 1.22 Scaling fermentation vessels by the 1:10 ratio method.

Table 1.25 Scale factors in translation of pilot plant data into production

Pilot plant	Factor	Production
Small	1. Sampling errors	Significant (nonuniformity)
Small	2. Process control response time	Large (nonuniformity)
Uniform	3. Agitation	Large (nonuniformity)
Easily controlled	4. Aeration/power input	Limitations of compressor capacity, peak load times
Difficult	5. Sterility (continuous feeds, inoculation port, valves, antifoam vessels)	Less difficult due to steam seals, jackets
Easily controlled	6. Water, steam, air quality	Difficult to control
Easily controlled	7. Heat transfer (cooling water)	Often limiting
Good	8. Reproducibility	Variable

1.7.6.3 Physical measurement The major physical process parameters that influence cellular function and process economics are temperature, pressure, agitator shaft power, impeller speed, viscosity, gas and liquid flow rates, foaming, tank volume/weight ratio, and heat generation.

Temperature is the most important parameter that is measured and controlled in most fermentation processes. Depending on the dimensions of the fermentor and the desired accuracy of regulation, thermistor (semiconductor resistance thermometer), metal resistance thermometer (nickel or platinum), or thermocouple controls are most commonly used. A metal resistance thermometer, which has the advantages of stability and linearity of response, is used in most fermentors today. In many large fermentors temperature is

Table 1.26 Basic process control parameters that can be measured in fermentation processes

Physical	Chemical	Biological
Temperature	pH	Cell concentration
Pressure	Dissolved O ₂	Enzyme activities
Power consumption	Exit O ₂ /CO ₂ concentration	DNA and RNA
Agitation speed	Redox potential	NADH
Viscosity	Substrate concentration	ATP
Air-liquid flow rate	Product concentration	Protein content
Turbidity	Ionic strength	-
Volume/weight of tank	-	-
Form detection	-	-
Heat generation	-	-

controlled by cooling water or by cooling water and steam as the heating source if necessary. Pressure monitoring is important during sterilization. Diaphragm gauges are usually used to monitor pressure. Pressure can easily be controlled by using regulating valves in the exhaust air line.

For large fermentors, a wattmeter measures the total power drawn by the agitator motor. A torsion dynamometer may also be used to measure shaft power input. The online measurement of viscosity and other rheological properties of culture broths can be quite difficult, primarily because of the non-Newtonian characteristics and solids content of most fermentation broths. One possible method is to measure power consumption at several different impeller speeds. Also, a dynamic method has been proposed in which shaft power input is monitored during and after a brief shutoff in agitator drive power. Most apparent viscosity measurements are performed offline using a Couette or Brookfield viscometer. A common method for measuring the flow rates of gases (air feed, exhaust gas) is the use of variable area flowmeters such as rotameters. Large gas flow can be measured by an electrical turbine flowmeter or by laminar flow. Thermal mass flowmeters with controller are increasingly popular for measuring and controlling airflow in laboratory and pilot plant fermentors. The control of the flow of liquid media is difficult because of the need to maintain sterility. Liquid flow rates can be monitored with electromagnetic flowmeters or capacitance probes, but most of these devices are expensive. The automatic measurement of predetermined volumes has proved satisfactory. Foaming is a common problem in many fermentation processes. Foam can be detected by either a capacitance or a conductivity probe. *Foaming* is controlled by mechanical foam destruction devices using single, rotary, or multiple rotating plates, ultrasonic irradiation, or the addition of sterile antifoam agents. The silicone-based antifoam agents are the most commonly used in the fermentation industries.

1.7.6.4 Chemical measurement The pH of a fermentation is commonly monitored with a steam-sterilizable pH electrode (combined sensor) connected to a pH meter for direct visual readout. Computer-based direct digital control (DDC) has replaced the analog titrator in many recent fermentation operations. Electrodes that can be repeatedly steam-sterilized in place are available for pH, redox potential (E_h), and dissolved O₂ and CO₂ partial pressures. The partial pressure of DO in the broth is found with either galvanic (potentiometric) or polarographic (amperometric or Clark) type electrodes,

which measure the partial pressure of the DO and not the dissolved O₂ concentration. A similar CO₂ probe produced by Ingold determines $p\text{CO}_2$ by measuring the pH of a standard bicarbonate solution, which is separated from the fluid by a gas-permeable membrane.

Methods for the online assay of volatile components and dissolved gases are also available. Several biosensors based on coupling the action of immobilized enzymes or cells with an analytical device can detect a particular product of the biocatalytic reaction. Also enzyme thermistors can be used for the detection of heat released by the enzyme-catalyzed reaction. Some of the compounds and ion species that have been assayed by immobilized thermistors or electrodes using immobilized enzymes have been mentioned (see Table 1.15).

A concern in the use of any biosensor employing enzymes, cells, or other biochemicals is, of course, deactivation of the sensor during reactor stabilization. Also, membrane fouling by cells or medium components and external mass transport resistance can cause shifts in calibration of the sensor.

Other exhaust gases such as CO₂, O₂, CH₄ (in anaerobic methane generation), and H₂ can be indicative of fermentative metabolic status. CO₂ content in fermentation gas streams is most commonly monitored using an infrared spectrophotometer, online process gas chromatographs; process mass spectrometers are being used to simultaneously measure O₂ and CO₂ contents of the inlet and outlet air streams in fermentations. Gas chromatography can continuously analyze several compositions of exhaust gases (O₂, CO₂, CH₄, H₂) and volatile components (ethanol, acetaldehyde, etc.). A gas semiconductor system is advantageous and changes its electrical conductivity when it comes into contact with combustible gases or organic vapors of volatile substances.

1.7.6.5 Biological measurement The reliable online determination of the number of cells or their weight is still the most difficult problem facing biotechnology. Growth is normally measured by determining the increase in the amount of dry matter (biomass) or in the number of cells. These two quantities do not necessarily correlate in a constant manner. Dry weight measured by drying overnight at 105 °C is accurate and comparatively cheap, but overestimates due to foreign compounds that cannot be washed out (e.g., precipitates of insoluble salts, solid constituents of the medium) are a hazard. Wet weight can be measured by filtering the cells; but all procedures for determining cell mass require a relatively large amount of manual work and cannot be automated without considerable expenditure. Measurements of the number of total cells by microscopic counting or the Coulter counter are very labor intensive. The number of live cells can be determined by plating out the culture and occasionally by specific staining. Photometric measurements (turbidometry) rapidly and simply yield results that can be correlated with growth. However, accuracy is lower as cell density and number of air bubbles increase and as solid constituents of the nutrients interfere.

More suitable methods developed recently are the flow cytometer or fluorometer. In *flow cytometry*, the cell sample stream is irradiated by a laser or other light source such as fluorescence, and light-scattering measurements are used to obtain information on the cell size distribution. Flow cytometry can be used to detect intracellular morphology and macromolecular composition by using a specific fluorescent dye, and to take simultaneous multiple measurements on individual cells. When the culture in a fluorometer fluoresces through a fluorescent bulb, the culture light passes out through the quartz window and through a second set of filters to a photomultiplier tube. The signal from the photomultiplier is amplified and recorded. ATP levels also change rapidly as a function of cellular metabolic activity, and thus cellular ATP content can be measured ascertained by means

of a *bioluminometer*, which measures luminescence produced by a reaction requiring ATP and the enzyme luciferase. *High resolution nuclear magnetic resonance (NMR)* measurements of ^{31}P have been used to determine intracellular ATP, ADP (adenosine diphosphate), sugar, phosphate, polyphosphate, and pH simultaneously. It is beyond the scope of this text to describe the individual parameters in detail.

1.7.6.6 Computer applications The automatic acquisition of data, data analysis, and the development of fermentation models using computers can be applied online for improved process control and optimization. Although the application of computers to process control is widely accepted in many firms in the chemical and petroleum industries, it has not yet been used widely in biotechnology. There are two major reasons for this slowness in development: (i) it is difficult to evaluate approaches to the reduction of fermentation cost using computers because the very complexity of the fermentation processes has prevented the full understanding of biosynthesis and the regulation of metabolic formation, and (ii) suitable biosensors are not yet available in sterile systems to measure the important variables to take advantage of computer capacity.

Data that are shown in Table 3.8 can be acquired directly at the fermentor with online sensors, and large quantities of measured results can be stored in the digital form for convenient access, analysis, and display at a later time. An alarm system can also be hooked up to the data acquisition system to inform an attendant that a failure of a system part has occurred and possibly also to initiate countermeasures. In data analysis, the data measured can be combined, and quantities such as oxygen utilization rate and respiratory quotient, yield coefficient, heat balance, and productivity can be analyzed instantaneously. When biomass is not continuously measured, the biomass yield can be calculated through the O_2 uptake rate. Measurements of inlet and exit gas flow rates and composition can also be used to calculate the average volumetric mass transfer coefficient, $K_L a$. Online estimation of biomass concentration and of specific growth rates during fermentation has been a central aim of data analysis. By using mathematical models, it is also possible to improve our knowledge of the process, which in turn permits the evaluation and optimization of conditions and strategies. A large number of models exist for batch and continuous fermentation, but each model is valid only for a certain specific process; none can be used universally.

1.7.6.7 Process control There are many advantages and objectives of computer-controlled fermentation processes. In fermentations for which substrates are the major costs, monitoring and controlling of raw material addition and total energy consumption can become far more cost-efficient. Computer-aided process optimization can also maximize volumetric productivity, product concentration, and conversion yield of substrate to product. Other potential advantages of computer-aided fermentation control include quick and efficient data management, storage and reproduction. Computer control can be divided into two kinds: digital set point control (DSC) or supervisory computer control (SCC), and DDC.

In *DSC*, regulation is comparatively slow but is adequate for many biotechnology processes. The presentation of essential elements of analog regulation is particularly advantageous in the initial phase of the incorporation of process computers. The measuring, controlling and regulating devices already present can be used. In the case of failure of the computer, a return can be made directly to the conventional management of the process. It is also possible to work first with the comparatively simple software, which specifies only desired values. The environmental variables are controlled by local single-loop controllers, and the environmental controller set points come from a digital computer.

Common practice in *DDC systems* is simultaneous use of several controllers, which can offer the flexibility of manipulating more than one process input to achieve control. For example, measurements of DO level and exit gas O_2 concentration allow online estimation of $K_L a$, whereupon it becomes possible to manipulate the agitation rate and/or the gas feed rate to control DO at the desired level. DDC requires digital-to-analog converters that possess variable outputs, and new strategies and algorithms of regulation in which the process takes place continuously, while digital computers operate discretely. In other words, data acquisition and signal output take place only at discrete points in time.

The software program in the computer commands the fermentation operation. Good software development can be the major task, and these costs are a significant part of the expense of installing a process computer. Recent downward trends in hardware prices certainly reinforce this area of research. An important consideration in selecting a hardware–software system is its ability to do multitasked operations (i.e., to run programs simultaneously). There are two different types of computer program: (i) utility programs, which start up the system and create files, and language programs to permit use of high level languages (FORTRAN, BASIC, APL, COBOL, etc.), and (ii) applications programs for accomplishing particular computations and other tasks.

The application of computers to the operation and maintenance of batch and continuous reactors is essential to implement controls or to do the calculations necessary to determine the desired control strategy. However, the number of sensors available to measure such key variables as cell concentration and product concentration is quite limited. Multivariable control and dynamic optimization so far have limited utility for most fermentation processes. New and robust sensors will have to be developed before more widespread automatic control of fermentation processes is available. Recent advances in molecular genetics and cell physiology may help us to develop more accurate process models to define various process controls. Operating strategies for recombinant microbial fermentations may closely resemble those for secondary metabolites, although the biochemical processes involved and their regulation are much different. When manufacturing a recombinant protein, it is usually best to delay the expression of the product until a suitable culture growth has been caused to occur, either by adding an inducer or by depleting an inhibitor of gene expression. Early expression of the product often inhibits cell growth and may accentuate any genetic instability problems.

1.7.6.8 SCADA control system Recently systems for controlling process variables applicable to a bioprocess have become increasingly sophisticated. These systems frequently employ digital systems such as programmable logic chips (PLCs), micro-processor based software control systems, or a hybrid arrangement. Advancements in processors, communication hardware, protocols, and archival software systems have transformed the concept of data management during bioprocessing from a luxury to a necessity.

The advent of sophisticated digital systems has given the bioprocess engineer the capability to repeatedly apply the same complex series of actions to any bioprocess. This has enabled large molecule pharmaceutical manufacturing to move toward the level of reproducibility that semiconductor processing now enjoys. Additionally, the use of digital systems to implement *supervisory control and data acquisition (SCADA)* now allows a smoother path to satisfying the requirements of *good manufacturing practise (GMP)* doctrines as well as *US Food and Drug Administration (USFDA)* requirements.

SCADA is a kind of software application program used for process control and gather real time data from remote locations for exercising this control on equipments and conditions. The SCADA System consists of hardware and software components. The hardware

collects and feeds data into a computer that has SCADA software installed in it. The data is then processed by the computer before presenting it in a timely manner. The function of SCADA is to record and log all events in a file that is stored in a hard disk or send them to a printer. If conditions become hazardous, SCADA sounds a warning alarm.

Input/output signal hardware, network, human machine interface, controllers, database, communication and software constitute an SCADA system. There are real-time automated and integrated control systems like its cooling by the computer itself for quick responding to the process changes within the processes' own time-frame.

New Brunswick Scientific (NBS) has developed three next-generation BioCommand[®] software packages designed to enhance your ability to monitor and control your fermentation and cell culture processes through the personal computer (PC). Three distinct BioCommand[®] packages are offered, providing the tools needed for research, optimization, and if needed, the security and audit trails to meet the regulatory requirements. BioCommand[®] Track and Trend, Batch Control and Batch Control Plus SCADA software packages provide (i) automatic data logging, (ii) remote monitoring and control capabilities, (iii) ability to monitor and supervise several fermentors and bioreactors from a single PC, and so on. This SCADA system for both reusable and single-use bioprocess applications can trigger different actions automatically based on events.

1.7.7 Scale-up challenges

Although many correlations and relationships have been established for the scale-up of chemical processes, these equations are not always applicable to their bioprocessing counterparts due to the nature of living systems and the differences in the principles of unit operations and unit processes between chemical and biochemical processes. The scale-up and optimization of the complicated biochemical activities of microorganisms and animal and plant cell systems present engineering challenges that are sophisticated and difficult. Many widely used fermentation processes were successfully scaled up on the basis of a constant volumetric oxygen transfer coefficient ($K_L a$) and power consumption per unit volume (P/V). The use of traditional empirical methods, such as P/V leads to an increase in mixing and circulation times at large scale. In addition, high oxygen demands and high viscosity can cause concentration gradients in oxygen, shear and pH, which can have a significant impact on fermentation yield. Therefore, the choice of scale-up criteria is not an easy task, given the potentially sensitive and diverse responses of cells to each of the transport phenomena influenced by impeller design, system geometry, scale, fluid properties and operating parameters.

The fed-batch, which is the high cell density cultivation of microbial strains, is the preferred industrial method for increasing the volumetric productivity of such bacterial products as nucleic acids, amino acids, and heterologous recombinant proteins. This type of feeding regime avoids problems associated with catabolic regulation, oxygen limitation, and heat generation that can occur during unlimited batch processes. Importantly, the buildup of toxic concentrations of metabolic by-products via so-called "overflow" metabolic routes can also be avoided. Overflow metabolism has been reported for *S. cerevisiae* as well as for *E. coli* and occurs at glucose concentrations above 30 mg/L. For *S. cerevisiae*, overflow metabolism is known as the "Crabtree effect" and the inhibitory by-product is ethanol but produced in a similar way to acetate in *E. coli*. In batch fermentation, overflow metabolism can be avoided by the use of a slowly metabolizable carbon source such as glycerol, but the preferred method is the use of a fed-batch process where growth can easily be controlled by substrate feed rate.

The scale-up challenges are compounded when the process involves batch fermentation. Due to the typical fragility of the engineered microorganisms, large-scale fermentation vessels must be designed with the ability to (i) remove the heat buildup that results from metabolic processes; (ii) manage agitation and mixing with minimal shear damage; (iii) effectively control the highly variable liquid flow rates and turn downs that are associated with batch fermentation; and (iv) execute safeguards and sterilization techniques to guard against potential contamination.

The engineering challenges are more acute when the fermentation process is used to make low-priced commodity chemicals including alcohol, amines, acids, solvents and surfactant, and so on. Because such products are not higher-value specialty chemicals, food additives and biopharmaceuticals, their production facilities often are forced to make engineering and bioreactor design trade offs for reducing cost constraints and leaner profit margins.

One of the most commonly made mistakes during the design of large-scale fermentation processes is the failure to adequately integrate the experience, expertise, and proven techniques developed by the pilot-plant engineers, facility microbiologists, and chemists into the criteria for the overall flowsheet, equipment specifications, process and instrumentation diagrams, and waste-handling systems. During the specification of commercial-scale equipment and controls, it is crucial to study and adapt the administrative and manual tasks generated during pilot-scale operations related to closed-vessel policies, material handling, cleaning, waste handling, and other operational aspects. A well-integrated team approach, with a common project view of the need to balance cost constraints against sterility needs, is essential.

While fermentation-based syntheses were once reserved for producing high-value specialty chemicals and biopharmaceuticals, commercial-scale bioprocess facilities are already producing vaccines and therapeutic pharmaceuticals (such as Amgen's Epogen and Wyeth's Mylotarg), food products (L-phenylalanine, a building block for NutraSweet) and food-grade additives (such as the algae-derived fatty acid DHA (docosahexaenoic acid) and ARA from Martek Biosciences, which is used as a nutritional additive). The development of large-scale production of recombinant therapeutic proteins with bacterial and mammalian cell cultures, supplanting older techniques for extracting proteins from blood and making vaccines in animal tissues as well as launching a whole new category of magic-bullet medicines: monoclonal antibodies. Other specialty and commodity biochemical facilities in BP and DuPont are teaming up to commercialize bio-based butanol as a gasoline blendstock in 2007. Genomatica and DuPont Tate & Lyle Bio Products successfully produced 1,4-butanediol (BDO) on a commercial scale in 2012.

Additional challenges may arise because emerging synthesis routes often exhibit a high degree of change throughout the scale-up and design stages. Also the design team must anticipate and manage changes to the design and construction specifications to minimize costs and keep the project on schedule.

Biomanufacturing is also becoming increasingly data dependent. The IT systems that support quality and process analytics, document manufacturing results, and store records for regulators share the legacy issues of all IT systems.

When producing pharmaceuticals and food additives, biosafety issues must be considered as product contact streams are regulated by the USFDA as well as by the US Department of Agriculture (USDA) in the case of biosynthesis. The US Environmental Protection Agency (USEPA) and the National Institutes of Health (NIH) have also issued guidelines for handling many of the commercial microorganism strains. In addition, in the United

States, Toxic Substances Control Act regulations establish procedures for commercializing new or modified strains.

The genetically or pathway-engineered organisms must be out of the surrounding environment. While such improvements are worthwhile, they may necessitate changes in equipment or utilities. For example, increased metabolic rates can enhance throughput, provided the higher heat generation can be controlled within the required temperature band, and agitation and delivery systems are sufficient to deliver the needed nutrients and oxygen to the more quickly multiplying organisms. While in all commercial-scale bioprocess facilities, (i) the upstream biosynthesis operation calls for rigorous sterility requirements, (ii) the downstream portion employs specific engineering unit operations to extract and purify the target product, and appropriately dispose of all waste streams. The particular engineering requirements and challenges associated with each of these two distinct portions differ, but must be tightly integrated during process design to ensure the most-cost-effective plant operation.

During fermentation, each of multiple fermentation vessels required by a commercial-scale facility will have its own particular design and operating requirements. These include the need to introduce the fermentation broth, sterile air, and sterilized nutrients. When air lift in the vessel cannot provide sufficient mixing, the fermenter may be equipped with low-shear agitation devices. Fermentation vessels must also be designed to ensure adequate heat-removal capabilities and promote cooling as needed. Sufficient safeguards must also be in place (both through design elements and operating procedures) to guard against contamination and cell mutation by double-block and bleed valves, and steam-in-place (SIP)/clean-in-place (CIP) systems.

Meanwhile, the variable flow rates associated with different stages of the cells metabolism and growth cycle, and the required cleaning cycles have tremendous design implications for process parameters including flow and pressure. All of these factors complicate the internal geometry in terms of baffles and agitators of the vessel, as well as the number, location and type of tank nozzles and ports needed.

Commercial-scale fermentation vessels must also be equipped with a variety of advanced instruments, sensors, and transmitters to monitor everything from pressure, level, and temperature inside the fermenter to pH, DO, and nutrient levels in the fermentation broth. The appropriate number and location of the analytical instruments and in-process checks must also be reconciled against capital and operating cost constraints, and sterilization concerns.

When the desired product ends up in the fermentation broth (excreted from the microorganisms or within the cell body), cells are destroyed; the product is separated and purified; and the dead cell bodies, unreacted carbohydrate feedstock/nutrients and by-products are removed, concentrated and neutralized before disposal. The dead cell bodies and other solid waste, and the high biological oxygen demand (BOD) aqueous streams produced throughout the process must be disposed of properly. The specific handling and disposal requirements are ultimately dictated by the biosafety classification of the microorganisms in the waste stream. Residual high-BOD aqueous waste streams are typically treated in onsite aerobic or anaerobic digesters. While aerobic digestion is economically applied for BOD up to 10,000 ppm, anaerobic digestion is generally used from 8,000 ppm and up.

Several issues that further deserve particularly close attention during the design and construction of large-scale bioprocess facilities are (i) the extreme sensitivity of the modified fragile organisms creates unique design and operating challenges, particularly when it comes to maintaining close control over all of the critical operating parameters within vessels; (ii) contaminants by phage infections and mutations within the bioengineered organism population, can lead to the disposal of the valuable fermentation batch and an

immediate shutdown for sterilization and cleanout. Designing systems that adequately filter airborne contaminants and bacteria, and remain dried (to avoid entrained condensate carrying bacteria through filters) requires design rigor that is crucial for facilities operating in hot, humid climates; (iii) commercial-scale fermentation facilities handle enormous volumes of water and steam (with varying composition and temperature) from fermentation, purification, evaporation and cleaning systems and thus recycle of water and energy reuse must be maximized; (iv) bioprocesses require removal of large volumes of water, and thus considerable operating savings can be realized by opting for today's highly efficient separations technologies, such as evaporation with mechanical vapor recompression and multiple-effect evaporators; (v) proper design of the waste-disposal facilities can also help to contain operating costs by further drying using standard press, plate, belt or drum dryers before being sent to a landfill for disposal.

The biosafety classification of the microorganism used in the fermentation process will determine the level of containment that is required for operations such as sampling, offgas venting and waste disposal to minimize the potential for biohazard risk to personnel and the environment. For the successful scale-up, the identification and modification of a suitable organism, followed by prudent pilot-scale studies certainly is crucial to success with bio-based manufacturing. A scale-up strategy that combines integrated teamwork with solid engineering efforts can minimize costly rework and delays, and help today's promising manufacturing routes based on renewable feedstocks to achieve their full commercial-scale potential on time and budget.

Summary

Effective interaction between biotechnologists and bio(chemical)engineers has been the key factor for both the technological innovation and the commercial success of the industrial microbiological processes. The primary objective of bioengineering practice is to develop processing systems that economically transform raw materials into marketable products using fermentation and downstream or product recovery processing.

The microbial and processing engineering factors that affect the commercial process are productivity and conversion efficiency, which are in turn controlled by the physical phenomena occurring in the reactor through mass and heat transfer capacities. Process constraints and limitations imposed by the instabilities of biological materials and by such new technologies as immobilized reactors, rDNA and hybridoma require the prevention of product denaturation and high expenditures on development.

When fermentation is complete, it is necessary to recover the desired end product; this involves separation of the cells and other particulates from the fermentation broth, but purification of biomolecules or metabolites with or without cell disruption may be required, as well. Such operations are referred to as downstream processing. Purification usually involves a series of adsorption/desorption or chromatography columns, and principles and applications in industrial scale were discussed. In finishing operations, the final volume is further reduced and the product is stored until end use. But at various points along the way, analyses are needed to facilitate the implementation of quality and safety considerations.

Recently systems for controlling process variables applicable to a bioprocess have become increasingly sophisticated employing digital systems such as PLCs, micro-processor based software control systems, or a hybrid arrangement. This has enabled large molecule pharmaceutical manufacturing to move toward the level of reproducibility that semiconductor processing now enjoys.

1.8 Molecular thermodynamics for biotechnology

Thermodynamics is a fundamental discipline that enables us to understand how energy is handled by living organisms. Many of the concepts are better understood by considering that the laws of thermodynamics are based on the stochastic behavior of large sets of molecules. However, cells are nonequilibrium systems in which information plays an essential role and thus, several nonstandard concepts should be explained why these very ordered systems proliferate actively in our planet despite their apparent disregard of the second law of thermodynamics. The *second law of thermodynamics*, commonly known as the law of increased entropy, states that in all energy exchanges, if no energy enters or leaves the system, the potential energy of the state will always be less than that of the initial state.

Until 1950, thermodynamics was not a prominent field in biotechnology, probably due to lack of data with respect to biomolecular properties, thermodynamic equilibrium positions, energy efficiency relations and the complexity of biological systems. Although most of the bioprocesses were not optimized as chemical processes, bioprocess techniques with recent developments in rDNA technology have increased significantly the application of thermodynamics in various fields of biotechnology (Table 1.27). The knowledge of the cell as a highly efficient cell factory in catalyzing/noncatalyzing reactions and minimizing energy consumption and side-reaction products has led to a reassessment of the principles in many classical chemical engineering principles. The improved understanding of molecular thermodynamic opens up an immense field of new possible applications for the chemical industry and biotechnology. All biotechnology-related business is now using biological information and techniques at the molecular level toward the discovery of new products and new processes. Thus, it has become important that physical, thermodynamic, and structural information are imperative to gain a deeper sense of the functional properties of biological macromolecules. The recent efforts in this field have resulted in models, simulation methods, and tools that allow not only solving basic science problems, but also contribute substantially to industrial research and development. Modified cells with tailor-made expression systems are now utilized for large-scale commercial production of chemicals, enzymes, and biopharmaceuticals.

As molecular thermodynamic can be applied to various fields, it is not easy to cover all, but only two important subjects on biotechnology: (i) Protein stability and protein folding and (ii) Downstream processes on crystallization and chromatography will be emphasized.

1.8.1 Protein folding and stability

Proteins, polymers of different amino acids joined by peptide bonds, serve many important physiological functions and have unique structural properties of proteins, especially on

Table 1.27 Applications of molecular thermodynamics in various fields of biotechnology

-
- Equilibrium studies in downstream processing
 - Bioprocess optimization
 - Biomolecule properties
 - Protein stabilization
 - Metabolite and biomass production
 - Cell transport
 - Agriculture and food production
 - Environment protection and biofuels
-

stability and folding. Protein structures are stabilized by noncovalent intramolecular interactions between amino acid side chains. Protein complexes are also formed by specific noncovalent intermolecular interactions. All biological processes depend on proteins being stable and in the appropriate folded conformation. It is important to know how proteins fold into their biologically active states, and how these active states are stabilized. A primary goal of protein engineering, rational drug design, and biopharmaceutical production is the development, production, and storage of stable proteins with full functionality. Although there have been rapid advances in structural biology and relating structure to biochemical function and mechanism, knowledge of protein structure alone does not ensure accurate prediction of stability, function, and biological activity. The complete characterization of any protein requires stability determination and the forces which lead to stability and correct folding. Protein stability is intimately connected with *protein folding* – proteins have to be folded into their final active state (and maintain it) to be stable. Protein folding is the process by which newly synthesized polypeptide chains acquire the three-dimensional structures necessary for biological function. The correct *three-dimensional structure* is essential to function, though some parts of functional proteins may remain unfolded.

Thermodynamic studies As shown in Figure 1.23, an important factor to be considered during protein folding is whether the process is thermodynamically or kinetically controlled. The information required for the proper folding is available in the linear polypeptide chain, but stable 3D structure of proteins can be affected by *miscoding* (error in protein synthesis) and/or *misfolding* (error in protein folding). Many theories on protein folding and stability have been proposed on theoretical and experimental studies, but the last step in Figure 1.23 as to whether protein folding is controlled by thermodynamics or kinetics is still unknown. Among many, two models of protein folding are currently being confirmed. In the diffusion collision model by Karplus and Weaver (1994), nucleation occurs at different regions of polypeptide chain forming microstructures that diffuse and coalesce to form substructures with the native conformation. In the nucleation-condensation model, the secondary and tertiary structures of the protein are made at the same time. Recent studies have shown that some proteins show characteristics of both of these folding models.

The folding of proteins is probably known as one of the most complex processes in biochemistry as it occurs in three important steps. The initial step occurs very quickly within about 0.01 s, followed by the formation of the molten globule in about 1 s, and then the final step forms the native protein structure in a much slower step within 500–2500 s. Its understanding implies the characterization from the unfolded state of all conformational changes such as intermediate and transition states that separate the unfolded polypeptide from its fully folded and active form. The situation is also rendered more complicated because most of the proteins are made up of several domains all having their own thermodynamic and kinetics parameters of folding.

Counter pressures are also exerted by the possible existence of disulfide bridges, which have to be appropriately paired through oxidative processes driven by the disulfide bonds generation machinery implicating oxido-reductases and isomerases as well as by the existence of the *cis* and *trans* possible configuration of the peptide bonds immediately preceding the prolyl residues. This *cis*–*trans* isomerization is dependent on a ubiquitous class of foldases known as peptidyl-prolyl *cis*–*trans* isomerases. Current theories of protein folding clearly indicated that the formation of stable protein structure after synthesis is a thermodynamically controlled process. Protein stability depends in the free energy change between the folded and unfolded states which is expressed by the following equation:

$$-RT \ln K = \Delta G = \Delta H - T\Delta S$$

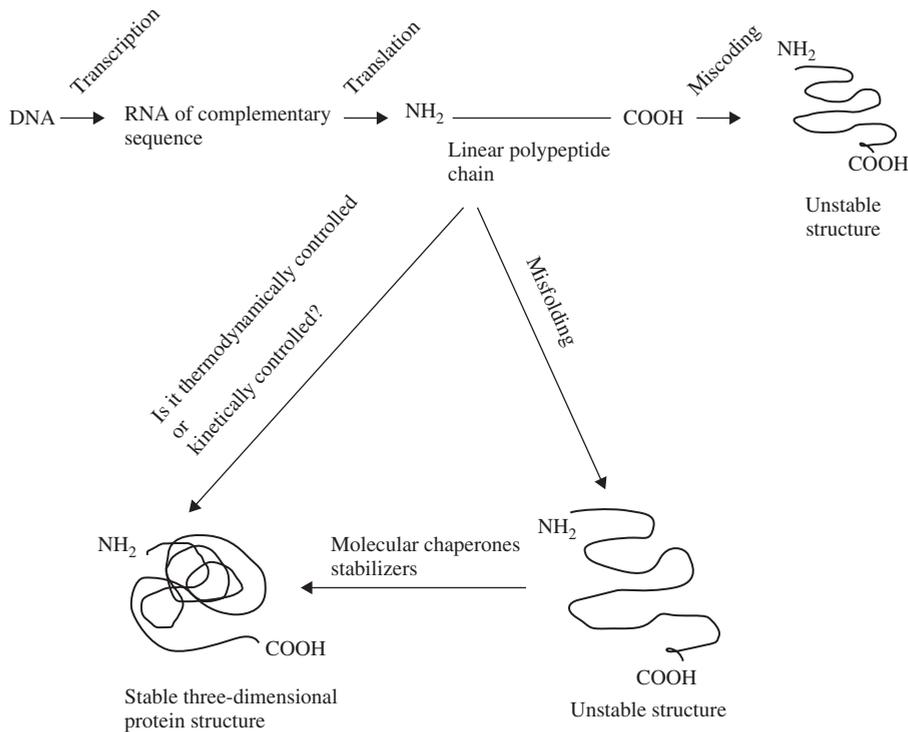


Figure 1.23 Different steps involved in synthesis of stable protein. *Source:* With kind permission from Springer Science+Business Media: Biotechnology and Bioprocessing Engineering, 8, 2003, 9–18, SN Gum-madi, Fig. 2.

where R represents the Avogadro number, K , the equilibrium constant, G , the free energy change between folded and unfolded, H , the enthalpy change and S , the entropy change from folded to unfolded. The enthalpy change, H , corresponds to the binding energy (dispersion forces, electrostatic interactions, VDW potentials and hydrogen bonding), while hydrophobic interactions are described by the entropy term, S . Proteins become more stable with increasing negative values of G , as the free energy of the unfolded protein (G_U) increases relative to the free energy of the folded or native protein (G_F). In other words, as the binding energy increases or the entropy difference between the two states decreases, the folded protein becomes more stable. The folded conformation of a domain is apparently in a relatively narrow free energy minimum, and substantial perturbations of that folded conformation require a significant increase in free energy. Measured by free energy, the maximum occurs when $S = 0$, while that measured by the equilibrium constant occurs when $H = 0$. These maximum stabilities can occur at quite different temperatures, but both are used in different situations. Regardless of which one is used, however, the stability of the folded state decreases at both higher and lower temperatures.

While factors such as binding interactions do obviously play a part in stabilizing the protein, they cannot account for a very significant portion of stabilization effects since similar phenomena occur in the unfolded state, the hydrophobic effect is probably the major stabilizing effect. The thermodynamics of protein stability is modeled quite well by the

Energy landscape theory, where the energy of a protein is a function of the topological arrangement of the atoms. The description of complex systems such as proteins requires more than such simplistic models. Each value in this surface describes the protein in a specific conformation, and there is an energy landscape for each state of the protein (e.g., neutral, charged, folded, intermediate, or unfolded).

The thermodynamic behavior of proteins as determined in various temperature-jump experiments is best described by stretched exponentials as opposed to Arrhenius's law, where the rate coefficient decreases with increasing speed as the temperature is reduced as follows,

$$\kappa(T) = \kappa_0 \exp\left(-\left(\frac{E}{kT}\right)^2\right)$$

This behavior corresponds to that of what are described as glasses or spin-glasses, which undergo a transition in which transition temperature depends on the characteristic observing time.

The random-energy model by Bernard Derrida (1981) correlates well with the rough energy landscape diagram for proteins. The random-energy model described the misfolding protein states on the energy landscape, with the misfolding minima acting as "traps" that slow down the protein molecules folding process; these traps become successively more difficult to escape as the temperature is lowered. The robustness of the protein native structure to conformational change is a consequence of the funneled nature of the energy landscape of a minimally frustrated protein. The geometry of this landscape cannot be significantly changed by the modification of a few isolated residues. Random heteropolymers on the other hand, have an energy landscape consisting of multiple funnels with each on leading to a different structure, making them more inclined to conformational change as a result of sequence modification.

Molecular chaperons Failure to fold into native structure produces inactive proteins that are usually toxic. To avoid these toxic species, cells appear to invest in a complex network of special proteins, known as *molecular chaperons*, which use ingenious mechanism to prevent protein aggregation and promote efficient folding. As protein molecules are highly dynamic, constant surveillance of chaperon is essential to ensure protein homeostasis (proteostasis). The folding process often begins cotranslationally, so that the N-terminus of the protein begins to fold, while the C-terminus portion of protein is still being synthesized by the ribosome. All cells have thus evolved various measures to cope with the presence of misfolded proteins. The two main alleviatory strategies employed by cells are probably either to prevent the misfolded proteins from aggregation and refold them to the native state by the help of molecular chaperons or to target them for degradation in case of unattainable native state. Specialized proteins called *chaperons* assist in the folding of many proteins. The chaperones are major prokaryotic and eukaryotic proteins, with the function of helping in the folding of nascent polypeptide chains, helping refolding of denatured proteins, and preventing aggregation of surface-exposed hydrophobic parts of proteins, having problems with folding. Chaperones help the proteins to fold, so they increase the speed of folding, by stabilizing unstable intermediates of the appropriate polypeptide chain, and decreasing the activation-energy barriers during folding. They do not change the thermodynamics of folding, that is, the ratio of folded and unfolded polypeptides. They only influence the kinetics of gyration in that they are often correlated with the enzymes. However, sometimes they are very similar to them, but sometimes are very different, as they are not too specific for the ligands, they help to fold, the substrates are very large, and their large-scale functions make them key-molecules of the cells. Mostly, they recognize

hydrophobic surfaces on the proteins, and prevent them from aggregation. Besides this function, chaperones can play an important role in signal transduction, in the maintenance of the organized state of the cytoplasm and other intracellular compartments, in the motions inside the cell, and some other vital functions of the cells.

Sometimes they are called *stress proteins*, or *heat-shock proteins (Hsps)*, because their synthesis increases (in most of the cases) after various forms of cellular stress, such as heat, cold, detergents, increase of ionic strength, changes in pH, toxic agents. However, the termini are not equivalent to each other, as some of the chaperones' level does not change upon stress.

Recent studies on certain neurodegenerative diseases such as *Alzheimer*, *transmissible bovine spongiform encephalopathy (BSE)*, commonly known as *mad cow*, variant *Creutzfeldt–Jakob disease* in humans, hemolytic anemia, *Parkinson's disease*, and so on, arises due to *protein misfolding*. This suggests that knowledge on protein folding and unfolding is very important. The process of protein folding, while critical and fundamental to virtually all of biology, still remains a mystery. *Macromolecular crowding* may be important in chaperone function. The crowded environment of the cytosol can accelerate the folding process, since a compact folded protein will occupy less volume than an unfolded protein chain. However, crowding can reduce the yield of correctly folded protein by increasing protein aggregation. Crowding may also increase the effectiveness of the chaperone proteins such as *GroEL*, which could counteract this reduction in folding efficiency.

Folding an isolated or expressed globular protein is a major biotech problem and often limits the commercialization of protein products. Many proteins, especially large ones, do not refold efficiently after being unfolded or, when they are over expressed in bacteria, form IBs that represent fairly homogenous, insoluble aggregates of the expressed protein.

More information on the various types and mechanisms of a subset of chaperones that encapsulate their folding substrates can be found in the *chaperonins*. Chaperonins are characterized by a stacked double-ring structure and are found in prokaryotes, in the cytosol of eukaryotes, and in mitochondria. Other types of chaperones are involved in transport across membranes, for example, membranes of the mitochondria and ER in eukaryotes. New functions for chaperones continue to be discovered, such as assistance in protein degradation, bacterial adhesion activity, and in responding to diseases linked to protein aggregation (e.g., *prion*).

Major chaperon classes Chaperons are usually classified by their molecular mass (*Hsp40*, *Hsp60*, *Hsp70*, *Hsp90*, *Hsp100*, and the *small Hsps*) in Table 1.28. They are known to be involved in many proteome-maintenance functions like *de novo* folding, refolding of stressed-denatured proteins, oligomeric assembly, protein trafficking and assistance in proteolytic degradation. The known chaperons involving in *de novo* protein folding and refolding are Hsp70s, Hsp90s, and chaperonins (Hsp60s) that are multicomponent molecules in promoting folding through ATP and cofactor-regulated binding and release cycles. These chaperons typically recognize hydrophobic amino acid side chains exposed by nonnative proteins that may functionally cooperate with *ATP-independent chaperons* (e.g., small Hsps) functioning as “*holdases*.”

The *Hsp70 chaperones*, which are essential in all eukaryotes, assist a large number of protein-folding processes, including *de novo* folding of polypeptides, refolding of misfolded proteins, solubilization of protein aggregates, degradation of proteins, translocation of proteins across membranes, assembly and disassembly of oligomeric complexes, and the regulation of stability and activity of certain natively folded proteins. In the ATP-dependent mechanism, *de novo* folding and refolding is promoted by kinetic

Table 1.28 The heat shock protein family of molecular chaperons

Chaperons	Protein family	Prokaryotes/eukaryotes	Function/structure
Hsp70	Dnak	Hsc73 (cytosol) BIP (endoplasmic reticulum) SSc1(mitochondria) Ct Hsp70 (chloroplasts)	ATP-dependent stabilization/Solved
Hsp60	GroEL	TRiC (cytosol) Hsp60 (mitochondria) Cpn60 (chloroplasts)	ATP-dependent protein folding /Solved
Hsp90	HtpG	Hsp90 (cytosol) Grp94 (endoplasmic reticulum)	ATP-driven reaction & maturation of steroid hormone receptors/Solved
Hsp100	-	ClpA, ClpX, HsIU	ATP-independent/Solved
Small Hsps (12–43 kDa)	-	-	ATP-independent/Some solved

partitioning ($K_{\text{fold}} > K_{\text{on}} > K_{\text{agg}}$), where folding, association and aggregation constants, respectively. Chaperon binding (or rebinding) to hydrophobic regions of a nonnative protein transiently blocks aggregation. The ATP-dependent reaction cycle of Hsp70 is regulated by chaperones of the Hsp40 family and nucleotide-exchange factors. Binding and release by Hsp70 is achieved by the allosteric coupling of a conserved amino-terminal ATPase domain with a carboxy-terminal peptide binding mechanism. Hsp70 system, a central player in protein folding and proteostasis control has proven effective in preventing toxic protein aggregation in disease models.

Chaperonins belong to a large class of molecules (about 800–900 kDa) that function by globally enclosing substrate proteins (up to about 60 kDa) for folding. Chaperonins are divided into two groups of common evolutionary origin: *group I chaperonins* are mostly found in prokaryotes, mitochondria, and plastids and *group II chaperonins* in archaea and the eukaryotic cytosol. Group I chaperonins, such as *E. coli GroEL*, are homo-oligomers with seven 57 kDa subunits per ring and the two rings contact each other in a 2:1 subunit arrangement, that is, one subunit of one ring interacts with two subunits in the other. Group II chaperonins, such as TRiC/CCT in the eukaryotic cytosol or the thermosome in archaea, are homo- or hetero-oligomers with eight or nine subunits of 57–61 kDa per ring, and their rings are exactly in register with 1:1 interring subunit contacts. For refolding of misfolded proteins, group I chaperonins cooperate with a homoheptameric cochaperone of 10 kDa subunits (*GroES* in *E. coli*), which closes the folding chamber like a lid by interacting with the apical domains. In contrast, group II chaperonins do not cooperate with a lid-forming cochaperone but instead have an insertion in the apical domain, which functions as a built-in lid. The *GroEL/GroES complex* in *E. coli* is the best characterized chaperonin complex, in which GroEL is a double-ring 14mer with a greasy hydrophobic patch at its opening and GroES is a single-ring heptamer binding to GroEL in the presence of ATP transition state analogs of ATP hydrolysis.

Hsp90 forms a proteostasis hub that controls numerous important signaling pathways in eukaryotic cells. Hsp90s like Hsp60s and Hsp70s can bind to misfolded proteins and

prevent their aggregation, but their main essential function is believed to be the interaction with a defined set of proteins in a native or near-native state. Many of these proteins are transcription factors and protein kinases involved in the control of cell homeostasis, proliferation, differentiation, and apoptosis. The *Hsp100/Clp* family of chaperones belongs to the superfamily of AAA+ domain-containing ATPases associated with various cellular activities. The AAA+ domain is characterized by sensor 1 and sensor 2 sequence motifs consisting of two subdomains. Most AAA+ proteins form oligomers with ATP bound close to the interface between subunits, and the neighboring subunit contributes the so-called arginine finger for ATP hydrolysis. Hsp100 proteins contain one or two AAA+ domains arranged in hexameric rings with a central pore through which substrate proteins can be threaded. Many Hsp100 proteins (*ClpA*, *ClpX*, *HslU* in *E. coli*) associate with *ring-forming peptidases* (*ClpP*, *HslV*), unfold proteins, and feed them into the proteolytic chamber. ClpB in *E. coli* and its relatives in lower eukaryotes (*Hsp104*) and plants (*Hsp101*) do not associate with peptidases but cooperate with the Hsp70 system to dissolve protein aggregates. The molecular mechanism of Hsp100 chaperones is best understood for *ClpX*. Two crystal structures of this covalently linked ClpX protein provide key insights into the conformational changes associated with the ATPase cycle and suggest a mechanism by which ATP hydrolysis is coupled to substrate translocation.

The Hsp100/Clp family of chaperones also belongs to the superfamily of AAA+ domain-containing ATPases associated with various cellular activities. The AAA+ domain is characterized by sensor 1 and sensor 2 sequence motifs and consists of two subdomains. Two crystal structures of this covalently linked ClpX protein provide key insights into the conformational changes associated with the ATPase cycle.

Many Hsp100 proteins (*ClpA*, *ClpX*, *HslU* in *E. coli*) associate with ring-forming peptidases (*ClpP*, *HslV*), unfold proteins, and feed them into the proteolytic chamber. ClpB in *E. coli* and its relatives in lower eukaryotes (*Hsp104*) and plants (*Hsp101*) do not associate with peptidases but cooperate with the Hsp70 system to dissolve protein aggregates. The molecular mechanism of Hsp100 chaperones is best understood for ClpX.

Small heat-shock proteins (sHsps) also exist in all three domains (Archaea, Bacteria, and Eukarya) and possess molecular chaperone activity by binding to unfolded polypeptides and preventing aggregation of proteins *in vitro*. These are a family of stress-inducible molecular chaperones that range in size from 12 to 43 kDa and that form oligomers consisting 9–50 subunits. The ability of sHsps to form oligomers contributes to their thermal stability and ability to avoid denaturation in response to high temperatures. At denaturing temperatures, sHsps can prevent the aggregation of proteins by binding to, and forming a stable complex with, folding intermediates of their substrate proteins. In some cases, sHsps can also promote renaturation of unfolded polypeptides. All proteins of this family contain the so-called α -crystallin domain (α C domain or ACD), a region of ~90 residues. This domain is considered an important hallmark of sHsps, independent of their origin and nature. sHsp shows extensive sequence variation and evolutionary divergence. Many sHsps have been shown to act in an ATP-independent fashion to bind up to an equal weight of nonnative protein to limit aggregation and to facilitate subsequent refolding by ATP-dependent chaperones.

In addition to chaperones, other enzymes are also involved in proper folding of some proteins within the cell. *Protein disulfide isomerases (PDI)* are involved in the proper formation of some disulfide bonds. Another set of such enzymes are the peptidyl prolyl *cis-trans* isomerases (PPI). PDI catalyze formation of disulfide bonds (—S—S—), that act like “staples” in a protein structure and these can often form in a complex pathway, for example, BPTI (bovine pancreatic trypsin inhibitor). Disulfide bond formation usually requires an oxidative environment (periplasm in bacteria, ER in eukaryotes), while the

cytoplasm is usually a reducing environment. *PPI* are ubiquitous enzymes – isomerases or rotamases that catalyze the cis–trans isomerization without breaking bonds. In proteins most peptidyl bonds are trans (omega=torison along the C–N bond=180) and this conformation is heavily favored in both denatured or folded forms. However, in extended chains, the peptide bond preceding a proline can be either in trans or in cis forms, with the trans form only slightly more favored than the cis form. In folded proteins, on the other hand, only about 7% of all prolyl-peptide bonds are cis. The ER is an important site for protein folding in eukaryotes. About 1/3 of all proteins in eukaryotes fold within the ER, especially all secretory and membrane proteins. ER is especially rich in chaperones, such as BIP, which belongs to the Hsp70 family.

Much progress has been made in understanding the molecular chaperons. Concerning the molecular mechanism, a clear picture is emerging for Hsp60s and Hsp100s. For Hsp100s, it is not clear how stalled substrates are dealt with and whether dissociation of the hexamer is an option or part of the mechanism. The picture is less clear for Hsp70s, Hsp60s, and sHsps.

Summary

All biotechnology-related business is now using biological information and techniques at the molecular level toward the discovery of new products and new processes. Thus, physical, thermodynamic, and structural information are thus imperative to gain a deeper sense of the functional properties of biological macromolecules. Molecular thermodynamic can be applied to various fields such as equilibrium studies in downstream processing, bioprocess optimization, biomolecule properties, protein stabilization, metabolite and biomass production, cell transport, agriculture and food production, and environment protection and biofuels. The recent efforts in this field have resulted in models, simulation methods, and tools that allow not only solving basic science problems but also contributing substantially to industrial research and development. Among many fields, protein stability and protein folding and downstream processes on crystallization and chromatography are very important in industrial applications. Protein stability is also important for many reasons in an understanding of the basic thermodynamics of the process of folding and increased protein stability may be a multibillion dollar value in food and drug processing, and in biotechnology and protein drugs. The process of protein folding, while critical and fundamental to virtually all of biology, remains a mystery. Moreover, perhaps not surprisingly, when proteins do not fold correctly (i.e., “misfold”), there can be serious effects, including many well-known diseases and failure to fold into native structure produces inactive proteins that are usually toxic. Incorrectly folded prion proteins must be able to deform their correctly folded analogs and to change their spatial structure. They transfer their own incorrect shape to the healthy proteins. When incorrectly folded, the protein has many regions containing β -sheets, structures and has a tendency to self-assemble into larger aggregates. These amyloids cannot be broken down and thus form deposits in the brain’s tissue (<http://phys.org/news/2013-08-deadly-molecular-mechanism-prion-protein.html#jCp>). Proteins fold through intermediate states that have exposed hydrophobic surfaces, and when the intermediates are present at high concentration, they have a strong tendency to aggregate. To avoid these toxic species, cell appears to invest in a complex network of special proteins, known as molecular chaperons, which use ingenious mechanism to prevent protein. The cell contains large protein oligomers called molecular chaperone that permits proteins to fold in protected environments aggregation and promote efficient folding. Besides this function, chaperones can play an important role in signal transduction, in the

maintenance of the organized state of the cytoplasm and other intracellular compartments, in the motions inside the cell, and some other vital functions of the cells.

1.8.2 Downstream processes on crystallization and chromatography

Downstream processing, which refers to the recovery and purification of biosynthetic products from natural sources such as animal or plant tissue or fermentation broth, consists of different unit processes. These depend on the product characteristics, purity level, and product localization in the cells (intracellular) or extracellular secreted into the medium. In addition to mechanical separations, the techniques used are vacuum drying, membrane filtration, precipitation, crystallization, chromatography, and lyophilization as discussed in the previous section. Downstream processing and analytical bioseparation both refer to the separation or purification of biological products, but at different scales of operation and for different purposes. Downstream processing implies manufacture of a purified product fit for a specific use, generally in marketable quantities, while analytical bioseparation refers to purification for the sole purpose of measuring a component or components of a mixture, that deal with sample sizes as small as a single cell. Downstream purification has the potential to become a critical bottleneck in large-scale production of expensive bioproducts.

Crystallization *Crystallization* process can be used to purify a substance or to remove water and salts in order to obtain a solid intermediate or product, and in drug substance formulation. Understanding the effects of the combinations of protein–protein, protein–solvent, and protein–solute interactions can provide insight into areas such as cellular processes, disease origins, and protein processing. The nature of a crystallization process is governed by both thermodynamic and kinetic factors, which can make it highly variable and difficult to control. Factors such as impurity level, mixing regime, vessel design, and cooling profile can have a major impact on the size, number, and shape of crystals produced.

Crystallization is the process of formation of solid crystals precipitating from a solution, molten material or more rarely deposited directly from a gas. Crystallization is also a chemical solid–liquid separation technique, in which mass transfer of a solute from the liquid solution to a pure solid crystalline phase occurs. Crystallization is an aspect of precipitation, obtained through a variation of the solubility conditions of the solute in the solvent, as compared to precipitation due to chemical reaction.

The crystallization process consists of two major events, *nucleation* and *crystal growth*. *Nucleation* is the step where the solute molecules dispersed in the solvent start to gather into clusters on the nanometer scale that become stable under the current operating conditions. These stable clusters constitute the nuclei, but when the clusters are not stable, they redissolve. Therefore, the clusters need to reach a critical size to become stable nuclei. Such critical size is dictated by the operating conditions (temperature, supersaturation, pH, salt, solvents, surface active agents, etc.). The difference between the actual value of the solute concentration at the crystallization limit and the theoretical (static) solubility threshold is called *supersaturation* and is a fundamental factor in crystallization dynamics. Supersaturation is the driving force for both the initial nucleation step and the following crystal growth, both of which could not occur in saturated or undersaturated conditions. It is at the stage of nucleation that the atoms arrange in a defined and periodic manner that defines the crystal

structure. The *crystal growth* is the subsequent growth of the nuclei that succeed in achieving the critical cluster size. Nucleation and growth continue to occur simultaneously while the supersaturation exists. Depending upon the conditions, either nucleation or growth may be predominant over the other, and as a result, crystals with different sizes and shapes are obtained. Once the supersaturation is exhausted, the solid–liquid system reaches equilibrium and the crystallization is complete, unless the operating conditions are modified from equilibrium to supersaturate the solution again.

Many compounds have the ability to crystallize with different crystal structures, a phenomenon called *polymorphism*. Each polymorphism is in fact a different thermodynamic solid state and crystal polymorphs of the same compound exhibit different physical properties, such as dissolution rate, shape (angles between facets and facet growth rates), melting point, and so on. For this reason, polymorphism is of major importance in industrial manufacture of crystalline products.

Protein crystallization, which has been mostly applied in protein structure analysis, has been recognized in principle as a method of protein purification. Within a crystal, protein molecules form a regular lattice able to exclude other proteins as well as misfolded protein molecules of the same type. Therefore, as routinely applied to small molecules, crystallization can also be used as a cheap and scalable purification procedure. The feasibility of protein purification by crystallization has been demonstrated for an industrial lipase or the model protein ovalbumin. However, the only biopharmaceutical routinely crystallized at industrial scale and with excellent recovery yields is insulin. Insulin is a small and extraordinarily stable peptide able to refold easily into its native structure even after exposure to organic solvents. It is crystallized late in the purification sequence where most of the impurities have already been removed. Additional benefits of protein crystallization from a formulation perspective are the higher stability of crystalline proteins in comparison to protein solutions, making crystalline formulations an attractive alternative with potentially longer shelf life, and the possibility to control delivery of a protein by making use of crystal dissolution kinetics. Many challenges of thermodynamics are in the design of a crystallization process on determinations of crystallization window, solubility curve, metastable zone, nucleation zone, nucleation, and growth kinetics as well as growth habit.

Chromatography The product purification process that is done to separate contaminants that resemble the product very closely in physical and chemical properties is expensive to carry out and requires sensitive and sophisticated equipment. This stage contributes a significant fraction of the entire downstream processing expenditure by using affinity, size exclusion, RPC, crystallization, and fractional precipitation especially for the therapeutic proteins. In principle, the separation methods exploit the differences in physicochemical, thermodynamic, or molecular properties between the target product and the contaminants. The separation is based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus changing the separation.

Chromatographic thermodynamics is the study of how to explain molecular separation behaviors in chromatographic methods thermodynamically. Large-scale chromatography operations continue to occupy a key position in the overall strategy for the downstream processing and purification of protein products for therapeutic use. In commercial manufacturing, a requirement exists to increase the scale of the chromatography operations, which are typically developed and optimized in small-scale experiments. Also, there are a broad range of next-generation sorbents and membranes commercially available

for process chromatography. These new chromatography media are often characterized by significantly improved performance compared to their classical chromatographies with higher dynamic binding capacities, higher operational flow rates, and specific and distinctive retention mechanisms. Nevertheless, traditional media based on agarose or polymers continue to be routinely used for new method development due to their proven suitability for protein purification in numerous Food and Drug Administration (FDA) or European Medicines Agency (EMA) approved applications.

The major drawback of chromatographic procedures is the high cost of adsorption media, which can cost more than US\$10,000 per liter of *Protein A* resin. Major breakthroughs to replace Protein A that are readily acceptable to regulatory authorities are unlikely in the short term to dramatically lower overall costs. However, recently modified manufacturing processes for anion and cation exchangers have significantly improved their performance. It is a matter of time before alternative to traditional chromatography for protein and antibody becomes available in industrial scale.

To quantify molecular thermodynamic properties and operating conditions, qualitative concepts based on the substantial experience can assist in translating the data into quantitative tools for designing separation methods and final process design. Physicochemical properties of biomolecules important in chromatography separation are molecular size, charge, pI, hydrophobicity, and affinity. Besides these, interactions of biomolecules among themselves in different environments are the key parameters in their partitioning in different phases. For example, the *osmotic second virial coefficient (SVC)* is a thermodynamic property of dilute protein solution, which characterizes pair-wise protein self-interactions. This SVC has been used to model and explain thermodynamically a number of separation techniques like crystallization, precipitation, aqueous two-phase separation, folding/refolding, and aggregation. However, some separation methods are very complex for modeling to obtain many physicochemical and thermodynamic parameters as input. Selection and design of downstream processing operations for biomolecules have thus been impossible in a systemic manner due to the lack of fundamental knowledge on the thermodynamic properties of the molecule to be separated.

Separation in ion-exchange chromatography occurs because of charge differences between product and contaminant molecules. As the net charge varies with the solution pH, the elution profile depends solely on operational pH and ionic strength. For a given operation condition, charge density than the net charge is the parameter affecting protein-partitioning behavior in ion-exchange chromatography. The separation principle of size-exclusion chromatography is based on molecular size, but concentration dependence of retention is a nonnegligible parameter at high concentration of biomolecules. The other chromatography techniques such as *hydrophobic interaction chromatography (HIC)* and *RPC* exploit the variable hydrophobic nature of biomolecules. HIC is based on the reversible interaction between the hydrophobic patches on the biomolecules and the mildly hydrophobic stationary phase at high salt concentration. Retentions of biomolecules in this system depend on the type and concentration of salt and density and type of hydrophobic ligand in the stationary phase.

The term *hydrophobicity* covers average *surface hydrophobicity*, location and size of hydrophobic patches on the biomolecule surface (named as surface hydrophobicity distribution). The basic retention process in RPC is basically the same as in HIC, but RPC matrices are generally more hydrophobic than HIC matrices; thus elution is done by organic solvents than aqueous solutions. The other affinity chromatography that is used in pharmaceutical protein purification is based on the biospecific affinity between the ligand attached on the stationary phase and the biomolecules in the liquid phase.

Summary

Downstream processing has a major impact on the economic performance of biochemical manufacturing processes, that may cost up to 80% of the total production costs; that is why a thorough and reliable development of separation processes is essential for the scale-up of economic processes for complex biochemical mixtures. Crystallization as an aspect of precipitation, obtained through a variation of the solubility conditions of the solute in the solvent can be useful to crystallize from downstream intermediates, in particular from crude homogenate, resulting in high product purity and scale-up to several kilograms per batch. Some products such as an IgG antibody were able to crystallize with a yield of 98%. This type of development enables the downstream processes, whereby time-consuming and expensive chromatographic steps can be replaced by the use of crystallization. In addition, protein crystallization has the potential to become a new economic tool for the stable formulation and storage of biopharmaceuticals.

Although new chromatography media are often characterized by significantly improved performance compared to their classical predecessors on higher dynamic binding capacities, higher operational flow rates, and specific and distinctive retention mechanisms, traditional media (e.g., based on agarose or polymers developed in the 1970s or even before) continue to be routinely used for the development of new methods due to their proven suitability for protein purification in numerous FDA or EMA approved applications. In these cases, the potential of modern ion exchangers and novel mixed-mode or multimode sorbents still remains poorly exploited. As a result, significant additional costs in production may occur and result in severe economic consequences.

1.9 Protein and enzyme engineering

As shown in the previous chapter, mutagens including ionizing radiations (e.g., X-rays, γ -rays), nonionizing radiations (e.g., UV light – which is strictly speaking not a mutagen) and various chemicals (e.g., sodium bisulfite, hydroxylamine or methoxylamine, nitrosoguanidine, benzene, ethidium bromide, etc.) have been used in the past to deliberately produce random mutations in the DNA of organisms in attempts to improve them – a process known as strain improvement. The mutants have then been screened and selected for improvements such as an increased yield. This is quite a success story since the yield of penicillin from industrial strains of the organism is millions of times higher than the original wild-type mold isolated from a moldy melon bought in a market. The success of this method depends on screening techniques to identify mutations that result in the desired properties. Both approaches should be used in parallel as regions of the molecule identified by *RM* may be used as targets for further rounds of site specific modification. *Site-directed mutagenesis (SDM)* will replace tedious traditional mutation procedures but this technique requires rDNA technology and also needs detailed knowledge of the three-dimensional structure of the enzyme. Since this information requires much research effort to obtain the data and this technique is an expensive undertaking, only improvements which are big enough to justify expensive R&D expenditures should be carried out. *Computer-aided molecular modeling* could provide a three-dimensional image of structures which will allow their manipulation in real time and visualization of molecular parts. A schematic diagram of protein engineering techniques is shown in Figure 1.24. Comparison of rational design (SDM) and directed evolution (RM) are shown in Figure 1.25.

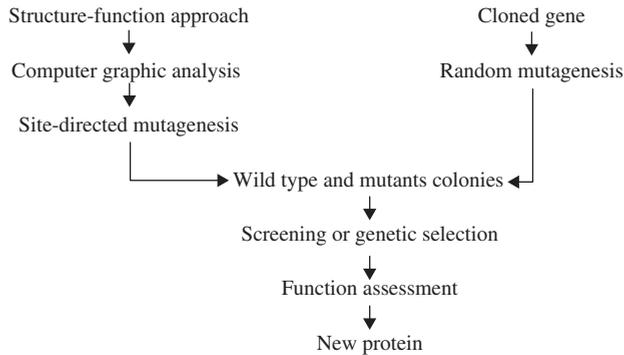


Figure 1.24 A schematic diagram of protein engineering techniques.

The desired result from those techniques never gave any guarantee (it was of the “hit-and-miss” nature) because mutations are random and most of them are disadvantageous to the organism. Nowadays, rDNA Technology offers ways of speeding up the process to make it far more specific. One of the rDNA technologies is *protein engineering*, which is the process of developing useful or valuable proteins. It is a young discipline involving many disciplines such as biology, protein chemistry, structural chemistry, and enzymology to define and exploit the relationship between protein structure and catalytic function. Two general techniques, *rational design* and *directed evolution*, for protein engineering are not mutually exclusive and are often applied. In the future, more detailed knowledge of protein structure and function, as well as advancements in high-throughput technology will greatly expand the capabilities of protein engineering.

In rational protein design, the scientist uses detailed knowledge of the structure and function of the protein to make desired changes. This technique is more specifically termed *in vitro mutagenesis* or *SDM*. This technique involves the alteration of single or a small number of known amino acids in a protein through the use of short oligonucleotide primers coding for the change so that its catalytic or other properties can be modified. This approach may also be applied to other proteins to modify antigenicity, digestibility, and the nutritional value. This generally has the advantage of being inexpensive and technically easy, since SDM techniques are well developed. However, its major drawback is that detailed structural knowledge of a protein is often unavailable, and even when it is available, it can be extremely difficult to predict the effects of various mutations. *Computational protein design algorithms* seek to identify novel amino acid sequences that are low in energy when folded to the prespecified target structure. While the sequence-conformation space that needs to be searched is large, the most challenging requirement for computational protein design is a fast, yet accurate, energy function that can distinguish optimal sequences from similar suboptimal ones.

Main methods in protein engineering are to: (i) isolate the gene coding for the protein/enzyme via mRNA and its conversion into cDNA, (ii) sequence the gene, (iii) decide on mutation that will “improve” the enzyme normally based on study of the enzyme’s three-dimensional structure and amino acid sequence as well as the computer modeling, (iv) use SDM to produce the desired change in codon(s) in the gene and subsequently the amino acid sequence of the enzyme molecule, and (v) test new enzyme for improvement.

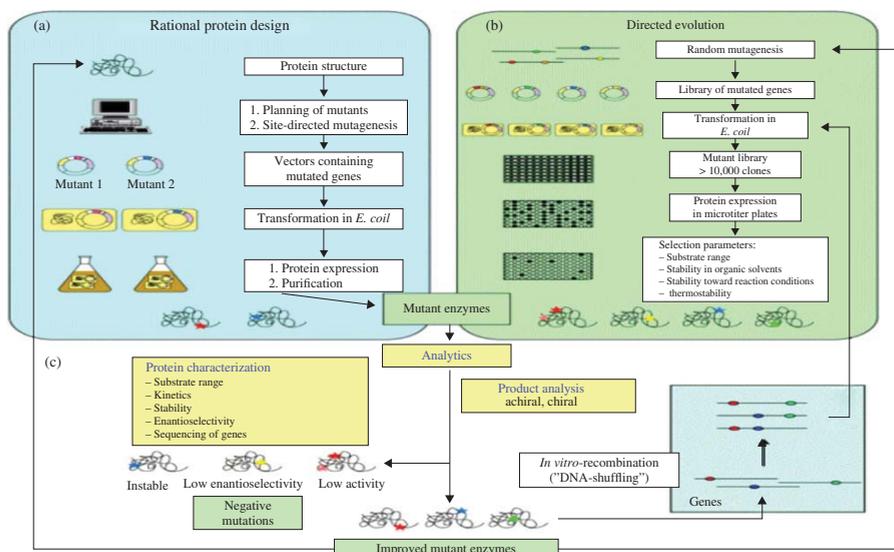


Figure 1.25 Comparison of rational design (site-directed mutagenesis, SDM) and directed evolution (random mutagenesis, RM). (a) In rational design, mutants are created on the basis of their protein structure and then prepared by SDM; after transformation in the host, the variant is expressed, purified and analyzed for desired properties. (b) Directed evolution starts with the preparation of mutant gene libraries by random mutagenesis (RM). They are then expressed in the host. Protein libraries are usually screened in microtiter plates using several parameters. Protein characterization and product analysis sort out desired and negative mutants. *In vitro* recombination by DNA shuffling, for example, can be used for further improvements. (c) Both protein engineering approaches can be repeated or combined until biocatalysis with desired properties is generated. Source: Bornscheuer and Pohl 2001. Reproduced with permission of Elsevier.

There are now several methods for carrying out SDM, including polymerase chain reaction (PCR)-based ones. The following one, called the *single-primer method* is based on the use of the phage vector M13.

Methods of SDM using M13 phage are to (i) isolate required enzyme gene, via mRNA and its conversion into cDNA, (ii) sequence the DNA of the gene (in order to decide on change required for primer in stage 5), (iii) splice gene into M13 vector dsDNA and transduce *E. coli* host cells, (iv) isolate ssDNA in phage particles released from host cells, (v) synthesize an oligonucleotide primer with the same sequence as part of the gene but with altered codon (mismatch/mispair) at desired point(s), (vi) mix oligonucleotide with recombinant vector ssDNA, (vii) use DNA polymerase to synthesize remainder of strand. (Oligonucleotide acts as a primer for the DNA synthesis). Then add ligase to make join between primer and new strand permanent dsDNA molecule, and (viii) transform *E. coli* cells and allow them to replicate recombinant vector molecule. DNA replication is semi-conservative, therefore two types of clone are produced each of which excretes phage particles containing ssDNA: type 1 containing the wild-type gene (i.e., unaltered) and type 2 containing the mutated gene. Ratio of the two types should be 1:1 but is not usually because *E. coli* “edits out” some of the mismatches, (ix) select mismatch clones bearing the mutation, and (x) if gene expression is required, use these clones to extract mutated DNA and insert it into an expression vector system with appropriate promoter, and so on, to produce the modified gene product (“designer protein”).

Directed evolution In directed evolution, *RM* is applied to a protein, a selection regime is used to pick out variants that have the desired qualities, and further rounds of mutation and selection are then applied. This method mimics natural evolution that generally produces superior results to rational design. An additional technique known as *DNA shuffling* mixes and matches pieces of successful variants in order to produce better results. This process mimics the recombination that occurs naturally during *sexual reproduction*. Directed evolution has a great advantage in that it requires no prior structural knowledge of a protein, nor is it necessary to be able to predict what effect a given mutation will have. The results of directed evolution experiments are often surprising in that desired changes are often caused by mutations that were not expected to have that effect. However, they require high-throughput, which is not feasible for all proteins. Large amounts of rDNA must be mutated and the products screened for desired qualities. The sheer number of variants often requires expensive robotic equipment to automate the process. A typical directed evolution experiment involves three steps: (i) *Diversification*: The gene encoding the protein of interest is mutated and/or recombined at random to create a large library of gene variants. Techniques commonly used in this step are *error-prone PCR* and DNA shuffling, (ii) *Selection*: The library is tested for the presence of mutants (variants) possessing the desired property using a screen or selection and isolated high-performing mutants by hand, while selections automatically eliminate all nonfunctional mutants, and (iii) *Amplification*: The variants identified in the selection or screen are replicated manyfold, and sequence their DNA in order to understand which mutations have occurred. These three steps are termed a “round” of directed evolution. Most experiments involve more than one round and at the end of the experiment, all evolved protein or RNA mutants are characterized using biochemical methods.

Directed evolution can be performed in living cells (*in vivo* evolution) or may not involve cells at all (*in vitro* evolution). *In vivo* evolution has the advantage of selecting for properties in a cellular environment, which is useful when the evolved protein or RNA is to be used in living organisms, but *in vitro* evolution is more often versatile in the types of selections that can be performed. *In vitro* evolution experiments can generate larger libraries

because the library DNA need not be inserted into cells, the currently limiting step. Most directed evolution projects seek to evolve properties that are useful to humans in an agricultural, medical, or industrial biocatalysis.

For the examples of engineered proteins, a protein with a novel fold, known as *Top7*, has been designed as well as sensors for unnatural molecules using computational methods. The engineering of fusion proteins has yielded *rilonacept* (IL-1 Trap), a pharmaceutical for the treatment of cryopyrin-associated periodic syndrome. Another computational method, IPRO (iterative protein redesign and optimization) successfully engineered the switching of cofactor specificity of *Candida boidinii* xylose reductase to increase or give specificity to native or novel substrates and cofactors. Computation-aided design has also been used to engineer complex properties of a highly ordered nano-protein assembly. A protein cage, *E. coli* bacterioferritin (EcBfr), which naturally shows structural instability and an incomplete self-assembly behavior by populating two oligomerization states is the model protein in this study.

Although many overproduced genetically engineered enzymes are commercially available, not many protein-engineered proteins are commercially available. Commercially marketed enzyme and protein are (i) *subtilisin* protease (from *Bacillus amyloliquefaciens*), which is the principal enzyme in the detergent enzyme preparation, Alcalase. This has been aimed at the improvement of its activity in detergents by stabilizing it at even higher temperatures, pH and oxidant strength, and (ii) The engineered fusion protein is *Rilonacept*, also known as IL-1 Trap (marketed by Regeneron Pharmaceuticals under the trade name Arcalyst), which is an IL-1 inhibitor for the treatment of cryopyrin-associated periodic syndrome. Further discussions on the engineered enzymes are in the Enzyme section in Part II.

Summary

Protein engineering, as the process of developing useful or valuable proteins, is a young discipline with much research taking place in the understanding of protein folding and recognition for protein design principles. Two general techniques for protein engineering, “rational” protein design and directed evolution are not mutually exclusive and both are often applied. Protein engineering aims at modifying the sequence of a protein, and hence its structure, to create enzymes with improved functional properties such as stability, specific activity, inhibition by reaction products, and selectivity toward nonnatural substrates. In the future, more detailed knowledge of protein structure and function, as well as advancements in high-throughput technology, may greatly expand the capabilities of protein engineering. New developments include advanced computational design, development of new and useful biocatalysts, and integration of functional biological parts with fabricated devices and construction of next generation biopharmaceuticals. Protein engineering also continues to provide valuable and fundamental understanding of natural protein construction and function that in turn will inevitably improve our ability to generate the next generation of novel proteins.

1.10 Genomics, proteomics, and bioinformatics

Genomics is to characterize the genome sequence of a number of model organisms that brought about a revolution in biology and medicine. Although the genetic blueprints of a number of model organisms are now available, the big challenge in deciphering the function

of the encoded proteins and how these contribute to the functioning of the organism as an entity remains. Functional genomic research uses the so-called post-genomic technologies to unravel the function of recently identified genes. The genomics researches include a comprehensive range of gene cloning, gene discovery/characterization, gene expression and protein expression, and characterization.

Functional genomics attempts to make use of the vast wealth of data produced by genomic projects such as genome sequencing projects to describe gene (and protein) functions and interactions and to answer questions about the function of DNA at the levels of genes, RNA transcripts, and protein products. The simple goal of functional genomics is to understand the relationship between an organism's genome and its phenotype. Unlike genomics, which is the static aspects of the genomic information such as DNA sequence or structures, functional genomics focuses on the dynamic aspects such as gene transcription, translation, and protein-protein interactions. A key characteristic of functional genomics studies is their genome-wide approach to these questions and generally involved in high-throughput methods rather than a more traditional "gene-by-gene" approach. The promise of functional genomics is to expand and synthesize genomic and proteomic knowledge into an understanding of the dynamic properties of an organism at cellular and/or organismal levels. This would provide a more complete picture of how biological function arises from the information encoded in an organism's genome. Understanding how a particular mutation leads to a given phenotype has important implications for human genetic diseases and this can eventually result in the discovery of a treatment or cure.

Functional genomics includes function-related aspects of the genome itself such as mutation and polymorphism analysis, as well as measurement of molecular activities. The latter comprise a number of "-omics" such as *transcriptomics* (gene expression), *proteomics* (protein expression), and *metabolomics*. Functional genomics uses mostly multiplex techniques to measure the abundance of many or all gene products such as mRNAs or proteins within a biological sample to quantitate the various biological processes and improve our understanding of gene and protein functions and interactions. Meanwhile, *Comparative genomics* is the study of the relationship of genome structure and function across different biological species or strains. Comparative genomics is an attempt to take advantage of the information provided by the signatures of selection to understand the function and evolutionary processes that act on genomes.

At the RNA level (*transcriptome profiling*), *microarrays* measure the amount of mRNA in a sample that corresponds to a given gene or probe DNA sequence. Probe sequences are immobilized on a solid surface and allowed to hybridize with fluorescently labeled "target" mRNA. The intensity of fluorescence of a spot is proportional to the amount of target sequence that has hybridized to that spot, and therefore to the abundance of that mRNA sequence in the sample. Microarrays allow for identification of candidate genes involved in a given process based on the variation between transcript levels for different conditions and shared expression patterns with genes of known function. *SAGE* (*serial analysis of gene expression*) is an alternate method of gene expression analysis based on RNA sequencing rather than hybridization. SAGE relies on the sequencing of 10–17 base pair tags, which are unique to each gene. These tags are produced from poly-A mRNA and ligated end-to-end before sequencing. SAGE gives an unbiased measurement of the number of transcripts per cell, as it does not depend on prior knowledge of what transcripts to study (as microarrays do).

At the protein level, *protein-protein interactions*, a *yeast two-hybrid* (Y2H) screen tests a "bait" protein against many potential interacting proteins ("prey") to identify physical protein-protein interactions. This system is based on a transcription factor,

originally GAL4, whose separate DNA-binding and transcription activation domains are both required in order for the protein to cause transcription of a reporter gene. In a Y2H screen, the “bait” protein is fused to the binding domain of GAL4, and a library of potential “prey” (interacting) proteins is recombinantly expressed in a vector with the activation domain. In vivo interaction of bait and prey proteins in a yeast cell brings the activation and binding domains of GAL4 close enough to result in the expression of a *reporter gene*. It is also possible to systematically test a library of bait proteins against a library of prey proteins to identify all possible interactions in a cell. New methods include protein microarrays, immunoaffinity chromatography followed by mass spectrometry (MS), dual polarization interferometry, microscale thermophoresis and experimental methods such as phage display and computational methods.

DNA microarray technology DNA microarray technology (also known as DNA arrays, DNA chips or biochips) shown in Figure 1.26 represents one of the latest breakthroughs and major achievements in experimental molecular biology. Although this

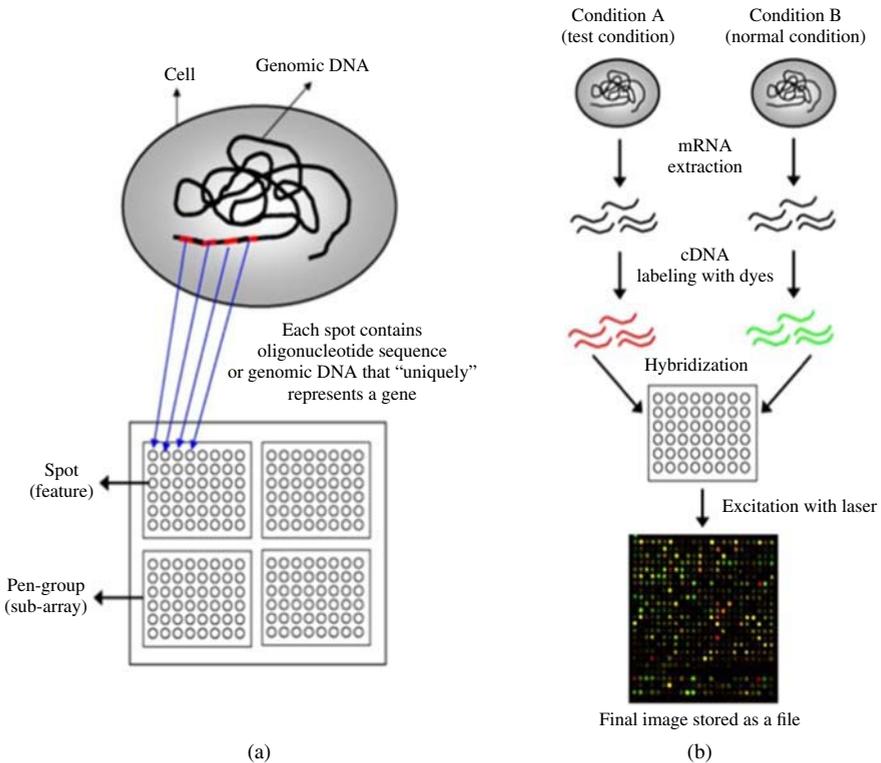


Figure 1.26 (a) A microarray may contain thousands of “spots.” Each spot contains many copies of the same DNA sequence that uniquely represents a gene from an organism. Spots are arranged in an orderly fashion into pen-groups. (b) Schematic of the experimental protocol to study differential expression of genes. Source: M. Madan Babu (2004) Computational Genomics: Theory & Application (ed. Richard P. Grant); Taylor & Francis ISBN: 978-1904933014; www.mrc-lmb.cam.ac.uk/.../microarray/figure1236.ppt. Reproduced with permission.

novel technology in which labeled nucleic acids could be used to monitor the expression of nucleic acid molecules attached to a solid support, the first article describing the application of DNA microarray technology (Brown and Botstein, 1999) to expression analysis was published in the scientific literature in 1995 by Dr. Patrick Brown at Stanford University (<http://hhmi.org/science/genetics/brown.htm>). Such widespread adoption of DNA microarray technology in both industry and many academic research laboratories was largely due to its aptitude to provide researchers the opportunity to quickly and accurately perform simultaneous analysis of literally thousands of genes in a massively parallel manner, or even entire genome of an organism, for example (Bacteria, Yeast, Virus, Protozoa, Mouse, or Human), in a single experiment, hence providing extensive and valuable information on gene interaction and function. DNA microarrays can be used to detect differences in the levels gene expression in different populations of cells on a genome-wide level. DNA microarray technology may be defined as a high-throughput and versatile technology used for parallel gene expression analysis for thousands of genes of known and unknown function, or DNA homology analysis for detecting polymorphisms and mutations in both prokaryotic and eukaryotic genomic DNA. Each identified sequenced gene on the glass, silicon chips or nylon membrane corresponds to a fragment of genomic DNA, cDNAs, PCR products, or chemically synthesized oligonucleotides of up to 70mers and represents a single gene. Usually a single DNA microarray slide/chip may contain thousands of spots each representing a single gene and collectively the entire genome of an organism. Each DNA spot contains picomoles (10^{-12} moles) of a specific DNA sequence, known as *probes* (or *reporters* or *oligos*). These can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA (also called antisense RNA) sample (called *target*) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target.

Microarray technology has evolved from Southern blotting, where fragmented DNA is attached to a substrate and then probed with a known DNA sequence. The first reported use of this approach was the analysis of 378 arrayed lysed bacterial colonies each harboring a different sequence, which were assayed in multiple replicas for expression of the genes in multiple normal and tumor tissue. This was expanded to analysis of more than 4000 human sequences with computer driven scanning and image processing for quantitative analysis of the sequences in human colonic tumors and normal tissue and then to comparison of colonic tissues at different genetic risk. These early gene arrays were made by spotting cDNAs onto filter paper with a pin-spotting device. The use of miniaturized microarrays for gene expression profiling was on a complete eukaryotic genome (*S. cerevisiae*) on a microarray in 1997.

The core principle behind microarrays is hybridization between two DNA strands, the property of complementary nucleic acid sequences to specifically pair with each other by forming hydrogen bonds between complementary nucleotide base pairs. A high number of complementary base pairs in a nucleotide sequence means tighter noncovalent bonding between the two strands. After washing off of nonspecific bonding sequences, only strongly paired strands will remain hybridized. Fluorescently labeled target sequences that bind to a probe sequence generate a signal that depends on the hybridization conditions (such as temperature), and washing after hybridization. Total strength of the signal, from a spot (feature), depends upon the amount of target sample binding to the probes present on that spot. Microarrays use relative quantization in which the intensity of a feature is compared to the intensity of the same feature under a different condition, and the identity of the feature is known by its position.

There are three basic types of samples that can be used to construct DNA microarrays, two are genomic and the other is transcriptomic, that measures mRNA levels. What makes them different from each other is the kind of immobilized DNA used to generate the array and, ultimately, the kind of information that is derived from the chip. The target DNA used will also determine the type of control and sample DNA that is used in the hybridization solution. Determining the level, or volume, at which a certain gene is expressed is called microarray expression analysis, and the arrays used in this kind of analysis are called "expression chips."

Basic steps in performing a DNA microarray experiment are: (i) The first step in the process of microarrays is the preparation of the target DNA, which can be done from the genomic source, or expressed sequence tags. (ii) The PCR is then used to amplify this DNA. (iii) This amplification step is vital in many applications and array formats. (iv) The DNA sequences are printed onto the microscope slides robotically, in a specific grid pattern. There are a number of methods for producing the microarray slides, and these are mainly done using robotic systems. They have their own advantages and disadvantages.

The organism is grown in two different conditions (a reference condition and a test condition). RNA is extracted from the two cells, and is labeled with different dyes (red and green) during the synthesis of cDNA by reverse transcriptase (RT). Following this step, cDNA is hybridized onto the microarray slide, where each cDNA molecule representing a gene will bind to the spot containing its complementary DNA sequence. The microarray slide is then excited with a laser at suitable wavelengths to detect the red and green dyes. The final image is stored as a file for further analysis (www.mrc-lmb.cam.ac.uk/.../microarray/figure1236.ppt). To determine which genes are turned on and which are turned off in a given cell, one must first collect the messenger RNA molecules present in that cell. One then labels each mRNA molecule by using a RT enzyme that generates a complementary cDNA to the mRNA. During that process fluorescent nucleotides are attached to the cDNA. The tumor and the normal samples are labeled with different fluorescent dyes. Next, the labeled cDNAs must be placed onto a DNA microarray slide. The labeled cDNAs that represent mRNAs in the cell will then hybridize – or bind – to their synthetic complementary DNAs attached on the microarray slide, leaving its fluorescent tag. A special scanner is then to measure the fluorescent intensity for each spot/areas on the microarray slides. If a particular gene is very active, it produces many molecules of messenger RNA, and thus, more labeled cDNAs, which hybridize to the DNA on the microarray slide and generate a very bright fluorescent area. Genes that are somewhat less active produce fewer mRNAs, thus, less labeled cDNAs, which results in dimmer fluorescent spots. If there is no fluorescence, none of the messenger molecules have hybridized to the DNA, indicating that the gene is inactive. One often uses this technique to examine the activity of various genes at different times. When co-hybridizing tumor samples (red dye) and normal sample (green dye) together, they will compete for the synthetic complementary DNAs on the microarray slide. As a result, if the spot is red, this means that specific gene is more expressed in the tumor than in normal (up-regulated in cancer). If a spot is green, then it means that gene is more expressed in the normal tissue (down regulated in cancer). If a spot is yellow, then that means that specific gene is equally expressed in normal and tumor.

Proteomics investigates the structure and function of proteins, the principal constituents of the protoplasm of all cells. The term proteomics was coined to make an analogy with genomics, the study of the genes. The *proteome* is the entire complement of proteins including the modifications made to a particular set of proteins, produced by an organism or system. While proteomics generally refers to the large-scale experimental analysis

of proteins, it is often specifically used for protein purification and *MS*. In many ways, proteomics runs parallel to genomics: Genomics starts with the gene and makes inferences about its products (proteins), whereas proteomics begins with the functionally modified protein and works back to the gene responsible for its production. The sequencing of the human genome has increased interest in proteomics because while DNA sequence information provides a static snapshot of the various ways in which the cell might use its proteins, the life of the cell is a dynamic process. This new data set holds great new promise for proteomic applications in science, medicine and most notably in pharmaceuticals.

After studying of genomics and transcriptomics, proteomics is considered the next step in the study of biological systems. It is much more complicated than genomics mostly because while an organism's genome is more or less constant, the proteome differs from cell to cell and from time to time. This is because distinct genes are expressed in distinct cell types, meaning that even the basic set of proteins which are produced in a cell needs to be determined. In the past this was done by mRNA analysis, but this was found not to correlate with protein content. It is now known that mRNA is not always translated into protein, and the amount of protein produced for a given amount of mRNA depends on the gene it is transcribed from and on the current physiological state of the cell. Proteomics thus confirms the presence of the protein and provides a direct measure of the quantity present. Not only does the translation from mRNA cause differences, but many proteins are also subjected to a wide variety of chemical modifications after translation. Many of these *post-translational modifications* are critical to the protein's function. One such modification is *phosphorylation*, which happens to many enzymes and structural proteins in the process of *cell signaling*. The addition of a phosphate to particular amino acids – most commonly serine and threonine mediated by serine/threonine kinases, or more rarely tyrosine mediated by tyrosine kinases – causes a protein to become a target for binding or interacting with a distinct set of other proteins that recognize the phosphorylated domain.

Ubiquitin is a small protein that can be affixed to certain protein substrates by enzymes called E3 ubiquitin ligases. Determining which proteins are poly-ubiquitinated can be helpful in understanding how protein pathways are regulated. This is therefore an additional legitimate “proteomic” study. In addition to phosphorylation and ubiquitination, proteins can also be subjected to methylation, acetylation, glycosylation, oxidation, and nitrosylation. Some proteins undergo all of these modifications, often in time-dependent combinations, that illustrate the potential complexity. Even cell may make different sets of proteins at different times, or under different conditions, any one protein can undergo a wide range of post-translational modifications, thus a “proteomics” study can become quite complex very quickly.

Applications of genomics and proteomics Analysis of different levels of gene expression in healthy and diseased tissues by proteomic approaches is as important as the detection of mutations and polymorphisms at the genomic level and may be of more value in designing a rational therapy. Protein distribution/characterization in body tissues and fluids, in health as well as in disease, is the basis of the use of proteomic technologies for molecular diagnostics. Proteomics will play an important role in medicine of the future which will be personalized and will combine diagnostics with therapeutics. Important areas of application thus include cancer (oncoproteomics) and neurological disorders (neuroproteomics) and crop improvement. A combinatorial approach of accelerated gene discovery through genomics, proteomics, and other associated -omic branches of biotechnology is proving to be an effective way to speed up the crop improvement programs worldwide. In the near future, swift improvements in -omic databases are becoming critical and demand immediate attention

for the effective utilization of these techniques to produce next-generation crops for the progressive farmers.

The markets for proteomic technologies are difficult to estimate as they are not distinct but overlap with those of genomics, gene expression, high-throughput screening, drug discovery, and molecular diagnostics. The largest expansion will be in bioinformatics and protein biochip technologies. The main objective and promise of Functional Genomics is to foster in-depth understanding of the processes that make up a living organism, which in turn will lead to new biomedical and pharmacological applications as well as biosynthetic and biotechnical developments.

The continuously increasing knowledge and data basis in the “-omics” field offers new prospects for the development of disease- or pathogen-specific biomarkers. Molecular diagnostics using such biomarkers provides the opportunity for early recognition of diseases combined with high sensitivity. However, the development of reliable diagnostic and prognostic markers and their validation is still a challenge. Next-generation sequencing genomic data are generated better, faster, and cheaper and different systems and technologies are emerging; both throughput and accuracy are the main targets for improvement. Next-generation sequencing opens the door to new diagnostic and prognostic applications as well as for food and agriculture including pathogen detection, and so on. Food proteomics is one of the most dynamic and fast-developing areas in food science in different safety aspects, such as food authenticity, detection of animal species in the food, and identification of food allergens and pathogens.

One of the most promising developments to come from the study of human genes and proteins has been the identification of potential new drugs for the treatment of disease. This relies on genome and proteome information to identify proteins associated with a disease, which computer software can then use as targets for new drugs. For example, if a certain protein is implicated in a disease, its 3D structure provides the information to design drugs to interfere with the action of the protein. A molecule that fits the active site of an enzyme, but cannot be released by the enzyme, will inactivate the enzyme. This is the basis of new drug-discovery tools, which aim to find new drugs to inactivate proteins involved in disease. As genetic differences among individuals are found, researchers expect to use these techniques to develop personalized drugs that are more effective for individuals. Proteomics is also used to reveal complex plant-insect interactions that help identify candidate genes involved in the defensive response of plants to herbivory.

Parallel analysis of the genome and the proteome facilitates discovery of post-translational modifications and proteolytic events, especially when comparing multiple species (comparative proteogenomics). Comparative proteomic analysis can reveal the role of proteins in complex biological systems, including reproduction. For example, treatment with the insecticide triazophos causes an increase in the content of brown planthopper (*Nilaparvata lugens* (Stål)) male accessory gland proteins (Acps) that can be transferred to females via mating, causing an increase in fecundity (i.e., birth rate) of females. To identify changes in the types of accessory gland proteins (Acps) and reproductive proteins that mated female planthoppers received from male planthoppers, researchers conducted a comparative proteomic analysis of mated *N. lugens* females. The results indicated that these proteins participate in the reproductive process of *N. lugens* adult females and males.

Limitations of genomics and proteomics studies Proteomics typically gives us a better understanding of an organism than genomics. First, the level of transcription of a gene gives only a rough estimate of its *level of expression* into a protein. Second, many proteins experience *post-translational modifications* that profoundly affect their activities; for

example, some proteins are not active until they become phosphorylated. Third, many transcripts give rise to more than one protein, through alternative splicing or alternative post-translational modifications. Fourth, many proteins form complexes with other proteins or RNA molecules, and only function in the presence of these other molecules. Finally, protein degradation rate plays an important role in protein content. Proteomics experiments conducted in one laboratory are not easily reproduced in another. A more common way to determine post-translational modification of interest is to subject a complex mixture of proteins to electrophoresis in “two-dimensions,” (so-called *two-dimensional gel electrophoresis*), which simply means that the proteins are electrophoresed first in one direction, and then in another, which allows small differences in a protein to be visualized by separating a modified protein from its unmodified form. Recently, another approach has been developed (*PROTOMAP*) that combines SDS-PAGE (polyacrylamide gel electrophoresis) with shotgun proteomics to enable detection of changes in gel-migration such as those caused by proteolysis or post-translational modification. For proteomic study, more recent techniques such as *matrix-assisted laser desorption/ionization (MALDI)* have been employed for rapid determination of proteins in particular mixtures and increasingly *electrospray ionization (ESI)*. The proteomic networks contain many *biomarkers* that are proxies for development and illustrate the potential clinical application of this technology as a way to monitor normal and abnormal cell development for guided development of clinical diagnostics by computer modeling.

New *fluorescence two-dimensional differential gel electrophoresis* can also be used to quantify variation in the 2D DIGE process and establish statistically valid thresholds for assigning quantitative changes between samples. Proteome analysis of *Arabidopsis peroxisomes* has been established as the major unbiased approach for identifying new peroxisomal proteins on a large scale.

Bioinformatics is the application of computer technology to the management and analysis of biological data. The result is that computers are being used to gather, store, analyze, and merge biological data. This field has developed out of the need to understand the code of life, DNA. Massive DNA sequencing projects have evolved and added to the growth of the science of bioinformatics. DNA codes for genes that code for proteins, which determine the biological makeup of humans or any living organism. Bioinformatics is an interdisciplinary research area, which is the interface between the biological and computational sciences. The ultimate goal of bioinformatics is to uncover the wealth of biological information hidden in the mass of sequence, structure, literature, and other biological data and obtain a clearer insight into the fundamental biology of organisms and to use this information to enhance the standard of life for mankind. It is being used now and in the foreseeable future in the areas of molecular medicine to help produce better and more customized medicines to prevent or cure diseases, it has environmental benefits in, identifying waste cleanup bacteria and in agriculture it can be used for producing high yield low maintenance crops. These are just a few of the many benefits bioinformatics will help develop.

Biological databases are archives of consistent data that are stored in a uniform and efficient manner. These databases are from a broad spectrum of molecular biology areas in which primary or archived databases contain information and annotation of DNA and protein sequences, DNA and protein structures and DNA and protein expression profiles. Secondary or derived databases contain the results of analysis on the primary resources including information on sequence patterns or motifs, variants and mutations, and evolutionary relationships. Information from the literature is contained in bibliographic databases, such as. It is essential that these databases are easily accessible and that an intuitive query system is provided to allow researchers to obtain very specific information

on a particular biological subject. The data should be provided in a clear, consistent manner with some visualization tools to aid biological interpretation.

Specialist databases for particular subjects such as *EMBL database* for nucleotide sequence data, *UniProtKB/Swiss-Prot protein database* and *PDBe* for 3D protein structure database. *SRS (sequence retrieval system)* is also a powerful, querying tool provided by the *EBI (The European Bioinformatics Institute)* that links information from more than 150 heterogeneous resources. The EBI provides a wide range of biological data analysis tools that fall into the following four major categories: (i) *similarity searching tools*, (ii) *protein function analysis*, (iii) *structural analysis*, and (iv) *sequence analysis*. Commonly used software tools and technologies in this field include *Java*, *XML*, *Perl*, *C*, *C++*, *Python*, *R*, *MySQL*, *SQL*, *CUDA*, *MATLAB*, and *Microsoft Excel*.

Metagenomics Metagenomics is the genomic analysis of unculturable microorganisms by direct extraction and cloning of DNA from an assemblage of microorganisms to discover new genes and make functional predictions. The development of next-generation DNA-sequencing technologies such as pyrosequencing systems have become the standard for ribosomal RNA identification (i.e., 16S, 18S, etc.) that do not require cloning or PCR amplification has allowed the large-scale metagenomic sequencing of environmental habitats such as aquatic environment, coral reefs, deep sea thermal vents, fermented food, animal, and human gut and cattle feces. Metagenomics has broad implications for human health and disease, animal production, and environmental health and has opened up a creative wealth of data, tools, technologies, and applications that allow us to access the majority of organisms that we still cannot access in pure culture (an estimated 99% of microbial life). Novel genes and gene products discovered through metagenomics include the first bacteriorhodopsin of bacterial origin; novel small molecules with antimicrobial activity; and new members of families of known proteins, such as an $\text{Na}^+(\text{Li}^+)/\text{H}^+$ antiporter, *recA*, DNA polymerase, and antibiotic resistance determinants.

Summary

Advancements in Genomics and Proteomics, together with integrating myriad disciplines from structural biology to bioinformatics, have brought tremendous changes in biotechnology, drug discovery, molecular diagnostics, practice of medicine, and food/agriculture in the post-genomic era – the first decade of the 21st century.

After genomics and transcriptomics, proteomics is the next step in the study of biological systems. It is more complicated than genomics because an organism's genome is more or less constant, whereas the proteome differs from cell to cell and from time to time. Distinct genes are expressed in different cell types, which means that even the basic set of proteins that are produced in a cell needs to be identified. In the past, this phenomenon was done by mRNA analysis, but it was found not to correlate with protein content. It is now known that mRNA is not always translated into protein, and the amount of protein produced for a given amount of mRNA depends on the gene it is transcribed from and on the current physiological state of the cell. Proteomics confirms the presence of the protein and provides a direct measure of the quantity present. A combinatorial approach of accelerated gene discovery through genomics, proteomics, and other associated -omic branches of biotechnology is proving to be an effective way to speed up the crop improvement programs worldwide.

1.11 Biosensors and nanobiotechnology

1.11.1 Biosensor

Biosensor is an analytical device in which a biologically derived sensing element is in intimate contact with a physiochemical transducer to give an electrical signal (Figure 1.27). The three basic components are biological element, transducer, and electronic component. The *sensitive biological element* can be derived from biological materials such as tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, and nucleic acids, and so on. Specific molecular recognition is a fundamental prerequisite, based on affinity between complementary structures such as enzyme-substrate, antibody-antigen and receptor hormone, and this property in biosensor is used for the production of concentration–proportional signals. To prepare biosensors, enzymes, cells and tissues, proteins, and nucleotides must be immobilized on the surface of the silicas, quartz, metals, carbons, semiconductors, and polymers. Immobilization can be done by simple *adsorption* (not normally used), *entrapment* (nonreactive gel, redox gel, conducting polymer, sol–gel network), *entrapment with cross-linking* (glutaraldehyde linking, polyethyleneglycol, polyethyleneimine) and *covalent coupling* to surface (direct), organosilanes, avidin-biotin anchors, and supporting membranes. Biosensor's selectivity and specificity highly depend on biological recognition systems connected to a suitable transducer. The main objectives are to develop rapid, specific, reliable, inexpensive, and compact devices.

Biosensors have been developed during the last two decades for environmental, industrial, and biomedical diagnostics (Table 1.31), but they are largely used in the biomedical area due to the largest market opportunity. Table 1.30 shows various applications of biosensors in different fields. Other applications are in food industries to detect contaminants, adulterants, nutritional compositions, evaluate product freshness, and monitoring quality control during the fermentation process. The market for biosensors application areas analyzed includes point-of-care testing, home diagnostics, environmental monitoring, research laboratories, process industry, and security and bio-defense. There are several applications of biosensors in food analysis. In food industry optic coated with antibodies are commonly used to detect pathogens and food toxins. The light system in these biosensors has been fluorescence, since this type of optical measurement can greatly amplify the signal. A range of immuno- and ligand-binding assays for the detection and measurement of small molecules such as water-soluble vitamins and chemical contaminants (drug residues), such as sulfonamides, have been developed for use on *SPR (surface plasmon resonance) sensor* systems, often adapted from existing enzyme-linked immunosorbent assay (ELISA) or other immunological assay. However, biosensor sector in the agri-food sector is very conservative

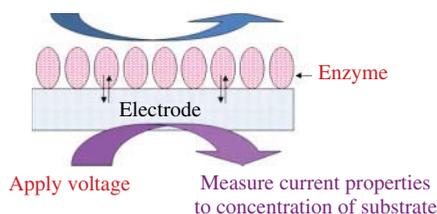


Figure 1.27 Principle of electrochemical biosensors. *Source:* J.F. Rusling, Department of Pharmacology, University of Connecticut Health Center.

Table 1.29 History of commercial biosensors

1962	First and most widely used commercial electrochemical glucose biosensor based on glucose oxidase (Dr. Leland C. Clark)
1975	Commercial glucose biosensor (Yellow Springs Inst.) and First microbe-based biosensor, first immunosensor
1976	First bedside artificial pancreas (Miles)
1980	First fiber optic pH sensor for <i>in vivo</i> blood gases (Peterson)
1982	First fiber optic-based biosensor for glucose
1983	First surface plasmon resonance (SPR) immunosensor
1984	First amperometric biosensor (glucose oxidase for glucose detection)
1987	Blood-glucose biosensor (MediSense ExacTech)
1990	Surface plasmon resonance (SPR) based biosensor (Pharmacia BIACore)
1992	Handheld blood biosensor (i-STAT)
1996	Launching of Glucocard
1998	Blood-glucose biosensor launch (LifeScan FastTake; Roche Diagnostics)
2008	HLAB-2020 biosensor for detection of <i>E. coli</i> O157:H7 and food allergens (Hanson Technologies) Lipid analyzer ICA-LG 400 (enzyme electrode) for measuring cholesterol, triglycerides, and phospholipids (Toyo Jozo)
2011	Biacore Q biosensor (label free SPR) for vitamin and food contaminant (General Healthcare, USA)
Current	Nanobiosensors using quantum dots, nanoparticles, nanowire, nanotube, and so on.

Table 1.30 Applications of biosensors in different fields*Clinical/medical*

- Glucose monitoring in diabetes patients.
- Analysis of electrolytes: Na^+ , K^+ , Cl^- , Ca^{2+} , pH.
- Analysis of metabolites: glucose, cholesterol, L-Lactate, urea.
- Drugs and neurotransmitters, and so on.

Bioprocess control and food analysis

- Alcohols, amino acids, sugars, carbon dioxide.
- Oxygen, pH, cell concentration, and so on.
- Routine analytical measurement of folic acid, biotin, vitamin B12, and pantothenic acid as an alternative to microbiological assay.
- Detection of pathogens, toxic metabolites such as mycotoxins, and so on.
- Determination of drug residues in food, such as antibiotics and growth promoters, particularly meat and honey.
- Measurement of fish freshness.

Environmental and agricultural

- Detection of heavy metals, pesticides, herbicides, organophosphate BOD, and so on.

Drug development

- Drug discovery and evaluation of biological activity of new compounds.
- Scientific detection of crime and warfare agents.
- Remote sensing of airborne bacteria to counter bioterrorist activities.

and the high cost of many commercial biosensors is not going to be an economically viable alternative.

The global market for biosensors in 2012 is estimated to US\$8.5 billion and projected to reach US\$16.8 billion by 2018 (<http://www.marketresearch.com/Industry-Experts-v3766/Biosensors-Global-Overview-6846583/>).

Modes of transduction include (i) *amperometric sensors* that monitor currents generated when electrons are exchanged between a biological system and an electrode, (ii) *potentiometric sensors* (measure the accumulation of charge density at the surface of an electrode), (iii) *optical biosensors* (correlate changes in concentration, mass, or number to direct changes in the characteristics of light), and (iv) other *physicochemical sensors* (monitor biological interactions through changes in enthalpy, ionic conductance, and mass). In *electrochemical biosensor* (Figure 1.27), many chemical reactions produce or consume ions or electrons, which in turn cause some change in the electrical properties of the solution, which can be sensed out and used as measuring parameter by (i) amperometric biosensor, (ii) conductimetric biosensor, and (iii) potentiometric biosensor. In *glucose biosensor*, glucose reacts with glucose oxidase (GOX) to form gluconic acid as well as two electrons and two protons. Glucose mediator reacts with surrounding oxygen to form H_2O_2 and GOX. This GOX can react with more glucose; thus, higher the glucose content, higher the oxygen consumption. The glucose content can be detected by the Pt-electrode.

Recently, arrays of many different detector molecules such as *electronic nose devices* have also been applied. The pattern of response from the detectors is used to fingerprint a substance. In the *Wasp Hound* odor-detector, the mechanical element is a video camera and the biological element is five parasitic wasps that have been conditioned to swarm in response to the presence of a specific chemical. Current commercial electronic noses, however, do not use biological elements. Many optical biosensors based on the phenomenon of SPR are evanescent wave techniques, that utilize a property of gold and other materials. A thin layer of gold on a high refractive index glass surface can absorb laser light, producing electron waves (surface plasmons) on the gold surface. SPR sensors operate using a sensor chip consisting of a plastic cassette supporting a glass plate, one side of which is coated with a microscopic layer of gold.

Piezoelectric sensors utilize crystals that undergo an elastic deformation when an electrical potential is applied to them. An alternating potential (AC) produces a standing wave in the crystal at a characteristic frequency. This frequency is highly dependent on the elastic properties of the crystal, such that if a crystal is coated with a biological recognition element, the binding of a (large) target analyte to a receptor will produce a change in the resonance frequency, which gives a binding signal. In a mode that uses surface acoustic waves (SAW), the sensitivity is greatly increased. This is a specialized application of the quartz crystal microbalance as a biosensor.

Biosensors found in various medical fields show much promise in diagnosing medical conditions and detecting genetic disorders, environmental monitoring of pollution and detection of hazardous chemicals, and in food analysis. Currently, one-shot glucose monitors are one of the most popular commercially available biosensor monitors. Similar handheld devices are being used as well to detect pesticides and pollutants in air and water. Currently, GM microorganisms play an increasingly significant role in improving the capacity of biosensors. Electrochemical and optical types of transducers have been widely employed in microbial biosensors, although bioluminescence and fluorescence methods have been highlighted lately. The *microbial fuel cell (MFC)*, which has been mainly applied in *BOD biosensors*, is a promising technology with respect to their applications in environmental monitoring and food analysis, including measurement of a variety of

common pollutants, products in fermenting processes, antibiotic residues, and toxins in food.

Applications for food contaminants Although biosensors are not commonly used for food microbial analysis, they have great potential in the detection of microbial pathogens and their toxins in food. They enable fast or real-time detection, portability, and multipathogen detection for both field and laboratory analyses. Several applications have been developed for microbial analysis of food pathogens, including *E. coli* O157:H7, *Staphylococcus aureus*, *Salmonella*, and *Listeria monocytogenes*, as well as various microbial toxins such as staphylococcal enterotoxins and mycotoxins. Biosensors have several potential advantages over other methods of analysis, including sensitivity in the range of nanogram per milliliter for microbial toxins and <100 colony-forming units per milliliter for bacteria. Fast or real-time detection can provide almost immediate interactive information about the sample tested, enabling users to take corrective measures before consumption or further contamination can occur. Miniaturization of biosensors enables biosensor integration into various food production equipment and machinery. Potential uses of biosensors for food microbiology include online process microbial monitoring to provide real-time information in food production and analysis of microbial pathogens and their toxins in finished food.

Biosensors can also be integrated into *Hazard Analysis and Critical Control Point* programs, enabling critical microbial analysis of the entire food manufacturing process. Hanson Technologies (USA) developed HLAB-2020 biosensor for detection of *E. coli* O157:H7 and food allergens and also introduced *Safe Vegetable Screening System (HSVS-1000)* for the ultra rapid detection of pathogens in fresh-cut produce without culturing. The elimination of culturing associated with many hours of delay in waiting for results represents a major advancement in food safety screening. The HSVS-1000 can simultaneously screen for multiple pathogens, allergens and toxins, including *E. coli* O157:H7 and *Salmonella*, in 2 h or less utilizing large sample sizes.

Many transducers used to detect food-borne pathogens are mostly electrochemical (amperometric), optical (luminescence), SPR, mass/acoustic (piezoelectric), and mass (cantilever), but require more work to prove their use with actual food samples. One such approach is the use of *antibody sandwich assays*, whereby antibodies specific for types of bacteria interact with the bacteria in the sample, a secondary antibody conjugated to a signal-generating moiety either by itself or with a substrate. While fiber optic biosensors that can detect fluorescence, amperometric biosensor method using a *horseradish peroxidase (HRP)*-labeled secondary antibody and HRP substrate to generate the signal can be used to *E. coli* O157:H7 within 1 h after receiving a milk sample, biosensor methods that incorporate a PCR by immobilizing an oligonucleotide specific for a gene of *E. coli* O157:H7 onto a mass/acoustic (piezoelectric) transducer were used in food samples.

Bacteriophage can also be used for the direct detection of *E. coli* in an array-format electrochemical (conductometric) assay. Several multiplexing biosensors using disposable microarray formats or PCR were tried to detect *E. coli* O157:H7, *S. typhimurium*, and *Legionella pneumophila*, and so on. PCR tests rely on the power of DNA polymerase to selectively amplify genetic fragments of any agents to make billions of copies, which can then be observed by one of several different methods. However, ELISA and PCR assays even with automation systems take time to run, that are usually labor intensive.

Analysis of food contamination is not only vital for the consumer protection, but also aid the global trade process and barrier. For toxins, pesticide and veterinary drug residues, physicochemical methods such as *liquid chromatography (LC)*–*tandem mass spectrometry (MS/MS)* are used to confirm the presence of these compounds, but these methods are

expensive, complicated to operate, and time-consuming. Current methods in this area have limitations; thus, future directions should optimize performance, develop portable biosensors for onsite monitoring, combine genetic and DNA approaches, nanotechnology, and phage-based biosensors for food-borne pathogens. Several applications of biosensors in food analysis are optic coated with antibodies that are commonly used to detect pathogens and food toxins. The light system in these biosensors has been fluorescence, since this type of optical measurement can greatly amplify the signal. The use of portable biosensors, such as commercial glucose biosensors on the market, will revolutionize medical diagnosis and environmental monitoring by allowing point-of-care and onsite testing. Few portable optical, *SPR* biosensors are already used in (i) detection of antibiotic residue in milk as well as toxins in shellfish, (ii) electrochemical (conductometric) biosensor system for bacterial concentration measurements in dairy products, and (iii) optical (fluorescence) array biosensor for botulinum neurotoxins (BoNTs). The form of analyte may range from macromolecule to a microelement and heterogeneous distribution of analyte in the food has made the situation worse for the analyst. In most cases, the analyst needs to separate the analyte from the food before detection.

Advances made until now in biosensor applications for the detection of food contaminants are quite varied, but advantages of biosensors are significant compared with other conventional methods such as *radioimmunoassay*, *enzyme-linked immunosorbent assay*, *fluorescence immunoassay*, and *luminescence immunoassay*. The main areas of development should include multiplexing, the ability to simultaneously analyze a sample with the potential of unrelated pathogens, toxins, pesticides, or drug residues. Chapter 9 in the first edition has described the details of biosensor for foods.

1.11.2 Nanobiotechnology and nanobiosensor

Nanobiotechnology is the application of *nanotechnology* to life sciences including the application of nanoscale tools to biological systems and the use of biological templates to create nanoscale products. Nanotechnology that focuses on the understanding and control of matter at approximately 1–100 nm (a nanometer is a billionth of a meter) has become one of the most promising scientific fields of research in recent decades and has the potential to provide new solutions on medical and food industries, for example, for disease-treatment delivery methods and biosensors for pathogen detection. Table 1.31 shows applications of nanobiosensors underway or commercialized in various fields.

Nanobiotechnology applications are broad: nanomedicine, nanobiosensors (biochips), nanofluidics, molecular self-assembly, intelligent drug delivery systems, and nanomachines, that become the convergence of engineering and molecular biology.

The application of nanotechnology to biosensor design and fabrication promises to revolutionize diagnostics and therapy at the molecular and cellular level. *Nanobiosensors* are based on a combination of nanotechnology with varying biosensing techniques with the aim of improving existing clinical practices and rapid detection of bacteria and viruses. The nanobiosensors are equipped with immobilized bioreceptor probes such as antibodies and enzyme substrate, and laser excitation is transmitted to photometric system in the form of optical signal (fluorescence).

Nanobiosensors will first revolutionize the future of disease diagnosis and other biomedical applications. The potential for monitoring *in vivo* biological processes within single living cells, for example, the capacity to sense individual chemical species in specific locations within a cell, will also greatly improve our understanding of cellular function, thereby revolutionizing cell biology. Existing nanoprobe have already demonstrated the capability of performing biologically relevant measurements inside single living cells.

Table 1.31 Applications of nanobiosensors underway or commercialized for biomedical, environments, cosmetics, packagings, and food analysis

Types	Applications
<i>Biomedicals</i>	
Submicron fiber-optic nanotubes	Monitoring pH, nitric acid
SPOT-NOSED on nanoelectrode	Diagnose diseases at early stages (with a layer of olfactory proteins)
Nanosphere lithography (NSL)	Detect streptavidin (1 pm concentrations) with Ag nanoparticles
Antibody based piezoelectric	Anthrax, HIV hepatitis
Optical with mouse anticytochrome	Detect cytochrome C in a single cell c antibodies
Multiple biomarker system	Vista's NanoBioSensor™ (NBS)
<i>Cosmetics</i>	
TiO ₂ + coating	Sun protecting ream
Lipid nanoparticles	Q10 cream
Nanoencapsulation	Tip-top up (omega 3 bread)/canola active oil
<i>Food packaging/composite/others</i>	
Nanofood packaging	Film (Bayer)
Nano clay particle	Beer bottle (Nano Co. Inc)
Nano cleaning	Emulsion disinfectant
Nano catalytic devise	Protect oil breakdown (www.oilfresh.com)
Nano feed	Nanoselenium for chicken (Attair Nanobiotech Ltd)
Carbon nanotubes in food packaging	Antimicrobial effects on <i>E. coli</i> , and so on.
<i>Food additives/contaminants</i>	
Carbon nanofibers (CNF)	Detection of food pathogens
Carbon nanotubes (MWCNTs-chitosan)	Detection for organophosphate insecticide
Acetylcholinesterase (AChE) on multiwall carbon nanotubes-chitosan	Detection of organophosphate and caramate pesticides
Magnetic nanoparticles conjugated with specific antibodies using interdigitated array microelectrode	Detection of <i>E. coli</i> O157:H7
Direct-charge transfer (DCT) nanowire Immunosensor	Detection of many pathogens including <i>Bacillus cereus</i> , and <i>Vibrio parahaemolyticus</i> .
Nanoporous silicon-based DNA biosensor	Detection of <i>E.coli</i> and <i>Salmonella</i>
Quantum dot (QD) nanobiosensors based on FOF1-ATPase	Detection of virus
Amperometric penicillin nanobiosensor using multi-walled carbon nanotubes (MWCNTs), hematein, and β-lactamase	Detection of penicillin in milk
<i>Animal husbandry/Aquaculture</i>	
Adhesin-specific nanoparticles	Removal of pathogens from livestock
Nanoparticles	Detection of toxins in feed, drug residues,
Nanoparticles/platinum-alumina cryogel	Prevention of diseases spread in animals
Nanowires	Detection of pollutants in aquaculture

Source: Adapted from Li YH and Tian X. 2012. *Sensors (Basel)*, 12, 2519–2153.

A fiber-optic nanosensor basically is a nanoscale probe that consists of a biologically or chemically sensitive layer that is covalently attached to an optical transducer. Various kinds of nanomaterials, such as *gold nanoparticles (GNPs)*, *carbon nanotubes (CNTs)*, *magnetic nanoparticles*, and *quantum dots*, are being gradually applied to biosensors because of their unique physical, chemical, mechanical, magnetic and optical properties, and markedly enhance the sensitivity and specificity of detection. GNPs show a strong absorption band in the visible region due to the collective oscillations of metal conduction band electrons in strong resonance with visible frequencies of light, which is called SPR. There are several parameters that influence the SPR. It is well known that well-dispersed solutions of GNPs display a red color, while aggregated GNPs appear a blue color. Based on this phenomenon, a GNPs-based biosensor to quantitatively detect the polyionic drugs such as protamine and heparin was established.

Despite the fact that biosensors are promising devices when it comes to fast and easy detection of analytes, their use has not yet been established in clinical routine to replace immunoassay techniques. Numerous publications and patents dealing with nanobiosensors have been published, but only a small part of the work reported was performed using real samples. The problem of nonspecific binding arising from complex sample matrices is yet to be solved. Biosensors based on *molecular switches* offer a promising tool to solve this problem, because only the analyte binding is able to generate a signal response and exclude cross-reactivity.

Biosensors are, in fact, artificial molecular switches that mimic the natural ones, which direct chemical responses throughout the cell. They help to build biologically based sensors for the detection of chemicals ranging from drugs to explosives to disease markers. All creatures from bacteria to humans have been monitoring their environments in order to survive with biomolecular switches, made from RNA or proteins. Molecular switches have been reported for optical and electrochemical transduction principles, which are currently the most commonly used transduction principles for biosensors. As disease-related marker profiles are still under investigation, specifications regarding a suitable biosensor instrument can hardly be given at the moment. However, almost certainly such a device should permit multiplex analysis to determine a marker profile in a sample in one measurement cycle. Therefore, a biosensor array would be required. At least as long as these profiles are not established, it could be useful to keep these arrays flexible. This would be supported by a packaging strategy in which each biosensor element is integrated in a single, array-compatible housing, allowing user-defined combination. This would make the arrays potentially adaptable to the respective application and hence make the underlying biosensor instrument more versatile. *RNA biochip* as biosensor elements that can be used to detect toxic metals such as cobalt, drug compounds such as theophylline, and natural compounds such as cyclic AMP and cyclic GMP in a single assay are under testing. The latest knowledge in nanobiotechnology and enzyme engineering will eventually lead to have low-cost, easy-to-operate in homes and doctor's office to diagnose patients and detect contaminants and biological attacks.

Food nanotechnology is an emerging area, and tens of millions of dollars are being spent in a global race to apply nanotechnologies in food production, processing, and packaging. Most applications of nanotechnology are currently in food packaging to improve plastic materials barriers, and incorporation of functionally bioactive components in packaging materials to extend shelf-life, improve food safety, and improve taste. Nanotechnology analysts estimated that about 150–600 nanofoods and 400–500 nanofood packaging applications are already on the markets (www.scribd.com/doc/9197096/Nano-food). The main areas of application include food packaging and food products containing nano-sized or nanoencapsulated ingredients and additives. CNTs used in food packaging

exhibited powerful antimicrobial effects on *E. coli*. A simple method for immobilization of acetylcholinesterase (AChE) on multiwall carbon nanotubes (MWCNTs)-chitosan composite was proposed to detect organophosphate and carbamate pesticides. Magnetic nanoparticles conjugated with specific antibodies have been used to detect *E. coli* O157:H7 using interdigitated array microelectrode in ground beef samples. A direct-charge transfer (DCT) nanowire immunosensor could be used to detect many pathogens including *Bacillus cereus*, and *Vivrio parahaemolyticus*. A nanoporous silicon-based biosensor product is produced by a company that is aimed at detecting the bacteria *E. coli* and *Salmonella* by analyzing their DNA. Quantum dot (QD) nanobiosensors based on F0F1-ATPase have been tried to detect avian influenza or other virus; fluorescence intensity of orange and green QD biosensors with and without virus can be used to detect virus when the ADP is added to initialize reaction. Another example is an amperometric penicillin biosensor with enhanced sensitivity; it was successfully developed by coimmobilization of MWCNTs, hematein, and β -lactamase on glassy carbon electrode using a layer-by-layer assembly technique. This nanobiosensor offered a minimum detection limit of 50 nm for penicillin V, much lower than conventional methods.

Summary

As biosensor technology advances, biosensors are now being developed for detection of microbial pathogens, cancer detection, and monitoring. In addition, biosensor technology is being applied to food analysis such as food pathogens, allergens, food and biomaterial quality testing, and basic research on molecular interactions. Biosensors offer several advantages over other analytical methods including rapid and even real-time measurements, high sensitivity, selectivity, and specificity even when a complex or turbid sample matrix is used. As the technology advances, producing lab-on-a-chip devices, these self-contained portable instruments will allow measurements outside the laboratory, in the field or at the bedside. Biochip technologies could offer a unique combination of performance capabilities and analytical features of merit, and allow simultaneous detection of multiple biotargets. Nanosizes of the probes minimize sample requirement and reduce reagent and waste requirement. For medical applications, this cost advantage will allow the development of extremely low cost, disposable biochips that can be used for in-home medical diagnostics of diseases without the need of sending samples to a laboratory for analysis.

Health aspects of nanotechnology and nanoparticles are described together in Section 1.13.

1.12 Quorum sensing and quenching

Quorum sensing (QS), which involves a regulation of genes in response to population density, is a cell-to-cell signaling mechanism used among various species of bacteria. In QS, bacteria release chemical signal molecules called autoinducers into the surrounding environment. When the number of cells is low, the concentration of autoinducers is also low, and the bacteria seem benign. However, when a certain threshold of cell density is reached, the autoinducers are internalized into the cell and the population of cells responds to the environment. The type of this response vary among different species of bacteria, and can vary from emitting fluorescent light, formation of biofilms, initiation of sporulation, production of antibacterial agents, motility, or conjugation (Table 1.32). A significant portion of bacterial genome (4–10%) and proteome (about 20%) can be influenced by QS.

Table 1.32 Examples of bacterial quorum sensing controlled processes and traits

Microorganism	Major signal molecules	Regulatory system	Benefits/traits
<i>Vibrio fischeri</i>	HAI-1, CAI-1 AI-2	LuxLM/LuxN LuxP/LuxQ	Bioluminescence emission, <i>V. harveyi</i> Symbiosis
<i>Bacillus subtilis</i>	ComX CSF (PhrC) PhrA, -E, -F, -K, -H	ComP/ComA Rap proteins	Competence, sporulation, biofilm formation, antibiotic production
<i>Myxococcus xanthus</i>	A-signal C-signal	SasSRN	Fruiting body formation or sporulation
<i>Pseudomonas aeruginosa</i>	3O-C12-HSL C4-HSL	LasI/LasR RhII/RhIR OscR (orphan)	Biofilm formation, virulence factors
<i>Staphylococcus aureus</i>	AIP-I, AIP-II, AIP-II, AIP-IV	AgrC/AgrA	Biofilm formation, virulence factors
<i>Streptococcus mutans</i>	CSP (ComC) XIP (ComS)	ComD/ComE ComR	Bacteriocins, biofilm formation, competence
<i>Streptococcus pneumoniae</i>	CSPs	ComD/ComE	Competence, fratricide, biofilm formation, virulence

Source: Adapted from Li YH and Tian X. 2012. *Sensors (Basel)*, 12, 2519–2153.

QS was first described in *Vibrio fischeri*, a luminescent marine bacterium. *V. fischeri* can be free-living or grow in light organs of their symbiotic hosts such as squid and fish. *V. fischeri* generate the light that has similar intensity to that of the moon and starlight above, protecting the host from predators by making them invisible to predators below. Thus, in this circumstance, the purpose of QS is symbiosis. In this bioluminescent system shown in Figure 1.28, (i) *LuxI* gene (encodes an autoinducer synthase (LuxI) produces the autoinducer *N*-homoserine lactone (*HSL*), (ii) *HSL* exits the cell and re-enters freely against gradient when external concentration reaches a threshold value, (iii) *HSL* binds to the *LuxR* gene (a transcription factor), (iv) *HSL*-*LuxR* complex binds upstream of the *LuxLCDABE* operon, and (v) increased transcription of luciferase system as well as the exponential increases in *LuxI* synthesis.

Another example of microorganism that exhibits QS mechanism is *Staphylococcus aureus*. Like in the case of *Vibrio fischeri*, *S. aureus* responds to its environment in population-dependent manner. In this case, however, the QS mechanism is involved with the pathogenesis of *S. aureus*. *S. aureus* found in approximately 30% of the adult population are part of normal flora of human intestinal tract. However, when these organisms or their extracellular products breach the epithelial layer, serious diseases can result. QS enhances the bacteria's pathogenicity by producing virulence factors. Also, it helps with formation of biofilms, groups of interacting cells, and enhances pathogenicity because biofilms are often resistant to antibiotic therapy and clearance by hosts.

Another microorganism that utilizes quorum sensing for its pathogenesis is *Pseudomonas aeruginosa*. *P. aeruginosa*, an opportunistic pathogen often isolated in nosocomial infections, secretes virulence factors which allow for its pathogenicity

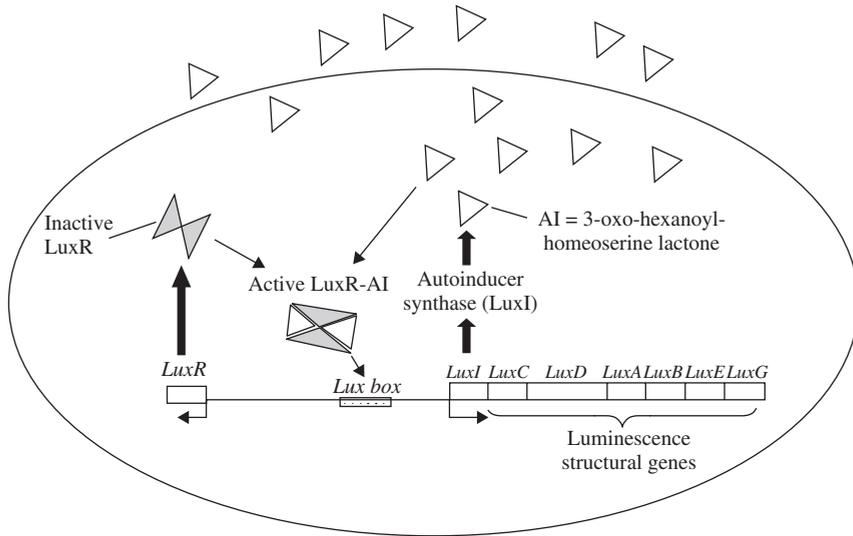


Figure 1.28 Model of quorum sensing in *Vibrio fischeri*. Source: Popham DL and Stevens AM 2006. Bacterial quorum sensing and bioluminescence, pp. 201–215. In *Tested Studies for Laboratory Teaching*, Vol. 27 (MA O'Donnell, ed.). Proceedings of the 27th Workshop/Conference of the Association for Biology Laboratory Education (ABLE). Reproduced with permission.

and the QS regulates gene expression of some of these extracellular factors. In addition, the QS plays a role in a development of biofilms, which greatly enhances the bacteria's pathogenicity.

As the QS system regulates expression of some virulence factors and contributes to the pathogenicity of bacteria, the QS is a possible candidate for an antibacterial drug target. As a result, there are numerous researches going on to use this system to reduce pathogenesis of bacteria.

Active research is underway to (i) discover new drugs that inhibit QS, so-called *quorum quenching* (QQ), thus generating a new type of antibiotic drug for the infectious pathogens, and (ii) inhibit QS system, to prevent food pathogens and food spoilages.

The extensive use of antibiotics brought the problem of emergence of drug resistance of pathogens to the medical society. The problem of drug resistance among pathogenic bacteria is facing more difficulties with the emergence of multidrug resistance strains and resistance even to the most developed drugs. Inevitably, an alternative to antibiotics is a necessity. Inhibiting QS is ideal as QS is not directly involved with survival of the pathogen. It does not seem to put much survival pressure on microorganisms to develop resistance against this type of inhibitors.

One system that was suggested as a potential antibacterial drug target is *two-component signal transduction system*, that is made up of two parts: a *sensor kinase* and a *response regulator*. The first part, a sensor kinase, responds to an environmental signal with autophosphorylation; the response regulator then interacts with the kinase to regulate the gene expression.

Among many genes that are regulated, genes that are involved in virulence and biofilms are also controlled. Blocking this system will thus work as an antibacterial drug.

Furthermore, this system works in a different method from conventional antibiotics, and this could provide alternatives to the drug-resistance problem.

Biofilm and quorum sensing The QS plays an important role in the development of biofilms. Formation of biofilms contributes to the pathogenicity of bacteria because it can assist in colonization by other pathogens, or can increase resistance to conventional antibiotics. For example, *P. aeruginosa* uses the quorum sensing to form biofilms in the lungs of cystic fibrosis patients and causes a major problem in treating chronic patients. It was discovered that mutations of *lasI* system of quorum sensing results in structurally altered biofilms. The biofilms contribute to the persistence and severity of *P. aeruginosa* (Kalia and Purohit, 2011). It also contributes to increased virulence because the presence of biofilms provides a chance for secondary pathogens to colonize the patients. With such roles that biofilms play in virulence of pathogens, it is clear that inhibition of biofilms will result in the reduced pathogenicity. Consequently, as a method to inhibit biofilms, the inhibition of the QS is being suggested. In a recent research, QS inhibitors were shown to increase *P. aeruginosa*'s susceptibility to antibiotics both *in vitro* and *in vivo* (Brackman et al., 2011).

Autoinducers The autoinducers involved in QS system differ in Gram-positive and Gram-negative bacteria. The molecule is known as oligopeptides in Gram-positive bacteria. The oligopeptides signals can be processed with side chain modifications. For instance, in *Staphylococcus* species, thiolactone rings are added to the oligopeptides. Also, in *B. subtilis*, isoprenyl groups are added to the oligopeptides. In Gram-negative bacteria, the QS uses *acylhomoserine lactones (AHLs)* as signaling molecules. The specificity of AHLs is determined by the differences in the length, degree of saturation, and substitution of the acetyl side chains. By understanding the mechanisms of autoinducers' antagonists, potent QS inhibitors can be designed.

Inhibitors To develop the inhibitors against the QS, it is essential to know what properties will result in the most ideal QS inhibitors. First, an ideal *quorum sensing inhibitors (QSI)* should be chemically stable and not be easily degraded by the host. It should also have low-molecular-mass and should significantly downregulate the expression of genes controlled by QS. This downregulation has to be highly specific. In other words, it should only downregulate the virulence-related genes regulated by QS, and not affect the expression of gene related to bacterial growth or survival. If the inhibitor negatively affects the expression of such genes, the survival pressure will push the pathogens to develop resistance against the inhibitors.

Three strategies can be applied for inhibition of QS. The first method is to block signal production. The second strategy is to inactivate signal molecules, which can be achieved in two ways: either use antagonist of autoinducers or degrade signals that are already created. The last method is to target the signal receptor.

Nanofactories Engineered biological nanofactories were suggested as a method to control on and off the QS (Fernandes et al., 2010). In other words, to inhibit pathogenesis, they can be used either to keep the QS off or turn on the QS even when the amount of bacteria is below the threshold level. This suggests that these nanofactories can induce the QS, and subsequently pathogenesis when the amount of bacteria would normally be insufficient to cause illness. The pathogens can then be detected and be cleared by the body's immune system. This "biological nanofactory" is made up of antibody for targeting and a fusion protein that produces autoinducer when bound to the target microorganism.

Summary

While some bacteria are communicating with neighboring cells (quorum sensing), others are interrupting the communication (QQ), thus creating a constant arms race between intercellular communication. QS in food microbiology and biotechnology became very important in the relationship between QS and food spoilage, enzyme and toxin production in food spoilage, and biofilm formation in food processing industries. Also this knowledge in the inhibition of biofilm formation and other QS interference mechanism naturally present in food and transgenic plants expressing the QS signal inactivating enzymes could be an alternative to control food spoilage and the potentially hazardous food-borne bacterial contamination.

Recent evidence extends the role of QQ to detoxification or metabolism of signaling molecules as food and energy source. While QQ has been explored as a novel anti-infective therapy targeting, quorum sensing evidence begins to show the development of resistance against QQ.

As antibiotic-resistant bacteria become a global threat to public health, novel therapeutics represent an important area of current scientific research. QS is a key regulatory system that controls the expression of virulence determinants, thus making QS an effective target for novel drug design as well as agricultural and industrial applications. QQ provides a strategy to disrupt QS, and in turn attenuates virulence determinants. The future of QS research lies in the discovery of additional communication signals, and thus QQ as a promising anti-infective strategy can be developed based on information obtained from QS studies for biotechnological and pharmaceutical applications.

1.13 Micro- and nano-encapsulations

Micro- or nanocapsules are small liquid, solid, gas, or living element coated by another substance in order to protect and separate the materials. *Micro-* or *nanoencapsulation* is a process in which tiny particles or droplets are surrounded by a coating to give small capsules with many useful properties. In its simplest form, a micro- or nanocapsule is a small sphere with a uniform wall around it. Core material can be released from micro- or nanocapsules when their walls burst under outside pressure, melt under heat influence, burn under high temperature, degrade under the influence of light, dry out, dissolve in a solvent or the core material is released gradually through the permeable wall of the microcapsule. Those capsules can be of various forms such as emulsion, suspension or powder with a variety of core materials, wall thicknesses, and particle sizes. Under the microscope single micro- or nanocapsules, clustered micro- or nanocapsules, multicore micro- or nanocapsules can be distinguished. Micro- or nanocapsules are often of spherical form, but micro- or nanocapsules of irregular forms are also known. The products obtained by a process of enclosing micron-sized particles of solids or droplets of liquids or gasses in an inert shell are called microparticles, microcapsules, and microspheres which differentiate in morphology and internal structure. When the particle size is below 1 μm , they are called *nanoparticles*, *nanocapsules*, *nanospheres*, and particles in diameter between 3 and 800 μm are known as microparticles, microcapsules, or microspheres (Figure 1.29).

Despite the similarity of both encapsulation, many micro- or nanocapsules bear little resemblance to these simple spheres. The core may be a crystal, a jagged adsorbent particle, an emulsion, a suspension of solids, or a suspension of smaller micro- or nanocapsules even with multiple walls.

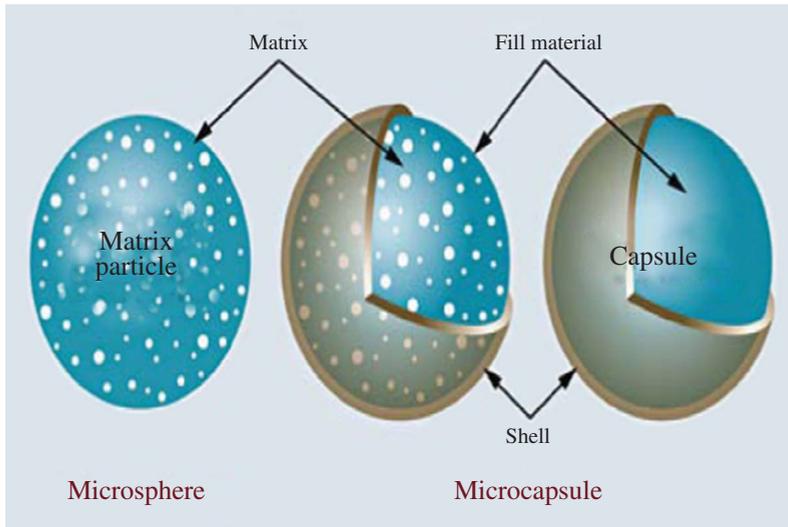


Figure 1.29 Particle size and morphology of microsphere and microcapsule.

Technologies of micro- and nanoencapsulation are currently providing solutions to complex issues such as sustained release and targeted delivery. The cell microencapsulation technology involves immobilization of the cells within a polymeric semipermeable membrane that permits the bidirectional diffusion of molecules such as the influx of oxygen, nutrients, growth factors, and so on that are essential for cell metabolism and the outward diffusion of waste products and therapeutic proteins. At the same time, the semipermeable nature of the membrane prevents immune cells and antibodies from destroying the encapsulated cells considering them as foreign invaders. The main motive of cell encapsulation technology is to overcome the existing problem of graft rejection in tissue engineering applications and thus reduce the need for long-term use of immunosuppressive drugs after an organ transplant to control side effects. Many varieties of both oral and injected pharmaceutical formulations are also microencapsulated to release over longer periods of time or at certain locations in the body. Aspirin tablets that can cause peptic ulcers and bleeding are often produced by compressing quantities of microcapsules that will gradually release the aspirin through their shells to avoid adverse effects such as gastric irritation or stomach damage by the drug.

The areas of applications are broad including food industry, pharmaceuticals, cosmetics, electronics, photography, agriculture, chemical industry, textile industry, graphic industry, biotechnology, and so on. However, micro- and nanoencapsulation technologies aiming at food and biotechnology are:

1. Biocatalysis in food processing and biotechnology on immobilization and microencapsulation of living cells, bioactive substances and functional food ingredients for controlled and effective delivery into food systems (e.g., antioxidant, anti-inflammatory, antimicrobial, anticancer compounds, enzymes, probiotics, etc.);
2. Improvement of quality, nutritional value, safety, and shelf-life of food (oxidation stability, etc.);

3. Design and utilization of food-grade micro- and nanoemulsions for delivery of nonpolar functional components (bioactive lipids, hydrophobic drugs, etc.);
4. Isolation, chemical modification, and purification of biopolymers (specifically cationic/anionic oligosaccharides and hydrophobic derivatives);
5. Biodegradable food-packaging materials (mainly cellulose and starch-based materials, biodegradable polymer films), and surface modification;
6. Polymer characterization by LC techniques and rheology;
7. In the case of food manufacturing, the current state of nanoencapsulation applications is not clear, though some products are already on the market. Fundamental research is ongoing for effective transfer of knowledge into innovation and to deal with considerations regarding risks, regulatory aspects, and consumer skepticism. Little is known about the unintended consequences of nanoparticles and how the particles interact with the environment and human body. No general conclusion can be made on the safety of nanofood and food contact materials incorporated with nanomaterials.

According to WHO, new data and measurement approaches are needed to ensure safety of products using nanotech can be properly assessed. Nanobiotechnology can easily fall into the premade trap of genetically modified foods (GMFs).

1.13.1 Microencapsulation

Microcapsules can be classified into three categories according to their morphology: (i) mononuclear (core–shell) capsules containing the shell around the core, (ii) polynuclear capsules which have many cores enclosed within the shell, and (iii) matrix types which are distributed homogeneously into the shell materials. In addition to these three morphologies, microcapsules can also be mononuclear with multiple shells, or they may form clusters of microcapsules.

Microparticles or microcapsules consist of two components, namely core material and coat or shell materials. Core material contains an active ingredient while coat or shell material covers or protects the core materials. Different types of materials such as active pharmaceutical ingredients, proteins, peptides, volatile oils, food materials, pigments, dyes, monomers, catalysts, pesticides, and so on, can be encapsulated with different types of coat materials such as ethylcellulose, hydroxyl ethylcellulose, carboxymethylcellulose, sodium alginate, poly-lactic-glycolic acid (PLGA), gelatin, polyesters, chitosan, and so on. To a large degree, the selection of the appropriate coating material dictates the resultant physical and chemical properties of the microcapsules. The coating material should be able to form a film that is cohesive with the core material, chemically compatible and nonreactive with the core material; it should also be able to provide the desired coating properties on strength, flexibility, impermeability, optical properties, and stability.

Most common applications of microencapsulation are in food ingredients as most flavorings are volatile. Encapsulation of these components can thus extend the shelf-life of products by retaining within the food flavors. Some ingredients are encapsulated to mask the organoleptic properties like taste or flavors of the products and even to last longer, as in chewing gum. The amount of encapsulated flavoring required is substantially less than liquid flavoring, as liquid flavoring is lost and not recovered during chewing. Flavorings that are comprised of two reactive components can be encapsulated individually, that add to the finished product separately so that they do not react and lose flavor potential prematurely. Some flavorings must also be protected from oxidation or other reactions caused by exposure to light.

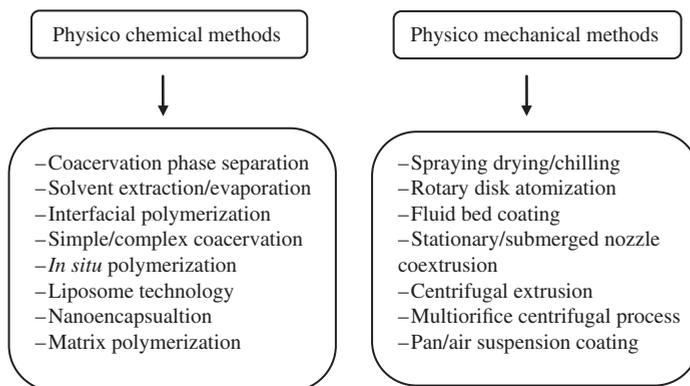


Figure 1.30 Manufacturing techniques of microcapsules.

Many factors are to be considered when selecting the encapsulation process and also many different techniques are available for the encapsulation of core materials. In general, microencapsulation processes are usually categorized into (i) Physicochemical methods and (ii) physical methods (Figure 1.30). However, these labels can be somewhat misleading, as some processes classified as mechanical might involve or even rely upon a chemical reaction, and some chemical techniques rely solely on physical events.

1.13.1.1 Physicochemical methods Capsules for carbonless paper and for many other applications are produced by a chemical technique called complex coacervation. This method takes advantage of the reaction of aqueous solutions of cationic and anionic polymers such as gelatin and gum arabic. The polymers form a concentrated phase called the complex coacervate. The coacervate exists in equilibrium with a dilute supernatant phase. As the water-immiscible core material is introduced into the system, thin films of the polymer coacervate coat the dispersed droplets of core material. The thin films are then solidified to make the capsules harvestable. In *interfacial polymerization (IFP)*, which is another chemical method of microencapsulation, the capsule shell is formed at or on the surface of the droplet or particle of dispersed core material by polymerization of the reactive monomers. After a multifunctional monomer is dissolved in the core material, this solution is dispersed in an aqueous phase. When a reactant to the monomer is added to the aqueous phase, polymerization quickly occurs at the surfaces of the core droplets, forming the capsule walls.

IFP can be used to prepare bigger microcapsules, but most commercial IFP processes produce smaller capsules in the 20–30 μm diameter range for herbicides and pesticide uses, or even smaller 3–6 μm diameter range for carbonless paper ink. *In situ* polymerization is a chemical encapsulation technique very similar to IFP. The distinguishing characteristic of *in situ* polymerization is that no reactants are included in the core material. All polymerization occurs in the continuous phase, rather than on both sides of the interface between the continuous phase and the core material, as in IFP. Examples of this method include *urea-formaldehyde (UF)* and *melamine formaldehyde (MF) encapsulation systems*. Polymer–polymer incompatibility, also called phase separation, is generally grouped with other chemical encapsulation techniques. This method utilizes two polymers that are soluble in a common solvent, and yet do not mix with one another in the solution. The polymers

form two separate phases, one rich in the polymer intended to form the capsule walls, the other rich in the incompatible polymer meant to induce the separation of the two phases. The second polymer is not intended to be part of the finished microcapsule wall, although some may be caught inside the capsule shell and remain as an impurity.

Solvent evaporation technique is currently used by companies including the NCR Company, Gavaert Photo - Production NV, and Fuji Photo Film Co., Ltd. to produce *microcapsules*. Three phases including core, coat material and *liquid manufacturing vehicle (LMV)* are present. The microcapsule coating is initially dissolved in a volatile solvent, which is immiscible with the LMV phase. A core material is dissolved in the coating polymer solution. The core coating material mixture is dispersed with agitation in the LMV phase to obtain the appropriate size microcapsule. The mixture is then heated to evaporate the solvent for polymer. Once the core material is dispersed in the polymer solution, the polymer shrinks around the core. In case the core material is dissolved in the coating polymer solution, a matrix type microcapsule is formed. After all the solvent for the polymer is evaporated, the liquid vehicle temperature is reduced to ambient temperature with agitation and then the microcapsules can be used in the suspension form, coated onto substrates or isolated as powders. The core materials may be either water soluble or water insoluble materials. A variety of film forming polymers can be used as coatings.

Liposome microencapsulation has been used mostly in pharmaceutical applications to achieve targeted delivery of vaccines, hormones, enzymes, and vitamins. However, technology has evolved in recent years to the point that it is now conceivable for liposome encapsulation to become a routine process in the food industry. In liposome entrapment, liposomes consist of an aqueous phase that is completely surrounded by a phospholipid-based membrane. When phospholipids such as lecithin are dispersed in an aqueous phase, the liposomes form spontaneously. Liposomes are vesicles consisting of a lipid bilayer enclosing an aqueous compartment at the center and thus are nontoxic and acceptable for foods. The lipid bilayer used in the liposomes are usually made of phospholipids, that are arranged in such a way that the hydrophilic head is exposed outside and the lipophilic tails are aliened inside. This makes the liposomes water-soluble molecules. Permeability, stability, surface activity, and affinity can be varied through size and lipid composition variations. They can range from 25 nm to several microns in diameter, are easy to make, and can be stored by freeze drying. As shown in Figure 1.31, liposomes are structurally classified on the basis of lipid bilayers such as *small unilamellar vesicles (SUVs: 20–200 nm)*, *multilamellar vesicles (MLVs: >0.5 μm)*, *giant unilamellar vesicles (GUVs: >1 μm)* and *large unilamellar vesicles (LUVs: 200 nm to 1 μm)*.

Liposome can be incorporated with drugs (antibiotics, antifungals), proteins/polypeptides (including antibiotics), and virus and bacteria can be incorporated in the liposomes. Hydrophilic drugs are incorporated within the central cavity which is hydrophilic, and lipophilic drug molecules are incorporated in the lipid bilayer. LUVs are the most appropriate liposomes for the food industry because of their high encapsulation efficiency, their simple production methods, and their good stability over time. The great advantage of liposomes over other microencapsulation technologies is the stability of liposomes to water-soluble material in high water activity application. Spray-dryers, extruders, and fluidized beds impart great stability to food ingredients in the dry state but release their content readily in high water activity application, giving up all protection properties. Another advantage of liposomes is the targeted delivery of their content in specific parts of the foodstuff. For example, it has been shown that liposome-encapsulated enzymes concentrate preferably in the curd during cheese formation, whereas nonencapsulated enzymes are usually distributed evenly in the whole milk mixture, which leads to very low (2–4%) retention of the flavor-producing enzymes in the curd.

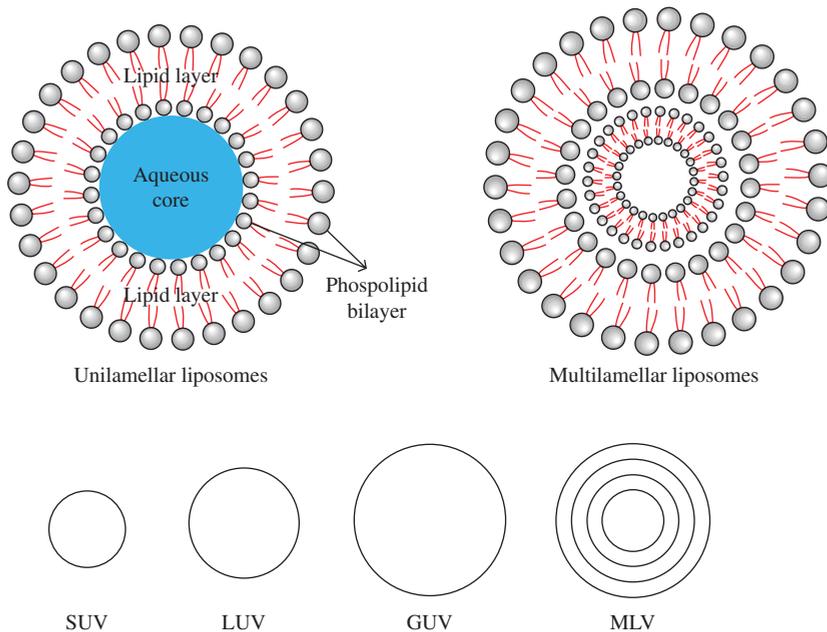


Figure 1.31 Schematic representation of basic structures and different types of liposomes. *Source:* Mishra GP, et al. 2011. Recent Applications of Liposomes in Ophthalmic Drug Delivery. Article ID 863734; doi:10.1155/2011/863734. Reproduced with permission from Journal of Drug Delivery.

Bromelain-loaded liposomes were also used as meat-tenderizer to improve stability of the enzyme during the food processing. Liposome-entrapped nisin retained higher activity against *Listeria*, which have improved stability in cheese production, proving a powerful tool to inhibit the growth of *Listeria* in cheese. Encapsulation of vitamin C also significantly improves the shelf life from a few days to up to 2 months, especially in the presence of common food components that would normally speed up decomposition, such as copper ions, ascorbate oxidase, and lysine. A cost-effective drying method of liposome microcapsules and a dry liposome formulation that readily reconstitutes upon rehydration would ensure a promising future to liposome encapsulation of food ingredients. Microfluidization has been shown to be an effective, cost-effective, and solvent-free continuous method for the production of liposomes with high encapsulation efficiency. The method can process a few hundred liters per hour of aqueous liposomes on a continuous basis. Liposome formulations are normally kept in relatively dilute aqueous suspensions that might be a serious drawback for the large-scale production, storage, and shipping of encapsulated food ingredients.

In an attempt to avoid the use of organic solvent in the production of liposomes, *supercritical fluids* like carbon dioxide, alkanes (C_2 to C_8) and nitrous oxide (N_2O) were used as the solvent for the phospholipids. In this process, supercritical fluid containing the active ingredient and the shell material are maintained at high pressure and then released at atmospheric pressure through a small nozzle. In this process called, *rapid expansion of supercritical solution (RESS)*, the sudden drop in pressure causes desolvation of the shell material, which is deposited around the active ingredient (core) and forms a coating layer.

Although this method is scientifically interesting, the encapsulation efficiency is limited at 15% and also very few polymers with low cohesive energy densities are soluble in supercritical fluids.

1.13.1.2 Physicomechanical methods Centrifugal force processes were developed in the 1940s to encapsulate fish oils and vitamins, protecting them from oxidation. In this method, an oil and water emulsion is extruded through small holes in a cup rotating within an oil bath. The aqueous portion of the emulsion is rich in a water-soluble polymer, such as gelatin, that gels when cooled. The resulting droplets are cooled to form gelled polymer-matrix beads containing dispersed droplets of oil that are dried to isolate. Similar in concept to centrifugal force processes, submerged nozzle processes produce microcapsules when the oil core material is extruded with gelatin through a two-fluid nozzle. The oil droplets are enveloped in gelatin as they are extruded through the nozzle and then the capsules are cooled to gel the walls before being collected and dried. Centrifugal extrusion processes generally produce capsules of a larger size from 250 μm up to a few millimeters in diameter. The core and the shell materials which should be immiscible with one another are pushed through a spinning two-fluid nozzle. This movement forms an unbroken rope which naturally splits into round droplets directly after clearing the nozzle. The continuous walls of these droplets are solidified either by cooling or by a gelling bath, depending on the composition and properties of the coating material.

Spray drying, either hot or cold media, is a mechanical microencapsulation method developed in the 1930s and is a low-cost commercial process, which is mostly used for encapsulating fragrances, oils, and flavors. Core particles are dispersed in a polymer solution, usually an oil or active ingredient immiscible with water and sprayed into a hot chamber. The resultant emulsion is atomized into a spray of droplets by pumping the slurry through a rotating disk into the heated compartment of a spray drier. The fine droplets are encapsulated within the core materials in the drying medium. As the hot or cold medium evaporates, the powder entraps the encapsulated core within the material. Spray chilling is applied mainly to retard volatilization during thermal processing of sensitive components including vitamins, minerals, and flavors. The capsules are collected through continuous discharge from the spray drying chamber.

Fluidized-bed drying, another mechanical encapsulation method, is restricted to encapsulation of solid core materials, including liquids absorbed into porous solids. *Fluidized-bed processing* involves drying, cooling, agglomeration, granulation, and coating of particulate materials. It is ideal for a wide range of both heat sensitive and nonheat sensitive products. Uniform processing conditions are achieved by passing a gas (usually air) through a product layer under controlled velocity conditions to create a fluidized state. Solid particles to be encapsulated are suspended on a jet of air and then covered by a spray of liquid coating material. The capsules are then moved to an area where their shells are solidified by cooling or solvent vaporization. The process of suspending, spraying, and cooling is repeated until the capsules' walls are of the desired thickness. This process is known as the Wurster process when the spray nozzle is located at the bottom of the fluidized bed of particles. Both fluidized-bed coating and the *Wurster process* are variations of the pan coating method. In pan coating, solid particles are mixed with a dry coating material and the temperature is raised so that the coating material melts and encloses the core particles, and then is solidified by cooling; or, the coating material can be gradually applied to core particles tumbling in a vessel rather than being wholly mixed with the core particles from the start of encapsulation. This technology is one of the few advanced technologies capable of coating particles with any kind of shell material including starches, emulsifiers, fats, complex formulations, enteric or powder coatings, yeast extract, and so on. Fluidized-bed

drying is suited for powders, granules, agglomerates, and pellets with an average particle size normally between 50 and 5000 μm . Very fine, light powders or highly elongated particles may require vibration for successful fluid bed drying.

Applications include the manufacture of chemicals, pharmaceuticals and biochemicals, polymers and food and dairy products. This technology offers important advantages over other methods of drying particulate materials. Particle fluidization gives easy material transport, high rates of heat exchange at high thermal efficiency while preventing overheating of individual particles. In nontherapeutic applications, cell microencapsulation technology has successfully been applied in the food industry for the encapsulation of live probiotic bacteria cells to increase viability of the bacteria during processing of dairy products and for targeted delivery to the gastrointestinal tract. In addition to dairy products, microencapsulated probiotics have also been used in nondairy products, such as Theresweet™ which is a sweetener. The pH, DO, storage temperature, species and strain, and concentration of lactic and acetic acids are some of the factors that greatly affect the probiotic viability in the product. The probiotic product should contain at least 10^6 – 10^7 cfu of viable probiotic bacteria per gram. Oral administration of microcapsules containing live bacterial cells has potential as an alternative therapy for several diseases. The microcapsules should have adequate membrane strength (mechanical stability) to endure physical and osmotic stress such as during the exchange of nutrients and waste products. The microcapsules should not rupture on implantation as this could lead to an immune rejection of the encapsulated cells. Also, while investigating the potential of using *alginate-poly-L-lysine-alginate* (APA) microcapsules loaded with bile salt hydrolase (BSH) overproducing active *Lactobacillus plantarum* 80 cells in a simulated gastrointestinal tract (GIT) model for oral delivery applications, the mechanical integrity and shape of the microcapsules were good. However, the *genipin cross-linked alginate-chitosan* (GCAC) microcapsules possess a higher mechanical stability as compared to APA microcapsules for oral delivery applications. Extensive research into the mechanical properties of the biomaterial to be used for cell microencapsulation is necessary to determine the durability of the microcapsules during production and especially for *in vivo* applications where a sustained release of the therapeutic product over long durations is required.

Another mechanical encapsulation process is spinning disk and centrifugal coextrusion; they are both atomization methods used in modified spray cooling encapsulation. The internal phase is dispersed into the liquid wall material and the mixture is advanced onto a turning disk. Droplets of pure shell material are thrown off of the rim of the disk along with discrete particles of core material enclosed in a skin of shell material. After having been solidified by cooling, the microcapsules are collected separately from the particles of shell material. Delivery of lipid-based health-promoting components such as *omega-3 fatty acids* (PUFA) while preserving taste is an important, and thus for effective delivery, functional lipids must be protected and stabilized against oxidative degradation. Encapsulation is needed for the prevention of off-taste or strong flavor above certain concentrations of functional active ingredient; for instance, green tea extract has a naturally bitter taste, but in higher concentrations, the bitterness is more intense. Therefore, green tea is sometimes encapsulated to mask the flavor. Centrifugal coextrusion processes generally produce capsules of a larger size, from 250 μm up to a few millimeters in diameter. The core and the shell materials, which should be immiscible with one another, are pushed through a spinning two-fluid nozzle. This movement forms an unbroken rope which naturally splits into round droplets directly after clearing the nozzle. The continuous walls of these droplets are solidified either by cooling or by a gelling bath, depending on the composition and properties of the coating material.

Extrusion process is a physical entrapment method using mostly sugars and starch to encapsulate volatile and unstable flavors. The process uses shear force to blend two materials linking them physically and involves the preparation of low moisture (5–10%) melt at 100–130°C and then the agglomerated starch entraps the flavor into encapsulated cavities. The carrier materials are added through the hopper, and a twin-screw extruder is used to grind the pellets into fine particles. The core material is then injected directly into the extruder to mix with the carrier materials that form complexes under shear and pressure.

As the mixture exits the extruder, the encapsulated mass is collected, dried, and milled to break up the aggregates into small particles. Two examples of extrusion for flavor encapsulation include the use of β -cyclodextrins and starch to entrap D-limonene and retention of β -carotene (58–97%) in wheat flour matrix.

One disadvantage of this technology involves the formation of large particles (typically 500–1000 μm), which limits the use of extruded flavors in application where mouth feel is a crucial factor. Also shell materials are limited for extrusion encapsulation. Some low temperatures process using mixtures of corn starch and fat or corn starch and polyethyleneglycol for the encapsulation of enzymes.

Coacervation is a relatively simple and promising technique balancing the electrostatic interaction between two components of the encapsulation emulsion to create water- and heat-resistant microcapsules. Very high payloads can be achieved up to 99% based on mechanical stress, temperature, or sustained release. A complex coacervation process begins with the suspension or emulsification of core material in either gelatin or gum arabic solution. When a core solution is mixed with an oppositely charged encapsulating material, a complex is formed, resulting in phase segregation and associative complexation. The characteristic size of the capsules formed can be altered by changing the pH and temperature, also by the bioactive component properties or the type of encapsulating agent.

A core material such as hydrophobic citrus oil can be dispersed in hydrophilic gelatin, creating a two-phase system. The coalescence of the polymeric colloid normally occurs around the suspended core oil particles, creating small microcapsules. The final process involves adding a suitable cross-linking agent such as glutaraldehyde or formaldehyde, adjusting the pH, and subsequently collecting, washing and drying the encapsulated particles. Coacervation works by the aqueous-phase separation of immiscible solutions such as oil and water or protein and ionic polysaccharides. Complex coacervation is possible only at pH values below the isoelectric point of proteins. At the isoelectric point of proteins, the pH values of the protein become positively charged. Comatrix encapsulation or inclusion complexation is one form of encapsulation in which cyclic polymers such as β -cyclodextrins are used to encapsulate other molecules. α -, β -, γ -*cyclodextrins* have been shown to encapsulate and stabilize lycopene, flavors, colors, and vitamins. Inclusion complexation in porous carbohydrate structures possible with both spray drying and/or extrusion processing was also used to entrap unwanted odors (deodorization) or bitter molecules. Inclusion encapsulation or complexation generally refers to the supramolecular association of a ligand (“encapsulated” ingredient) into a cavity-bearing substrate (“shell” material). The encapsulated unit is kept within the cavity by hydrogen bonding, VDW forces or by the entropy-driven hydrophobic effect.

Despite coacervation’s intrinsic advantage and unique properties compared to the other common encapsulation processes, a major problem is the high cost of the process, complex, and cross-linking agent, glutaraldehyde is a harmful toxicity, and so must be carefully used. Other cheaper processes such as spray drying can be used or a process in which a transglutaminase is used to crosslink the proteins in the shell material.

Table 1.33 Examples of microencapsulated meats, enzymes, and cells

Products	Technology	Purpose
<i>Meats</i>		
Pigment	Spray-rying	Color retention
Volatile flavor	Encapsulation	Flavor retention
Acidulate/antioxidant	Spray-drying/fluidized bed	Direct acidification
Antimicrobials	–	Shelf-life of dry sausages
Bacteriophage	–	Safety for animal pathogens
<i>Enzymes</i>		
Lactase	Liposome/Polyvinylalcohol hydrogel, and so on.	Lactose hydrolysis
Glucoamylase	Aginate beads, and so on.	Maltodextrin hydrolysis
Glucose isomerase	Alginate, and so on.	Fructose syrup
Invertase	Polyvinylalcohol hydrogel, and so on.	Invert sugar syrup
α -Amylase	Sodium alginate, and so on.	Starch hydrolysis
Inulinase	Alginate, and so on.	Sucrose hydrolysis
Chymosin	Liposome, and so on.	Continuous milk coagulation
Peptidases/Proteinases	Liposome, and so on.	Accelerated cheese ripening
Peptidase (recombinant)	Alginate + Chitosan	Accelerated cheese ripening
Flavorzyme	Liposome	Accelerated cheese ripening
<i>Cells</i>		
<i>Lactobacillus casei</i>	Alginate beads	Enhanced survival to heat
Lactobacilli	Spray-dried alginate	Enhanced survival to heat
<i>Bifidobacterium longum</i>	Encapsulated, and so on.	Enhanced survival to freezing
Lactococci	Alginate beads, and so on.	Protection against bacteriophage

Source: Author's compiled data; Champagne, C., L. Saucier, and B. H. Lee. 2009. Immobilization of cells and enzymes for fermented dairy or meat products: In: Encapsulation of Food Bioactive Ingredients and Food Processing (Eds: N.J. Zuidam and V. A. Nedovic), pp. 400, Springer, USA.

Some microencapsulated cells and enzymes have the potential to address many problems in food processing, among which some are already commercially used, particularly for flavor acceleration of cheeses and meats, stability of probiotics and protective cultures in foods (Table 1.33). Many works on encapsulation of probiotics have mainly focused on maintaining viability of probiotic bacterial cells at low pH and high bile concentrations, as well as during spray drying, freeze drying, and storage. Much research has focused on microencapsulation technologies and the manipulation of encapsulation parameters, such as coating material types and their concentrations and the use of multiple coating layers. By these efforts, few encapsulated probiotic cultures stable for up to one year or more at room temperature are on the markets.

1.13.2 Nanoencapsulation

While microencapsulation is similar to *nanoencapsulation* aside from it involving larger particles and having used for a longer period of time than nanoencapsulation, nanoencapsulation is a new technique of miniaturized microencapsulation using *nanocomposite*, *nanoemulsion*, and *nanoextrusion*. Nanoencapsulation is the coating of various substances within another material at sizes on the nanoscale. Nanoparticles are colloidal

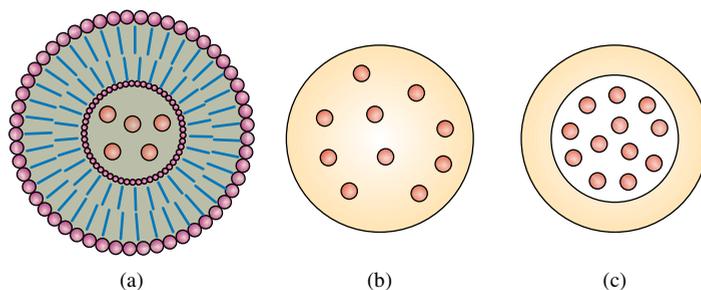


Figure 1.32 Schematic structure of (a) liposome, (b) nanosphere, and (c) nano-capsule. *Source:* Orive, G., et al. 2009. Reproduced with permission of Nature Publishing Group.

sized particles in ranging from 10 to 1000 nm diameter composed with nanocapsules and nanospheres including liposome (Figure 1.32). While nanocapsules are vesicular systems in which the bioactive compound is confined to a cavity surrounded by a polymer membrane, nanospheres are matrix systems where that compound is uniformly dispersed. Their small sizes are better suited for targeted delivery of drugs, nutrients, or bioactive compounds in small quantities to specific sites. As nanoencapsulation can improve the solubility and pharmacokinetic profiles of many insoluble drugs, targeted drug delivery is greatly enhanced, bioavailability to the target tissues and cells are significantly improved, while toxicity is reduced. Nanoencapsulation thus has the potential to enhance bioavailability, improve controlled release, and enable precision targeting of the bioactive compounds in a greater extent than microencapsulation.

A liposome already described in microencapsulation is an artificially prepared vesicle composed of a lipid bilayer, that can be used as a vehicle for administration of nutrients and pharmaceutical drugs. Liposomes are composed of natural phospholipids, and other mixed lipid chains with surfactant properties (e.g., egg phosphatidylethanolamine). Liposomes can be prepared by disrupting biological membranes such as by sonication. The major types of liposomes are the MLV, the SUV, the LUV, and the cochleate vesicle. Liposomes are different than micelles and reverse micelles composed of monolayers. Micelles are useful for encapsulating non-water soluble drugs to be administered intravenously. Nanospheres and nanocapsules are basically small vesicles used to transport materials. Nanospheres are typically solid polymers with drugs or active ingredients embedded in the polymer matrix. Nanocapsules are a shell with an inner space loaded with the ingredient of interest. Both systems are useful for controlling the release of a drug or bioactive molecule and/or protecting it from the surrounding environment. Dendrimers which are highly branched polymers with a controlled three-dimensional structure around a central core might be the most versatile of all nanocarriers.

In food nanoencapsulation, protection of bioactive compounds such as vitamins, antioxidants, proteins, lipids, and carbohydrates can better be achieved for the production of enhanced and stable functional foods. Flavors, oils, and other lipophilic materials are used in systems such as in salad dressings or mayonnaise where long-term stability is desirable. Nanoencapsulation is important for “*Nutraceutical*,” a combined word of “nutrition” and “pharmaceutical.” It is a food or food product that provides health and medical benefits, including the prevention and treatment of disease. While nutraceuticals are products isolated or purified from foods that are generally sold in medicinal forms capsules or tablets, *functional foods* are usually associated with food and consumed as a part of

foods. The effectiveness of nutraceuticals in preventing disease depends on preserving the bioavailability of bioactive ingredients until their release at targeted sites. Reducing the particle size may improve the bioavailability, delivery properties, and solubility of the nutraceuticals due to more surface area per unit volume and thus their biological activity. The bioavailabilities of these nutraceuticals are supposed to increase as a nanocarrier allows them to enter the bloodstream from the gut more easily.

Some of the nanoencapsulated hydrophilic nutraceuticals are ascorbic acid, polyphenols, and so on, and lipophilic compounds are insoluble in water but soluble in lipids and organic solvents. Nanoencapsulated lipophilic nutraceuticals include *lycopene*, *β -carotene*, *lutein*, *phytosterols*, and *DHA*. The solubility of the bioactive ingredients determines the release rate and release mechanism from a polymeric matrix system. Hydrophilic compounds show faster release rates and their release kinetics is determined by the appropriate combination of diffusion and erosion mechanisms. Lipophilic compounds often resulted in incomplete release due to poor solubility and low dissolution rates by an erosion mechanism. Although lipophilic compounds are highly permeable through the intestine via active transport and facilitated diffusion, hydrophilic compounds have low permeability that are absorbed only by active transport mechanism. Nanocarrier food systems such as lipid or natural biopolymer-based capsules are most often utilized for encapsulation, among which *nanoliposomes*, *archaeosomes*, and *nanocochleates* are three types of lipid-based nanocarrier systems that have applications in pharmaceutical, cosmetic, and food industries. Natural polymers such as albumin, gelatin, alginate, collagen, chitosan, α - and β -lactalbumin were used for the formulation of nano delivery systems. Whey protein has also been used as nanocarrier to improve the bioavailability of nutraceuticals, nanodrops mucosal delivery system of vitamins, and nano-based mineral delivery system. Nanoencapsulation of probiotics is desirable to develop designer probiotic bacterial preparations that could be delivered to certain parts of the gastrointestinal tract where they interact with specific receptors. These nanoencapsulated designer probiotic preparations may also act as *de novo* vaccines, with the capability of modulating immune responses. Biopolymer assemblies stabilized by various types of noncovalent forces have recently shown considerable progress. A starch-like nanoparticle can also help to stop lipids from oxidizing and therefore improve the stability of oil-in-water emulsions. The health benefits of anticancer compound *curcumin*, the natural pigment that gives the spice turmeric its yellow color, could be enhanced by encapsulation in nanoemulsions. Nanoemulsions could improve stability and oral bioavailability of *epigallocatechin gallate* and curcumin. A stearin-rich milk fraction was used, alone or in combination with α -tocopherol, for the preparation of oil-in-water sodium caseinate-stabilized nanoemulsions. Immobilization of α -tocopherol in fat droplets, composed by high melting temperature milk fat triglycerides, provided protection against degradation.

1.13.2.1 Nanoencapsulation techniques In general, the physicochemical properties such as particle size, size distribution, surface area, shape, solubility, encapsulation efficiency, and releasing mechanisms were reported to be altered by the encapsulation technique and delivery system. The appropriate encapsulation techniques must be based on the required size, physicochemical properties, nature of the core material, and wall material. Thus, the techniques used to achieve nanoencapsulation are more complex than microencapsulation, probably due to the difficulty in attaining a complex capsule and core material as well as the demands of releasing rates of nanoencapsulates. Various techniques developed and used for microencapsulation purpose such as emulsification, coacervation, inclusion complexation, emulsification–solvent evaporation, nanoprecipitation, and supercritical fluid

technique are all considered nanoencapsulation techniques since they can produce capsules in the nanometer range (10–1000 nm).

Nanoencapsulation techniques use either “top-down” or “bottom-up” approaches for the development of nanomaterials. “*Top down*” methodologies which consist in decreasing the size of macrostructures down to the nano-size scale or by “*Bottom-up*” techniques in which arrangements of atoms, molecules, or single particles are induced. A top-down approach such as emulsification and emulsification-solvent evaporation involves the application of precise tools that allow size reduction and structure shaping for desired application of the nanomaterials being developed. In the bottom-up approach such as the supercritical fluid technique, inclusion complexation, coacervation, and nanoprecipitation, materials are constructed by self-assembly and self-organization of molecules. These are influenced by many factors including pH, temperature, concentration, and ionic strength. These nanoencapsulation techniques can be used for encapsulation of various hydrophilic and lipophilic bioactive compounds. Emulsification, coacervation, and supercritical fluid technique are used for the encapsulation of both hydrophilic and lipophilic compounds, but inclusion complexation, emulsification-solvent evaporation, and nanoprecipitation techniques are mostly used for lipophilic compounds.

Nanoemulsions, which are nanoscale droplets of multiphase colloidal dispersions formed by dispersing of one liquid in another immiscible liquid by physically induced rupturing. Different size ranges of nanoemulsions less than 100, 100–500, and 100–600 nm have been reported, but the most appropriate ones are having the size ranges of less than 100 nm and processing different properties than ordinary emulsions. Nanoemulsions are liquid-in liquid dispersions with small droplets, typically in the range of 20–200 nm and are more thermodynamically stable. Nanoemulsions result from high kinetic energy input induced by shearing, which results in high energy emulsified small droplets making them stable against sedimentation or creaming. Stability of nanoemulsions can be enhanced by adding emulsifiers, which may be incorporated using high-shear homogenization. High-shear homogenization makes it possible for manufacturers to lower levels of surfactants in products.

Nanoemulsions are used in cosmetics, personal-care formulations and in some chemical industries and other nanoemulsions used for the delivery of micronutrients include the use of liposomes to deliver vitamins (A, D, E, and K) and carotenoids. Nanoemulsions consisting of soybean-derived triglycerides and egg yolk phospholipids were used for parenteral high energy feeding. When dried, spray-dried emulsions of fish oil resulted in nanoparticles increased size in the range of 210–280 nm. Nanoemulsion can be prepared with mechanical (high-pressure homogenization, microfluidization, ultrasonication) and nonmechanical (solvent diffusion).

In high-pressure homogenization, the coarse dispersion of oil, aqueous phase, and emulsifier is passed through a small inlet orifice at pressures between 500 and 5000 psi. Microfluidization uses a very high pressure of up to 2000 psi to force the liquid through the interaction chamber consisting of microchannels of a special configuration, where the emulsion feeds through configuration. The mechanism of nanoemulsion generation by ultrasonication is likely attributed to bubble cavitation and the collapse of the cavities provides sufficient energy to increase surface area of droplets. Although there is high potential of ultrasonication for research purpose, industrial applications do not appear to be practical and high-pressure homogenization or microfluidization is often preferred. Nanoemulsions formed by nonmechanical methods (solvent diffusion technique) have been used to prepare nanoemulsion (90–120 nm) of α -tocopherol first dissolved in an organic solvent. The resulting coarse dispersion is passed through a high-pressure homogenizer and then the solvent is removed from fine emulsion by evaporation. This method

has a limitation of using large amount of organic solvent to prepare and of removing organic solvent by expensive equipment before consumption.

Nanomaterials are divided in three categories: (i) nanoparticles, (ii) nanoplates (like silver, gold, ZnO, etc.), and (iii) nanocomponents. Nanoparticles are mostly used in the medical area to find disease in human body and many nanomaterials are commercially available. The subjects are out of this text; only related topics on food and agriculture are discussed. Nanocomposites are materials that are made based on different components like nanoclay or CNTs (to provide conductivity to other materials).

Nanoparticles (also known as *nanomaterials*) in Figure 1.33 are manufactured for use in an array of applications such as cosmetics, material coatings, biomedical, optical, electronic, toxicology, food, agriculture, and environmental remediation as well as a fuel additive. They are being increasingly investigated for use in medical applications such as drug delivery and release. Nanoparticles often possess unexpected optical properties as they are small enough to confine their electrons and produce quantum effects. This is the size scale where the so-called quantum effects rule the behavior and properties of particles. Although many of their effects have been well documented, some of their mechanisms of action are not fully understood. Nanoparticles, because of their small size, are better suited for targeted delivery of nutrients in small quantities to specific sites. Flavor and oils and other lipophilic materials are used in systems such as in salad dressings or mayonnaise where long-term stability is desirable.



Figure 1.33 Representation of nanoparticles (millionths of a millimeter in size). *Source:* F005/0791 Nanoparticle, artwork LAGUNA DESIGN/SCIENCE PHOTO LIBRARY Nanoparticle, computer artwork.

Biocompatible and biodegradable biopolymers can be used to form delivery nanoparticles, which can maximize loading efficiency of nutrients in the particles, for site-specific delivery in the GIT. Nanoparticles emulsions and hydrogels can be made from egg white, soybean, and whey proteins. Mucosal delivery systems were also created from peptides derived from proteins and plasmids. Nanoparticles are easily dispersed in oil-based suspensions used in consumer products such as the delivery of omega-3 from fish oils. Nano-sized emulsions are kinetically stabilized monolayer of uniformly polydispersed spherical particles with a large surface area. The future application of nanoparticles technology in the areas of micronutrient and nutraceutical delivery will depend largely on the type of active molecule/ions and the product format, sprays or gels.

However, concern has arisen that widespread long-term nanoparticle use may “trickle down” into the environment, food, unforeseen effects on plant or animal or even human health.

Among two nanoparticles, *polymeric nanoparticles (PNs)*, which include nanospheres and nanocapsules, are solid carriers ranging from 10 to 1000 nm in diameter made of natural or artificial polymers, which are generally biodegradable and in which therapeutic drugs can be adsorbed, dissolved, entrapped, encapsulated, or covalently linked to the particles. The synthetic materials used to prepare nanoparticles include *poly(lactic acid) (PLA)*, *poly(glycolic acid) (PGA)*, *poly[lactide-co-glycolide (PLGA)*, *poly(alkylcyanoacrylate)* and *polyanhydride poly[bis(p-carboxyphenoxy)]propane-sebacic acid (PCPP-SA)*; natural polymers such as chitosan, alginate, and gelatin have also been tested.

When systemically administered nanoparticles are generally more stable than liposomes but are limited by poor pharmacokinetic properties, and a poor ability to cross the blood–brain barrier. Similar to liposomes, the surface of nanoparticles can be coated with molecules to increase blood–brain barrier permeability and improve pharmacokinetics and even enable targeting for delivery and imaging purposes. More recently, other configurations of nanocarriers such as *solid lipid nanoparticles (SLNs)*, micelles and dendrimers have been tested for brain drug delivery. SLNs consist of solid lipid matrices stabilized by surfactants to oral and parenteral drug delivery systems with low cytotoxicity and good physical stability. These combine the advantages of lipid emulsion and polymeric nanoparticle systems while overcoming the temporal and *in vivo* stability issues that trouble the conventional as well as PNs drug-delivery approaches. Other advantages are the avoidance of organic solvents, a potential wide application spectrum (dermal, per os, intravenous) and the high-pressure homogenization as an established production method.

1.13.2.2 Applications Nanomaterials and nanotechnologies are expected to yield numerous health and health care advances, such as more targeted methods of delivering drugs, new cancer therapies, methods of early detection of diseases, nanobiosensors as well as numerous benefits in agrifoods and environments (Table 1.34). Well over 100 nanoproducts are on the markets, but they also may have unwanted effects. Increased rate of absorption is the main concern associated with manufactured nanoparticles. In particular, nanoparticle research holds potential in areas such as diagnostics, cancer detection, and targeted drug delivery. However, their safety has been questioned, particularly through the discovery of toxic nanoparticles in sunscreens. The reactivity of nanoparticles is difficult to assess, so properties are still relatively unknown.

Nanoparticles in biomedicine *Nano-biochip* is one more dimension of lab-on-a-chip technology in which magnetic nanoparticles bound to a suitable antibody are used to label specific molecules, structures, or microorganisms. GNPs tagged with short segments of

Table 1.34 Various applications of nanobiosensors and nanoparticles

Types	Applications
(a) Nanobiosensor/Biochips	<ul style="list-style-type: none"> — Pregnancy test (human chorionic gonadotrophin (hCG) in urine)* — Glucose test (diabetes patients)* — Infectious disease (from red blood cell)* — LAB-ON-A-CHIP (for genetic analysis, bacteria/viruses, cancers, blood DNA, etc.)* — Nanochemical sensor (for detecting harmful chemicals and biological weapons)* — Smart nanosensors for grain quality monitoring — Nano electronic nose (for recognizing smells, etc.)
(b) Nanocomposites/ Agrochemicals	<ul style="list-style-type: none"> — Nanoclay particle beer bottle* — Nanocleaning/nanoemulsion disinfectant* — Many paints/varnishes* — Nanoselenium feed for chicken* — NanoFungicide, NanoPlant Growth Regulator (PrimoMaxx, Syngenta, etc.)* — Nanofertilizers/pesticides — Nanofuel catalysts* — Nanopowered catalytic device (for deep flying oil)*
(c) Nanoencapsulation/Nanofoods	<ul style="list-style-type: none"> — Food ingredients and additives (AquaNova; Bioral omega-3 nanocochleates, Synthetic BSF lycopene, etc.)* — Beverage (oat chocolate/vanilla, etc.)* — Flavors/colors and enzymes* — Detection of food pathogens, virus, toxin (antibody fluorescence-based methods, etc.) — Structural control for texture, heterologous mixtures of emulsions and suspensions — Nanosilver antibacterial kitchenware (Nano Care Tech, etc.)/food contact material (A-Do Global)* — Food packaging (Durethan KU2-2601)/NanoZnO plastic wrap (SongSing Nanotech)* — Edible nano wrappers* — Chemical release food packaging — Nanomanipulation for seeds
(d) Nanocosmetics/Others	<ul style="list-style-type: none"> — Sunscreen cream with TiO₂, ZnO* — Nanorepair Q10 cream* — Nanocoatings (sunglass, fabrics, etc.)*
(e) Drug delivery/Environment/ Pathogens	<ul style="list-style-type: none"> — Delivery and directing of drugs to tumors and growing new organs — Detecting and filtering toxins out of water supplies — Cleaning up heavy metals and organic chemicals

*Commercialized products.

Source: From: (1) Authors compiled data; (2) Ljabadeniyi, O. A. 2012. *Afr J. Biotechnol.* 11:15258–15263; (3) Fathi, M. et al. 2012. *Trends Food Sci Technol.* 23: 13–27; (4) Rai, V. et al. 2012. *J. Biomater. Nanobiotech.* 3: 315–324.

DNA can be used for detection of genetic sequence in a sample. Multicolor optical coding for biological assays has been achieved by embedding different-sized quantum dots into polymeric microbeads. Nanopore technology for analysis of nucleic acids then converts strings of nucleotides directly into electronic signatures.

Nanoparticles have been a boon in *delivering drugs* to specific cells. The overall drug consumption and side-effects can be lowered significantly by depositing the active agent in the morbid region only and in no higher dose than needed. This highly selective approach such as dendrimers and nanoporous materials reduces costs and human suffering. Another example is to use block copolymers, which form micelles for drug encapsulation. They could hold small drug molecules transporting them to the desired location. Other small nanoelectromechanical systems are being investigated for the active release of drugs. Some potentially important applications include cancer treatment with iron nanoparticles or gold shells. To provide controllable release of bioactive compounds and local delivery of potentially therapeutic molecules, PLA, PGA, PLGA, poly(alkylcyanoacrylate) and PCPP-SA natural polymers such as chitosan, alginate, and gelatin have also been tested. When systemically administered, nanoparticles are generally more stable than liposomes but are limited by poor pharmacokinetic properties and a poor ability to cross the blood–brain barrier. PLGA nanoparticles and microparticles (particle size >1000 nm) have been successfully used to deliver drugs for the treatment of neurodegenerative disorders.

Nano electronic nose (e-nose) based on natural olfactory receptors using nanotechnologies could also help to develop the right smells for their foods, sniff out rotting ingredients, pick up the smells produced by bacteria and pathogens, and detect cancer infections, gastrointestinal disorder, liver diseases, and so on. As nano-nose applications can be applied to agrifoods, health, and environmental monitoring, this technology will revolutionize the electronic nose industry with its compact size and practical applicability. As compared with existing artificial e-nose devices and current e-nose devices based on metal oxide semiconductors or conducting polymers, nanowire chemiresistors are seen as critical elements in the future miniaturization of e-noses. Prototype nano e-nose was recently designed to detect harmful airborne agents such as pesticides, biological weapons, gas leaks, and other unwanted presences. The development has clear applications in military, industry, and agricultural area (www.neapplications.com/nanosensors-are-key-electronic-nose-prototype).

Nanoclays and nanofilms in packaging as barrier materials are used to prevent spoilage and prevent oxygen absorption. Plastic polymers containing or coating with nanomaterials are used to improve mechanical or functional properties. This is the largest share of the current short-term market for nanotechnology applications in food sector. Nanoparticles with silver and zinc oxide are used for antimicrobial/antifungal surface coatings. Nanocarrier systems for delivering of nutrients and supplements in the form of liposomes or biopolymers-based nanoencapsulated substances are commercially available.

Nanofood and agriculture Commercially available nano-ingredients and nanomaterial additives include nanoparticles of iron or zinc, and nanocapsules containing ingredients like omega 3, or producing stronger flavors and colorings. In food packaging, nanoparticles are used to detect bacterial contamination, absorb oxygen or release preservatives to food, surface coating of bottles for ketchup or dressings, and so on. Untested nanotechnology is being used in more than 100 food products, food packaging, and contact materials currently on the shelf, without warning or new FDA testing. A list of some food products currently containing nanoproducts besides in Table 1.34 include: Canola Active Oil (Shemen, Haifa, Israel), Nanotea (Shenzhen Become Industry Trading Co. Guangdong,

China), Fortified Fruit Juice (High Vive.com, USA), Nanoceuticals Slim Shake (assorted flavors, RBC Lifesciences, Irving, USA), NanoSlim beverage (NanoSlim), Oat Nutritional Drink (assorted flavors, Toddler Health, Los Angeles, USA), and “Daily Vitamin Boost” fortified fruit juice (Jamba Juice Hawaii, USA) and nanocapsules containing tuna fish oil (a source of omega 3 fatty acids) in “Tip-Top” Up bread (Enfield, Australia). Polylysine, a food-grade polypeptide, can be added to the oil droplets to help protect from oxidation. Polylysine is much smaller than the phytoglycogen octenyl succinate nanoparticles, allowing it to fill in the gaps between phytoglycogen octenyl succinate nanoparticles.

The effects of two commonly used nanoparticles containing zinc oxide and cerium oxide were also for their effects on the growth of soybean. Zinc oxide is a common component of cosmetics and ultimately ends up as a contaminant of solid waste generated by sewage treatment. This waste is widely used as an organic fertilizer. Cerium oxide is used in some diesel fuels to improve combustion and reduce particulate emissions. The plants grown in the presence of zinc oxide nanoparticles actually grew slightly better than control plants grown in the absence of nanoparticles. However, zinc built up in the edible parts of the plants, which included the leaves and the beans. Zinc oxide nanoparticles have been shown to be toxic to mammalian cells grown in the laboratory, but effects in humans remain to be examined fully. After nanoparticles are found in foods, including bread, cornflakes, biscuits and caramels, their safety has been questioned, particularly through the discovery of toxic nanoparticles in sunscreens. As the reactivity of nano-sized particles is difficult to assess, properties are still relatively unknown. Nanoparticles have also been known to pass through cell membranes and cause possible harm to otherwise healthy cells. The carbon nanoparticles were extracted from the food sources surrounded by polymerized sugar; thus some demonstrated that even at high concentrations, the nanoparticles had little or no toxicity.

1.13.2.3 Safety issues of nanoparticles

Health aspects on nanotechnology Many applications of nanomaterials are expected to food and additive preparations such as in (i) encapsulation of additives/nutrient, flavors and colors, and enzymes, (ii) structure control for texture, emulsions and suspensions, and (iii) detection of food pathogens, virus, and toxins. Consumers are increasingly concerned about pesticide and antibiotic residues, food allergies, GMF, irradiation, and food adulteration, and so on, and now more concern about health implications of unintended consequences of nanoparticles.

Very little is known about how the particles interact with the environment and human body, but there is evidence that nanoparticles might ferry toxins right past the body’s normal defense. Nanoparticles are readily inhaled and ingested and at least some will cross the skin, and may gain access to tissues and cells. The Royal Society in the United Kingdom mentioned that nanotechnology poses health and environmental risks great enough to justify banning. Also there is a lack of analytical methods and predictive model to evaluate the safety of nanofood and food contact materials incorporated with nanomaterials. Currently, a high degree of uncertainty exists as to whether regulatory system and statute would give attention to the “nano” scale. According to WHO, new data and measurement approaches needed to ensure safety of products using nanotech can be properly assessed, in which nanobiotechnology can easily fall into the premed trap of GMO.

Although possible dangers of nanoparticles have been discussed and concerns are now arising that nanomaterial may prove toxic to humans or the environment, it is not simply uncertain on what effects, if any, nanoparticles will have on the *environment, health, and safety (EHS)*. This nanotechnology should not completely halt on EHS grounds, since

nanotechnologies are extremely beneficial to both the environment and human health in the long term.

When materials are made into nanoparticles, their surface area to volume ratio increases. The greater specific surface area (surface area per unit weight) which can make the particles very reactive or catalytic, so that they can easily pass through cell membranes in organisms that may cause unwanted effects to the lungs as well as other organs. Their interactions with biological systems are relatively unknown. However, the particles must be absorbed in sufficient quantities in order to pose health risks. Although the results in 2008 showed that iron oxide nanoparticles caused little DNA damage and were nontoxic, but zinc oxide nanoparticles were slightly worse and titanium dioxide caused only DNA damage. While CNTs caused DNA damage at low levels, copper oxide was found to be the worst offender.

Whether cosmetics and sunscreens containing nanomaterials pose health risks also remains largely unknown at this stage. However considerable research has demonstrated that zinc nanoparticles are not absorbed into the bloodstream *in vivo*. Diesel nanoparticles have been found to damage the cardiovascular system in a mouse model. Concern has also been raised over the negative health effects of respirable nanoparticles from certain combustion processes. However, other research showed that most nanoparticles do not pose a serious threat to human and environmental safety, and thus in theory nanoparticles should be benign.

Some nanoparticles have the unique ability to easily pass through cell walls and can permeate the blood–brain barrier and may also be bactericidal, that can highly damage into ecosystems where bacteria are at the bottom of the food chain. Thus, this safety issue is currently done on a case-by-case basis, but risk assessment methods need to be kept up to date as the use of nanomaterials expands, especially as they find their way into consumer products.

Summary

Microencapsulation system offers potential advantages over conventional drug delivery systems and also established as unique carrier systems for many pharmaceuticals (targeted drug delivery systems). Although a wide range of encapsulated products have been developed and successfully marketed in the pharmaceutical and cosmetic industries, microencapsulation has a comparatively much smaller market in the food industry. Also the development time is rather long involving multidisciplinary cooperation and the low margins typically achieved in food ingredients are a deterrent factor. Despite the significant advances in the field of microencapsulation, still many challenges need to be rectified during the appropriate selection of core materials, coating materials, and process techniques. A wide variety of different types of delivery systems are available for functional bioactives, but each type of delivery system has its own specific advantages and disadvantages. Techniques such as microencapsulation, microemulsions, colloidal particulation, and nanostructuring may be adequate for the delivery of bioactive components at health-optimizing doses, at appropriate site, using the proper natural triggers. Future work will improve encapsulation and coencapsulation delivery of functional foods like live probiotic bacterial cells. Simulated human gastrointestinal models are making the evaluation of functional bioactives much robust allowing for *in vitro* simulation of harsh conditions in the GIT.

Colloidal dispersions of soft matter systems are used to create atomic systems. Nanoparticles are easily dispersed in oil-based suspensions used in consumer products such as the delivery of omega-3 from fish oils. Nano-sized emulsions are kinetically stabilized

monolayer of uniformly polydispersed spherical particles with large surface area. Colloidal particles and nanoemulsions could be used as delivery systems for micronutrients and nutraceuticals. Future application of nanoparticles technology in the areas of micronutrient and nutraceutical delivery will depend largely on the type of active molecule/ions and the product format, sprays or gels.

Nanotechnology may offer many benefits for human health and the environment. The properties of nanoparticles make them excellent candidates for advanced medication and new drug delivery methods, as well as for curing diseases like cancer and AIDS. Nanotechnologies can also help monitor pollution, lower energy requirements, and reduce the use of harmful cleansing chemicals. This technology can also be used to enhance food flavor and texture, to reduce fat content, or to encapsulate nutrients to ensure they do not degrade during a product's shelf life.

Nanoparticles emulsions and hydrogels can be made from egg white, soybean, and whey proteins. Nanoparticle-sized colloidal dispersions are used in a broad range of products including foods, cosmetics, paints, and drugs. Nanoparticle structures are created with technologies and processes that can accommodate soft materials. Nanomaterials can be used to make packaging that keeps the product fresher for longer. Intelligent food packaging, incorporating nanosensors, could even provide consumers with information on the state of the food inside and alert consumers when a product is no longer safe to eat. Sensors can warn before the food goes rotten or can inform us the exact nutritional status contained in the contents. Food nanotechnology advances offers important challenges for both government and industry. The food processing industry must ensure consumer confidence and acceptance of nanofoods.

Currently available information of risks associated to manipulation of nano-sized products is limited, and there are not internationally agreed regulatory parameters related to manipulation of nano-sized food materials. Thus, many products reach markets without prior evaluation on safety aspects.

Very little information is available on the development of foodstuffs which contain nanoparticles and on their physicochemical properties used as additives during their transit through the gastrointestinal tract. Until now, consumers lean to be more unwilling to nanofood applications than other uses. Regulatory aspects led by the United States of America and The National Nanotechnology Initiative (NNI) and other countries are centered on the uses and the applications of nanotechnology in three main areas: (i) Research and technology on the development of products at the atomic, molecular or macromolecular scales in the length of 1–100 nm, (ii) creating and using structures that have novel properties and functions because of their small sizes, and (iii) ability to control or manipulate on the atomic scale. The EU organization Strategy for Nanotechnology asserts that nanotechnology has the potential to enhance quality of life and industrial competitiveness, and thus lobbies aggressively for minimal legislation on nanotechnology. Current laws state that anyone producing or importing nanomaterials into Europe is required to provide written notification to public authorities; that requires the manufacturer to conduct research illustrating the properties and dangers of the product.

As the long-term impact of nanomaterials on the natural environment and human health is unknown, it is difficult to comprehensively regulate this technology in a single piece of legislation that would capture its risks. Nanotechnology should rather be regulated by a series of laws which govern the exposure of nanotechnology on specific areas: food, environment, medicine, and agriculture. Mishandling of previous GM food debates will put nonfoods at a disadvantage and nanofoods might be the next "next GMO." The possible solution to current problem will be the proper labeling on nanoproducts and legislation.

Bibliography

- Albæk, M. O., K. Gernaey, M. S. Hansen, S. Morten, and S. M. Stocks. 2011. Modeling enzyme production with *Aspergillus oryzae* in pilot scale vessels with different agitation, aeration, and agitator types. *Biotechnol. Bioeng.* 108: 1828–1840.
- Anbar, M., O. Gul, R. Lamed, U. O. Sezerman and E. A. Bayer. 2012. Improved thermostability of *Clostridium thermocellum* endoglucanase Cel8A by using consensus-guided mutagenesis. *Appl. Environ. Microbiol.* 78: 3458–3464.
- Andersson, D. I and D. Hughes. 2010. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat. Rev. Microbiol.* 8: 260–271.
- Ansari, A. H., and Q. Husain. 2013. Potential applications of enzymes immobilized on/in nano materials: a review. *Biotech. Adv.* 30: 512–523.
- Antunes, L. C, R. B. Ferreira, M. M. Buckner, and B. B. Finlay. 2010. Quorum sensing in bacterial virulence. *Microbiology* 156: 2271–2282.
- Ates, O., E. T. Oner, and K. Y. Arga. 2011. Genome-scale reconstruction of metabolic network for a halophilic extremophile, *Chromohalobacter salexigens* DSM 3043. *BMC Syst. Biol.* 5: 12–25.
- Atkinson, B., and F. Mavituna. 1991. *Biochemical Engineering and Biotechnology Handbook*, 2nd ed. Macmillan, New York.
- Augustin, M. A. and Y. Hemar. 2009. Nano- and micro-structured assemblies for encapsulation of food ingredients. *Chem. Soc. Rev.* 38: 902–912.
- Azarnia, S., B. H. Lee, V. Yaylayan, and K. N. Kilcawley. 2010a. Proteolysis development in enzyme-modified Cheddar cheese using natural and recombinant enzymes of *Lactobacillus rhamnosus* S93. *Food Chem.* 120: 174–178.
- Azarnia, S., D. St-Gerais, C. Champagne, and B. H. Lee. 2010b. Effect of encapsulated recombinant aminopeptidase of *Lactobacillus rhamnosus* on the accelerated Cheddar cheese. *Food Biotech.* 24: 135–149.
- Bailey, J. E. and D. F. Ollis. 1986. *Biochemical Engineering Fundamentals*, 2nd ed. McGraw-Hill, New York.
- Basha, E., H. O'Neill, and E. Vierling. 2012. Small heat shock proteins and α -crystallins: dynamic proteins with flexible functions. *Trends Biochem. Sci.* 37: 106–117.
- Berkemeyer, M. and F. Nothelfer. 2011. Protein crystallization in downstream processes. *BioProcess Int.* 9: 36.
- Bornscheuer, U. T. and M. Pohl. 2001. Improved biocatalysts by directed evolution and rational protein design. *Curr. Opin. Chem. Biol.* 5: 137–143.
- Brackman, G., P. Cos, L. Maes, H. J. Nelis, and T. Coenye. 2011. Quorum sensing inhibitors increase the susceptibility of bacterial biofilms to antibiotics *in vitro* and *in vivo*. *Antimicrob. Agents Chemother.* 55: 2655–2661.
- Brown, P. and D. Botstein. 1999. Exploring the new world of the genome with DNA microarrays. *Nat. Genet. Suppl.* 21: 33–37.
- Bumgarner, R. 2013. UNIT 22.1. Overview of DNA microarrays: types, applications, and their future. In *Current Protocols in Molecular Biology*. John Wiley & Sons, Inc., New York. doi: 10.1002/0471142727.mb2201s101.
- Champagne, C., L. Saucier, and B. H. Lee. 2009. Immobilization of cells and enzymes for fermented dairy or meat products. In *Encapsulation of Food Bioactive Ingredients and Food Processing*, N. J. Zuidam and V. A. Nedovic, pp. 400. Springer, USA.

- Chassaing, H., J. V. Norgaard, and A. J. Van Hengel. 2007. Proteomics based approach to detect and identify major allergens in processed peanuts by capillary LC-Q-TOF (MS/MS). *J. Agric. Food Chem.* 55: 4461–4473.
- Clements, F. and K. Bayer. 2006. Improvement of bioprocess monitoring: development of novel concepts. *Microb. Cell Fact.* 5: 19–26.
- Cowan, T. 2010. *Biotechnology in Animal Agriculture: Status and Current Issues.* (www.cnie.org/NLE/CRSreports/10Oct/RL33334.pdf).
- Das, M., N. Saxena, and P. D. Dwivedi. 2009. Emerging trends of nanoparticles application in food technology. Safety paradigms. *Nanotoxicology* 3: 10–18.
- Davies, H. M. 2010. Review article: commercialization of whole-plant systems for biomanufacturing of protein products: evolution and prospects. *Plant Biotechnol. J.* 8: 845–861.
- Debès, C., M. Wang, G. Caetano-Anollés, and F. Gräter. 2013. Evolutionary optimization of protein folding. *PLoS Comput. Biol.* 9: e1002861. doi:10.1371/journal.pcbi.1002861.
- Derrida, B. 1981. Random-energy model: an exactly solvable model of disordered systems. *Phys. Rev. B.* 24: 2613–2626.
- Dhanasekharan, K. 2006. Design and scale-up of bioreactors using computer simulation. *BioProcess Int.* 4: 34–42.
- Dion, M. A., M. Luykx, L. M. Ruud, J. B. Peters, S. M. van Ruth, and H. Bouwmeester. 2008. A review of analytical methods for the identification and characterization of nano delivery systems in food. *J. Agric. Food Chem.* 56: 8231–8247.
- Dobretsov, S., M. Teplitski, and V. Paul. 2009. Mini-review: quorum sensing in the marine environment and its relationship to biofouling. *Biofouling* 25: 413–427.
- Dufosse, L., M. Fouillaud, Y. Caro, S. A. S. Mapari, and N. Sutthiwong. 2014. Filamentous fungi are large-scale producers of pigments and colorants for the food industry. *Curr. Opin. Biotechnol.* 26: 56–61.
- Duncan, J. V. 2011. The communication challenges presented by nanofoods. *Nat. Nanobiotechnol.* 6: 683–688.
- Dutta, R. 2008. *Fundamentals of Biochemical Engineering*, Springer.
- Duvall, M. N. 2012. FDA Regulation of Nanotechnology, February, available at www.shb.com/newsevents/2012/FDARegulationofNanotechnology.pdf.
- Eldakak, M. S., S. I. Milad, A. I. Nawar, and J. S. Rohila. 2013. Proteomics: a biotechnology tool for crop improvement. *Front. Plant Sci.* 4: 1–12.
- Elsaesser, A. and C. C. Howard. 2012. Toxicology of nanoparticles. *Adv. Drug Deliv. Rev.* 64: 129–137.
- Farrell, P., J. Sun, M. Gao, H. Sun, B. Pattara, A. Zeiser, and T. D'Amore. 2012. Development of a scaled-down aerobic fermentation model for scale-up in recombinant protein vaccine manufacturing. *Vaccine* 30: 5695–5698.
- Fathi, M., et al. 2012. Nanoencapsulation of food ingredients using lipid based delivery systems. *Trends Food Sci. Technol.* 23: 13–27.
- Fernandes, R., V. Roy, H. C. Wu, and W. E. Bentley. 2010. Engineered biological nanofactories trigger quorum sensing response in targeted bacteria. *Nat. Nanotechnol.* 5: 213–217.
- Ferrer-Miralles, N., J. Domingo-Espín, J. Luis Corchero, E. Vázquez, and A. Villaverde. 2009. Review: microbial factories for recombinant pharmaceuticals. *Microb. Cell Fact.* 8: 17–25.
- Foo, J. L., C. B. Ching, M. W. Chang, and S. S. J. Leong. 2012. The imminent role of protein engineering in synthetic biology. *Biotechnol. Adv.* 30: 541–549.

- Freitag, R., and F. Hilbrig. 2012. Isolation and purification of recombinant proteins, antibodies and plasmid DNA with hydroxyapatite chromatography. *Biotechnol. J.* 7: 90–102.
- Gagnon, P. 2010. Monoliths open the door to key growth sectors. *BioProcess Int.* 8: 20–23.
- Garrido, C., J. M. Cantoral, M. Carbu, V. E. Gonzalez-Rodriguez, and F. J. Fernandez-Acero. 2010. New proteomic approaches to plant pathogen fungi. *Curr. Proteomics* 7: 306–315.
- Gbassi, G. K. and T. Vandamme. 2012. Probiotic encapsulation technology: from microencapsulation to release into the gut. *Pharmaceutics*, 4: 149–163.
- Gillian, R., H. Hadi Lioe, F. Stengel, A. J. Baldwin, and J. L. P. Benesch. 2013. Small heat-shock proteins: paramedics of the cell. *Top. Curr. Chem.* 328: 69–98.
- Goldenberg, D. P. 2013. Protein folding and assembly. *Encyclopedia of Biological Chemistry*, 2nd ed. pp. 625–631. Elsevier.
- Goldstein, D. A. and J. A. Thomas. 2004. Biopharmaceuticals derived from genetically modified plants. *Q. J. Med.* 97: 705–716.
- Gómez-López, V. M., M. S. Medina-Martínez, M. A. Santana. 2012. Q. Sensing. In *Decontamination of Fresh and Minimally Processed Produce*, 1st ed. V. M. Gómez-López, Ed., John Wiley & Sons, Inc.
- Gottschalk, U. 2011. The future of downstream processing. *Pharm Technol.* 35: s36–s41.
- Grande, C., F. Torres, C. M. Gomez, O. P. Troncoso, J. Canet-Ferrer, and J. Martínez-Pastor. 2009. Development of self-assembled bacterial cellulose-starch nanocomposites. *Mater. Sci. Eng.* 29: 1098–1104.
- Graveland-Bikker, J. F. and C. G. De Kruifa. 2006. Unique milk protein based nanotubes: food and nanotechnology meet. *Trends Food Sci. Technol.* 17: 196–203.
- Gresham, D., M. J. Dunham, and D. Botstein. 2008. Comparing whole genomes using DNA microarrays. *Nat. Rev. Genet.* 9: 291–302.
- Grieshaber, D., R. MacKenzie, V. Janos, and E. Reimhult. 2008. Electrochemical biosensors – sensor principles and architectures. *Sensors* 8: 1400–1458.
- Gruhl, F. J., B. E. Rapp, and L. Kerstin. 2012. Biosensors for diagnostic applications. *Adv. Biochem. Eng. Biotechnol.* 109: 1–34.
- Guisan, J. M. Ed. 2006. Immobilized cells and enzymes. In *Methods in Biotechnology*, 2nd ed. Humana Press Inc., New Jersey.
- Gummadi, S. N. 2003. What is the role of thermodynamics on protein stability? *Biotechnol. BioProcess Eng.* 8: 918.
- Hartl, F. U., A. Bracher, and M. Hayer-Hartl. 2011. Molecular chaperones in protein folding and proteostasis. *Nature* 475(7356): 324–332.
- Hibbett, D. S., M. Binder, J. F. Bischoff, M. Blackwell, P. F. Cannon, O. E. Eriksson, et al. 2007. A higher level phylogenetic classification of the *Fungi*" (PDF). *Mycol. Res.* 111: 509–547.
- Hong, K. W., C. L. Koh, C. K. Sam, W. F. Yin, and K. G. Chan. 2012. Quorum quenching revisited – From signal decays to signalling confusion. *Sensors* 12: 4661–4696.
- Houdebine, L. M. 2009. Production of pharmaceutical proteins by transgenic animals. *Comp. Immunol. Microbiol. Infect. Dis.* 32: 107–121.
- Huang, Y., S. Chen, X. Bing, C. Gao, T. Wang, and B. Yuen. 2011. Nanosilver migrated into food-simulating solutions from commercially available food fresh containers. *Packag. Technol. Sci.* 24: 291–297.

- Janson, J. C. Ed. 2011. Protein Purification: Principles, High Resolution Methods, and Applications, 3rd ed. John Wiley & Sons, Inc., NY.
- Jones, A., M. Lamsa, T. P. Frandsen, T. Spendler, P. Harris, A. Sloma, F. Xu, J. B. Nielsen, and J. R. Cherry. 2008. Directed evolution of a maltogenic α -amylase from *Bacillus* sp. TS-25. *J. Biotechnol.* 134: 325–333.
- Kalia, V. C. and H. J. Purohit. 2011. Quenching the quorum sensing system: potential antibacterial drug targets. *Crit. Rev. Microbiol.* 37: 121–140.
- Karplus, M. and D. L. Weaver. 1994. Protein folding dynamics: the diffusion-collision model and experimental data. *Protein Sci.* 3: 650–668.
- Keasling, J. D. 2012. Synthetic biology and the development of tools for metabolic engineering. *Metab. Eng.* 14: 189–195.
- Key, N. S. and C. Negrier. 2007. Coagulation factor concentrates: past, present, and future. *Lancet* 370: 439–448.
- Khan, A. A. and M. A. Alzohairy. 2010. Recent advances and applications of immobilized enzyme technologies: a review. *Res. J. Biol. Sci.* 5: 565–575.
- Krivoruchko, A., V. Siewers, and J. Nielson. 2011. Opportunities for yeast metabolic engineering: lessons from synthetic biology. *Biotechnol. J.* 6: 262–276.
- Kula M. R., et al. 1982. Purification of enzymes by liquid–liquid extraction. *Adv. Biochem. Eng.* 24: 773–778.
- Langridge, P. and D. Fleury. 2011. Making the most of ‘omics’ for crop breeding. *Trends Biotechnol.* 29: 33–40.
- Lanza, A. M., N. C. Crook, and H. S. Alper. 2012. Innovation at the intersection of synthetic and systems biology. *Curr. Opin. Biotechnol.* 23: 1–6.
- Lee, B. H. and A. Szilagyi. 2012. Chapter 46: Lactose: Uses in Industry and Biomedical Importance. pp. 806–820. Royal Society Chemistry, UK.
- Li, Y. H. and X. Tian. 2012. Quorum sensing and bacterial social interactions in biofilms. *Sensors (Basel).* 12: 2519–2538.
- Li, K. T., J. Zhou, S. J. Wei, and X. Cheng. 2012. An optimized industrial fermentation processes for acarbose production by *Actinoplanes* sp. A56. *Bioresour. Technol.* 118: 580–583.
- Ljabadeniyi, O. A. 2012. Safety of nanofood: a review. *Afr. J. Biotechnol.* 11: 15258–15263.
- López, A., R. Gavara, and J. Lagaron. 2006. Bioactive packaging: turning foods into healthier foods through biomaterials. *Trends Food Sci. Technol.* 17: 567–575.
- Madan Babu, M. 2004. Computational Genomics: Theory & Application. R. P. Grant, Ed., Taylor & Francis. ISBN: 978-1904933014; www.mrc-lmb.cam.ac.uk/.../microarray/figure1236.ppt.
- Maga, E. A. 2005. Genetically engineered livestock: closer than we think? *Trends Biotechnol.* 23: 533–535.
- Martinko, J. M and M. T. Madigan. 2005. Brock Biology of Microorganisms, 11th ed. Prentice Hall, Englewood Cliffs, NJ.
- May, T. and K. Pohlmeier. 2011. Improving process economy with expanded-bed adsorption technology. *BioProcess Int.* 9: 32–36.
- Mayer, M. P. 2010. Gymnastics of molecular chaperones. *Mol. Cell* 39: 321–331.
- McGrath, T. E., C. T. Elliott, and T. L. Fodey. 2012. Biosensors for the analysis of microbiological and chemical contaminants in food. *Anal. Bioanal. Chem.* 403: 75–92.

- Mishra G. P., et al. 2011. Recent applications of liposomes in ophthalmic drug delivery. *J. Drug Delivery* 2011 Article ID 863734; doi: 10.1155/2011/863734.
- Moore, D., G. D. Robson, and A. P. J. Trinci. Eds. 2011. *21st Century Guidebook to Fungi*, 1st ed. Cambridge University Press. ISBN: 978-0521186957.
- Mymrikov, E. V., A. S. Seit-Nebi, and B. G. Nikolai. 2011. Large potentials of small heat shock proteins. *Physiol. Rev.* 91: 1123–1159.
- Nielsen, J. and J. Keasling. 2011. Synergies between synthetic biology and metabolic engineering. *Nat. Biotechnol.* 29: 693–695.
- Niina, J., H. Ronkainen, B. Halsall, and W. R. Heineman. 2010. Electrochemical biosensors. *Chem. Soc. Rev.* 39: 1747–1763.
- Nooralabettu, K. P. 2010. *Downstream Processing-A New Horizon in Biotechnology*. Prentice Hall of India Pvt. Ltd, New Delhi.
- Nyrén, P. (2007). The history of pyrosequencing. *Method. Mol. Biol.* 373: 1–14.
- Obembe, O. O., J. O. Popoola, S. Leelavathi, and S. V. Reddy. 2011. Advances in plant molecular farming. *Biotechnol. Adv.* 29, 210–222.
- Onwulata, C. I. 2012. Microencapsulation and functional bioactive foods. *J. Food Process. Preserv.* 36: 1–23.
- Orive, G., E. Anitua, J. L. Pedraz, and D. F. Emerich. 2009. Biomaterials for promoting brain protection, repair and regeneration. *Nat. Rev. Neurosci.* 10: 682–692.
- Otles, S. and B. Yalcin. 2012. Review on the application of nanobiosensors in food analysis. *Acta Sci. Pol. Technol. Aliment.* 11: 7–18.
- Oud, B., J. Antonius, A. van Maris, J. M. Daran, and J. T. Pronk. 2012. Minireview: genome-wide analytical approaches for reverse metabolic engineering of industrially relevant phenotypes in yeast. *FEMS Yeast Res.* 12: 183–196.
- Paul, M. and J. K. Ma. 2011. Plant-made pharmaceuticals: leading products and production platforms. *Biotechnol. Appl. Biochem.* 58: 58–67.
- Peters, J., T. Minuth, and W. Schroeder. 2005. Implementation of a crystallization step into the purification process of a recombinant protein. *Protein Expr. Purif.* 39: 43–53.
- Pischetsrieder, M. and R. Baeuerlein. 2009. Proteome research in food science. *Chem. Soc. Rev.* 38: 2600–2608.
- Popham, D. L. and Stevens, A. M. 2006. Bacterial quorum sensing and bioluminescence. In *Tested Studies for Laboratory Teaching*, M. A. O'Donnell, Ed., Vol. 27. Proceedings of the 27th Workshop/Conference of the Association for Biology Laboratory Education (ABLE). pp. 201–215.
- Prakash, S., C. Tomaro-Duchesneau, S. Saha, and A. Cantor. 2011. Review: The gut microbiota and human health with an emphasis on the use of microencapsulated bacterial cells. *J. Biomed. Biotechnol.* Article ID 981214, 12 pages. DOI: 10.1155/2011/981214.
- Prasad, N. K. 2012. *Downstream Process Technology. A New Horizon in Biotechnology*, pp. 370. PHI Learning Pvt. Ltd.
- Punt, P. J., N. van Vieson, A. Conesa, A. Albers, J. Mangnus, and C. van den Hondel. 2002. Filamentous fungi as cell factories for heterologous protein production. *Trends Biotechnol.* 20: 200–206.
- Rai, V., S. Acharya, and N. Dey. 2012. Implications of nanobiosensors in agriculture. *J. Biomater. Nanobiotechnol.* 3: 315–324.
- Raina, S., D. De Vizio, M. Odell, M. Clements, S. Vanhulle, and T. Keshavarz. 2009. Microbial quorum sensing: a tool or a target for antimicrobial therapy? *Biotechnol. Appl. Biochem.* 54: 65–84.

- Rhim, J. W. and P. K. Ng. 2007. Natural biopolymer-based anocomposite films for packaging applications. *Crit. Rev. Food Sci. Nutr.* 47: 411–433.
- Richau, K. H. and R. A. L. van der Hoorn. 2010. Studies on plant pathogen interactions using activity based proteomics. *Curr. Proteomics* 7: 328–336.
- Rios, M. 2012. A decade of microbial fermentation. *BioProcess Int.* 10: 14–17.
- Ro, D. K., E. M. Paradise, M. Quellet, K. J. Fisher, K. L. Newman, et al. 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 440: 940–943.
- Scarselli, M., M. M. Giuliani, J. Adubobie, M. Pizza, and R. Rappuoli. 2005. The impact of genomics on vaccine design. *Trends Biotechnol.* 23: 84–91.
- Sekhon, B. S. 2010a. Food nanotechnology – an overview, *Nanotechnol. Sci. Appl.* 3: 1–15.
- Sekhon, B. S. 2010b. Food nanotechnology – an overview. *Nanotechnol. Sci. Appl.* 114: 1–15.
- Semo, E., E. Kesselman, D. Danino, and Y. D. Livney. 2007. Casein micelle as a natural nano-capsular vehicle for nutraceuticals. *Food Hydrocoll.* 21: 936–942.
- Shamik, S., A. Whalley, J. McLaughli, F. Brello, B. Bishop, and A. Banerjee. 2011. Fermentation process technology transfer for production of a recombinant vaccine component. *BioPharm Intern.* 24: 30–39.
- Singer, S. J., and G. L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. *Science* 175(4023): 720–731.
- Singh, R. K., M. K. Tiwari, R. Singh, and J. K. Lee. 2013. From protein engineering to immobilization: Promising strategies for the upgrade of industrial enzymes. *Int. J. Mol. Sci.* 14: 1232–1277.
- Smejkal B., B. Helk, J. M. Rondeau, S. Anton, A. Wilke, P. Scheyerer, J. Fries, D. Hekmat, and D. Weuster-Botz. 2013. Protein crystallization in stirred systems – scale-up via the maximum local energy dissipation. *Biotechnol. Bioeng.* 110: 1956–1963.
- Soto, C. 2012. Transmissible proteins: expanding the prion heresy. *Cell* 149: 968–977.
- Sozer, N. and J. L. Kokini. 2009. Nanotechnology and its applications in the food sector. *Trends Biotechnol.* 27: 82–89.
- Staley, J. T., Ed. 1989. *Bergey's Manual of Systematic Bacteriology*, Vol. 3, Williams & Wilkins, Baltimore.
- Stevens, A. M. and E. P. Greenberg. 1997. Quorum sensing in *Vibrio fischeri*: essential elements for activation of the luminescence genes. *J. Bacteriol.* 179: 557–562.
- Su, L., W. Jia, C. Hou, and Y. Lei. 2011. Microbial biosensors: a review. *Biosens. Bioelectron.* 26: 1788–1799.
- Takors, R. 2012. Scale-up of microbial processes: Impacts, tools and open questions. *J. Biotechnol.* 160: 3–9.
- Tiede, K., A. Boxall, S. P. Tear, J. Lewis, H. David, and M. Hasselov. 2008. Detection and characterization of engineered nanoparticles in food and the environment. *Food Addit Contam. Part A* 25: 795–821.
- Toldra, F. and L. M. L. Nollet. Eds. 2013. *Proteomics in Foods: Principles and Applications*. Springer, New York.
- Vidhyalakshmi, R., R. Bhakayaraj, and R. S. Subhasree. 2009. Encapsulation “The future of probiotics” – a review. *Adv. Biol. Res.* 3(4): 6–103.
- Walsh, G. 2005. Therapeutic insulins and their large-scale manufacture. *Appl. Microbiol. Biotechnol.* 67: 151–159.

- Wang, N. S. and G. Stephanopoulos. 1984. Computer applications in fermentation processes. *CRC Crit. Rev. Biotechnol.* 2: 1–103.
- Willey, J. M., L. M. Sherwood, and C. J. Woolverton. 2008. Prescott, Harley, and Klein's Microbiology, pp. 1088. McGraw-Hill Higher Education.
- Wongchoosuk, C., A. Wisitsoraatb, A. Tuantranontb, and T. Kerdcharoen. 2006. Portable electronic nose based on carbon nanotube-SnO₂ gas sensors and its application for detection of methanol contamination in whiskeys. *Sensors Actuators B* 147: 392–399.
- Woodley, J. M. 2013. Protein engineering of enzymes for process applications. *Curr. Opin. Chem. Biol.* 17: 310–316.
- Wu, N. and A. M. Clausen. 2007. Fundamental and practical aspects of ultrahigh pressure liquid chromatography for fast separations. *J. Sep. Sci.* 30: 1167–1182.
- Xia, S., S. Xu, X. Zhang, and F. Zhong. 2007. Effect of coenzyme Q10 incorporation on the characteristics of nanoliposomes. *J. Phys. Chem. B* 111: 2200–2207.
- Ye, V. M. and S. K. Bhatia. 2012. Metabolic engineering for the production of clinically important molecules: omega-3 fatty acids, artemisinin, and taxol. *Biotechnol. J.* 7: 20–33.
- Yuan, Y., Y. Gao, J. Zhao, and L. Mao. 2008. Characterization and stability evaluation of beta-carotene nanoemulsions prepared by high pressure homogenization under various emulsifying conditions. *Food Res. Int.* 41: 61–68.
- Zeng, L. R. and J. P. Xie. 2011. Molecular basis underlying LuxR family transcription factors and function diversity and implications for novel antibiotic drug targets. *J. Cell. Biochem.* 112: 3079–3084.
- Zhao, R., J. Sun, P. Torley, D. Wang, and S. Niu. 2008. Measurement of particle diameter of *Lactobacillus acidophilus* microcapsule by spray drying and analysis on its microstructure. *World J. Microbiol. Biotechnol.* 24: 1349–1354.
- Zhao, Y., M. Ye, Q. Chao, N. Jia, Y. Ge, and H. Shen. 2009. Simultaneous detection of multifoed-borne pathogenic bacteria based on functionalized quantum dots coupled with immunomagnetic separation in food samples. *J. Agric. Food Chem.* 57: 517–524.
- Zwietering, M. H., I. Jongenburger, F. M. Rombouts, and K. van 't Riet. 1990. Modeling of the bacterial growth curve. *Appl. Environ. Microbiol.* 56: 1875–1881.

Read more: Components of Plant & Bacterial Cells As Compared to Animal Cells | eHow.com http://www.ehow.com/info_8470668_components-cells-compared-animal-cells.html#ixzz1oVEg6WME

2

Concepts and Tools for Recombinant DNA Technology

Biotechnology which is synonymous with *genetic engineering* or *recombinant DNA (rDNA)* is an industrial process that uses the scientific research on DNA for practical applications. rDNA is a form of artificial DNA that is made through the combination or insertion of one or more DNA strands, therefore combining DNA sequences, within different species, that is, DNA sequences that would not normally occur together. To understand the significance and the various applications of biotechnology, a basic knowledge of rDNA technology is indispensable. In order to understand this, one must remember how a fragment of DNA, representing a genetic code is involved in proteins synthesis, that is, mRNA transcription from this DNA fragment followed by translation involving ribosomal RNA (rRNA) and transfer RNA (tRNA) that carries the amino acid. To produce large quantities of a functional protein artificially one must first clone the DNA fragment corresponding to the gene using recombinant technology. rDNA technology has many applications in medicine to produce valuable pharmaceutical drugs, and so on and agriculture for genetically modified crops, which will be discussed in Part III.

2.1 Concepts of macromolecules: function and synthesis

2.1.1 DNA replication

One of the characteristic features of living organisms is their ability to grow, develop, and reproduce. Prokaryotes do not contain a nucleus, but hereditary materials can be transferred from one strain of bacteria to another asexually by means of simple binary fission in which DNA replication is followed by a septum formation, which divides the cell into two. Incomplete sets of genetic materials can be transferred between bacteria by

three mechanisms of gene transfer: *conjugation*, *transduction*, and *transformation*, but no individuals are produced from such transfers.

In all eukaryotic cells, cellular fusion is the first step in sexual reproduction. The two cells that participate are *gametes*, and the resulting fusion cell as a *zygote*. Gametic fusion is followed by nuclear fusion, with the result that the zygote nucleus contains two complete sets of genetic determinants. Sexual fusion results in a doubling of the number of chromosomes as the nuclei of the gametes, each containing n chromosomes, fuse to form the nucleus of the zygote, which consequently contains $2n$ chromosomes. The halving of the chromosome number is a universal accompaniment of sexuality, which is brought about by a special process of nuclear division, termed *meiosis*. During cell division there is complete duplication of the genome (the total complement of genes in a cell), and this phenomenon is based on the replication of DNA. Although the action of the DNA polymerases is simple and well understood, the process is complex, and many questions remain to be answered.

The polymerization of DNA is catalyzed by enzymes called *DNA polymerases*. In addition to the deoxynucleoside triphosphates [deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), deoxythymidine triphosphate (dTTP)] that serve as substrates, two molecules of nucleic acid are required: one is the DNA template to which substrate deoxynucleoside triphosphate molecules pair according to the rules of base pairing; and second is the RNA primer, to which the nucleotides are attached as a consequence of the polymerization. Three different DNA polymerases catalyze the addition of nucleotides from the 5' end to the 3' end of the DNA template, following the RNA primer. Since DNA polymerase requires a single-stranded template, the double-stranded chromosome must be separated, at least locally, before replication can occur. Such separation forms a bubble in the chromosome at a specific site, termed the *replication origin (ori)*, at which replication always initiates. Replication of a closed circular double helix of DNA produces a loop, termed the *supercoiled twist* (θ structure) and formed by the two replication forks at the opposite directions of origin in the molecule that will soon meet and stop the process of replication. The required functions of strand separation, primer synthesis and elimination of twists are mediated by a variety of proteins, along with DNA polymerases, that comprise a loose multienzyme complex termed the replication apparatus.

This process, which entails at least 13 different proteins, takes place with remarkable speed and accuracy; approximately 3000 nucleotides are polymerized per second (at 37 °C) and only about one mistake (incorrect pairing) per 10^{10} nucleotides copies is made. For the strands to separate completely a considerable expenditure of energy would be required, and it is now thought that only a part of the DNA unfolds to form a replication fork. The double helix is rapidly unwound by the enzyme *DNA helicase*, and the resulting single strands are prevented from recombining by helix destabilizing proteins, which bind strongly to single-stranded DNA. Other proteins, including DNA unwinding enzyme I (topoisomerase I) and Rep protein, collectively called *unwinding proteins*, actively separate the DNA strands with concomitant hydrolysis of ATP.

Another protein, *DNA gyrase (topoisomerase II)*, which requires ATP hydrolysis, prevents the formation of twists by periodically breaking a phosphodiester bond in one opposite strand. Later this enzyme reforms the same bond. The activities of the various proteins that mediate the processes of strand separation and elimination of twists are as follows: (i) primer RNA is synthesized by a RNA polymerase (R pol); then (ii) DNA is polymerized to the primer by DNA polymerase III (pol III); (iii) a preceding primer RNA is hydrolyzed while DNA is being polymerized in its place by the exonuclease and DNA polymerase I (pol I); and (iv) the completed short segment of DNA is joined to (v) the continuous strand by DNA ligase (Figure 2.1).

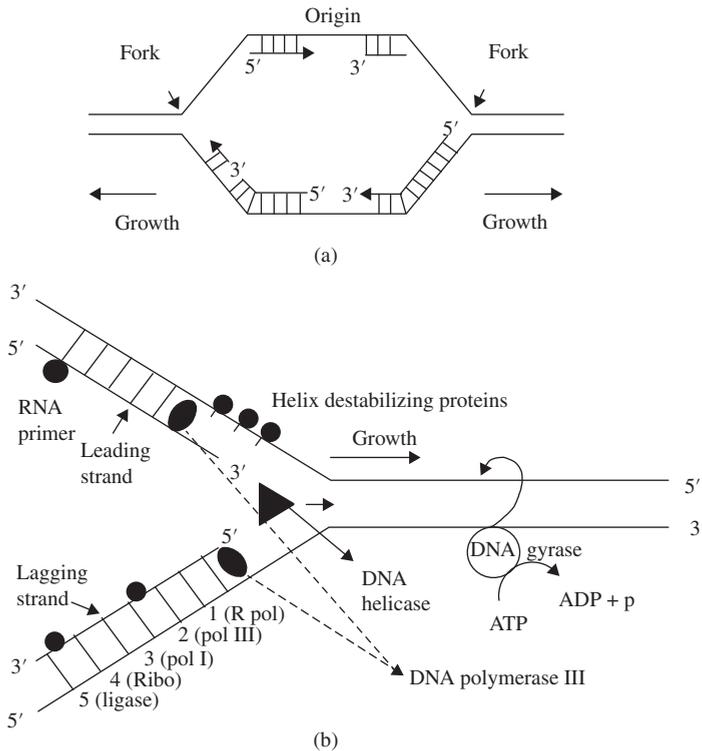


Figure 2.1 Schematic representation showing the essential features of bacterial DNA replication.

Once the strands are separated, short pieces of RNA complementary to a portion of the single-stranded regions are synthesized on each single strand by a special enzyme primase. The RNA primer is not produced by the general classes of RNA polymerases found in the cell but by a primase that can initiate chain elongation *de novo*. Primases recognize specific sequences on single strands of DNA. Recognition proteins, called *N-proteins*, choose the origin sites on the DNA at which primases act. The RNA primer is later removed by ribonuclease and the gaps filled in with DNA polymerase I. The fragments are then sealed by DNA ligase to form a continuous strand, and this is accompanied by the hydrolysis of adenosine triphosphate (ATP).

The strand that is replicated in the same direction as the movement of the replication forks, run from 5' to 3', is termed the *leading strand*. The DNA on the leading strand is synthesized continuously, whereas the DNA on the lagging strand, which runs in the opposite direction, is copied in a discontinuous fashion in a series of short bursts. Discontinuous copying results in the formation of Okazaki fragments, short sections of DNA, containing about 1000–2000 nucleotide residues and attached to RNA. These sections are later joined together by the action of DNA ligase to produce a continuous DNA strand.

The mechanism of synthesis of DNA in eukaryotes is probably similar to the process in prokaryotes. There are major complications, however: DNA in eukaryotes is organized in several linear chromosomes and is much longer than that found in prokaryotes; moreover, the rate of DNA polymerase movement is very much slower in prokaryotes than in

eukaryotes. To compensate for this, a eukaryotic cell contains more than 20,000 molecules of the enzyme, which allows a much larger number of replication forks to form (≥ 2000). Smaller Okazaki fragments (40–300 bases) are also formed. Therefore, a very much faster rate of DNA replications is achieved compared with that of *Escherichia coli*.

A model for eukaryotic replication forks based on viral DNA replication in animal cells (simian adenovirus SV 40) demonstrated two distinct polymerases: polymerase σ , which synthesizes the leading strand, and polymerase α , which synthesizes the lagging strand. The lagging strand is looped around its polymerase, allowing the enzyme to move in the direction of the replication fork, in a similar fashion to that for prokaryotic replication.

2.1.2 Roles of RNA

The various RNAs that participate in normal cell function serve the purpose of reading and implementing the genetic instructions of DNA. Three major classes of cellular RNA [messenger RNA (mRNA), rRNA, and tRNA] are all involved in translation, but only mRNA carries the information that specifies the primary structure of proteins.

Ribosomes are the most abundant constituent of cells actively engaged in protein synthesis. Since about half the mass of ribosomes is RNA, *ribosomal RNA (rRNA)* is the most abundant type of cellular RNA, accounting for 75% of total RNA. The ribosomes of prokaryotic and eukaryotic cells are distinct and different in shape, size, subunit size and molecular composition (Figures 2.2 and 2.3). The ribosomes of prokaryotes and chloroplasts showed sedimentation coefficients of about 70 S, while those of eukaryotes are larger (≈ 80 S). All ribosomes are composed of two functionally and structurally distinct subunits, which reversibly dissociate *in vivo* during protein synthesis, and *in vitro* when the Mg^{2+} concentration of the medium is lowered from 10^{-2} to 10^{-4} M. The 50 S subunit of the 70 S ribosomes contains two species of rRNA and about 34 ribosomal proteins, while the 60 S subunit of the 80 S ribosome contains three species of rRNA and about 45 ribosomal proteins. The small ribosomal subunit (30 S in prokaryotes, 40 S in eukaryotes) contains one species of rRNA and 21–33 ribosomal proteins. Ribosomes possess a multitude of activities necessary for protein synthesis including peptidyltransferase activity, codon-directed binding of aminoacyl-tRNAs, binding mRNA, initiation, elongation and termination factors, and GTPase activity (Figure 2.2).

Since protein synthesis in eukaryotes occurs on ribosomes in the cytoplasm, some intermediate molecule must be involved in transferring information from the nucleus to the cytoplasm. RNA processing occurs in the nucleus of eukaryotes. In virtually all organisms, the large rRNAs of the two ribosomal subunits are derived by the processing and modification of a large, common precursor RNA molecules (Figure 2.3).

Transfer RNAs (tRNAs), the smallest type (4 S), are formed in the cytoplasm and serve for the translation of the genetic code at the ribosome. tRNAs have several features in common. All are single chains containing between 73 and 93 ribonucleotides, with a high proportion of unusual bases, which arise by posttranscriptional modification of the standard bases. Many of these modified bases are involved in maintaining tertiary structure. Nearly half the nucleotides are base-paired to form double-helical stems from which loops of unpaired bases protrude. The T ψ C loops (derived from the sequence ribothymide-pseudouridine-cytosine), the anticodon loop (which interacts with a codon of three nucleotides in mRNA), and the dihydrouracil loop, are three loops (Figure 2.4).

Nearly all tRNAs have a cloverleaf structure (Figure 1.37), although it is known that some animal mitochondrial tRNAs lack the dihydrouracil loop. The cells also contain more than one tRNA species for most of the amino acids, and these are termed *isoaccepting tRNAs*. In both prokaryotes and eukaryotes, tRNAs are derived from longer precursor

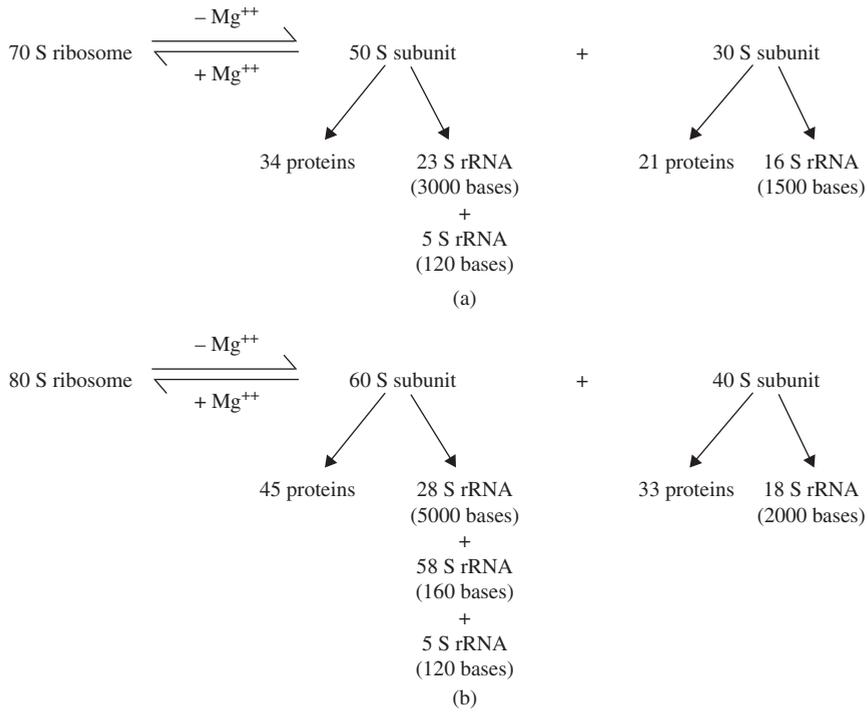


Figure 2.2 The components of (a) prokaryotic and (b) eukaryotic ribosomes.

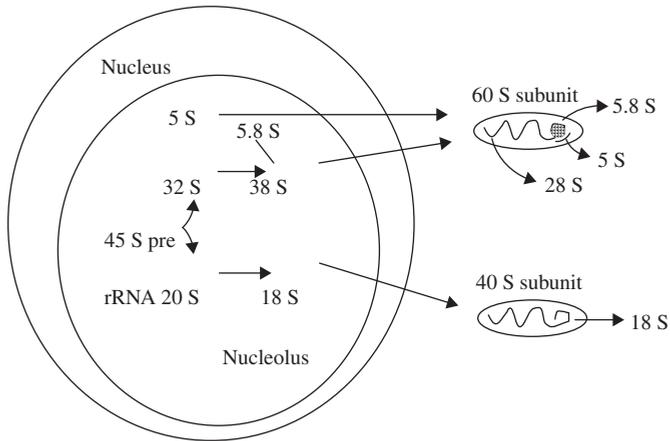


Figure 2.3 The biosynthesis of ribosomal subunit in eukaryotic cells.

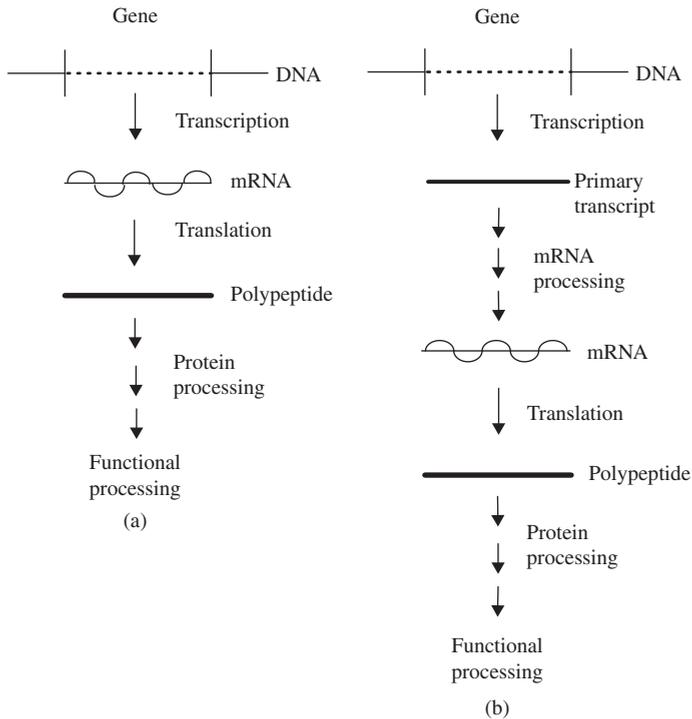


Figure 2.5 The structural differences and gene expression in (a) prokaryotic and (b) eukaryotic mRNA.

coding region of the mRNA, which contains the codons corresponding to the amino acid sequence of the protein (Figure 2.5). The beginning of the sequence is an initiator codon (AUG), and the mRNA molecule also terminates in a noncoding sequence at the 5' end, which is termed the *leader sequence*. Parts of this sequence are involved in the binding of ribosomes to the mRNA. The 5' leader sequences are usually longer in eukaryotes than in prokaryotes. These 3' and 5' noncoding sequences are also called *untranslated regions (UTRs)*.

All cytoplasmic eukaryotic cellular mRNA molecules possess at the 5' end a catabolite activator protein (CAP) structure that is involved in the interaction of mRNA with the ribosome. Most eukaryotic cytoplasmic mRNA molecules also contain a sequence of 100–200 adenylyl units at the 3' end which are added posttranscriptionally. This sequence, the polyadenylated or poly(A) tail, appears to be involved in protecting the mRNA from nucleolytic degradation; it is not absolutely essential for translation. The poly(A) tails provide a convenient way for purifying mRNA using affinity chromatography: oligo-dT or oligo-U is attached to cellulose beads, which are packed into a chromatography column (Figure 2.5).

2.1.3 Detailed aspects of protein synthesis

It is important to understand the principles of the regulation of protein synthesis. As elaborated in previous sections, all three stages of protein synthesis – transcription, translation,

Most promoters differ slightly from the consensus with respect to the precise sequence between them and the point of initiation. These variations are presumed to determine promoter strength or frequency of RNA initiation. Thus, the nearer the -35 and -10 sequences are to these optima, the stronger the promoter.

In *E. coli* growing in a rich medium at 37°C , the strongest promoters initiate a transcription as frequently as every 4 s, while the weakest ones may act as infrequently as a few times per hour. The *E. coli* cell contains only about 12 molecules of lac repressor, and its gene is transcribed only once every 30 min. About 10,000 ribosomes must be made in each generation, and thus each rRNA is transcribed about once per second. These differences arise through variations in promoter strength.

Until recently, *E. coli* was thought to possess a single type of RNA polymerase holoenzyme capable of transcribing all genes. However, recent studies on the heat-shock response have shown that more than one type of sigma (σ) subunit might occur, thereby further modulating promoter strength. In *E. coli* there are about 17 heat-shock proteins, whose expression is triggered by the product of the hptR gene when the cell is exposed to a high temperature (an abrupt increase of a few degrees in the environmental temperature of a cell). This gene responds to a heat-shock substitute factor (relative molecular mass, 32,000). This factor (termed σ^{32}) associates with normal core RNA polymerase to produce the holoenzyme $\alpha_2\beta\beta'\sigma^{32}$, which binds to the promoter elements of the heat-shock genes, but not those of the other genes. The promoter of heat-shock genes has an entirely different -10 sequences and a slightly different -35 sequence.

There is other clear evidence for the involvement of multiple sigma factors in *Bacillus subtilis* (an aerobic spore former), using sigma factor switching to bring about the transition from vegetative growth to sporulation. At least four sigma proteins (σ^{28} σ^{29} σ^{37} σ^{55} ; designated by their molecular weight $\times 10^{-3}$) and addition forms such as σ^{43} have been reported. This bacterium can produce at least four different forms of RNA polymerase by modulating the frequency of transcription from various promoters. As the proportion of the forms changes, the expression of genes is modulated. The equivalent sequences for σ^{43} promoters in *B. subtilis* are TTGACA (-35) and TATAAT (-10). This close resemblance to the *E. coli* sequences at least partly explains the expression of *B. subtilis* genes in *E. coli* or vice versa.

Initiation begins when the RNA polymerase holoenzyme binds to the promoter, causing the strands of a DNA double helix to separate (melt). The major contact points between the promoter and the polymerase lie in the -10 and -35 regions of the promoter (Figure 1.34). The closed promoter complex is rapidly converted into an open promoter complex by the disruption of the interstrand H bonds over a region of 17 base pairs within the sequence bound by the enzyme. The first (or initiating) nucleoside triphosphate, ATP or guanosine triphosphate (GTP), binds to the subunit of the polymerase and forms a base pair at the start point. A second nucleoside triphosphate is then bound and the first phosphodiester bond is formed, and polymerization begins with the sense strand as template similar to the process of DNA replication in the $3'$ -to- $5'$ direction. When approximately the first 12 nucleotides have been added in the growing mRNA chain, the σ subunit of the polymerase dissociates from the transcription complex. The core enzyme in the cytosol catalyzes a new round of initiation to complete elongation. All control of transcription is affected by the frequency of its initiation and termination; and attenuation (a special form of termination) plays an important role in regulating transcription (Figure 2.7).

Similar to initiation, termination of transcription is signaled by specific sequences of DNA called *terminators*. In *E. coli* two termination mechanisms exist: one is dependent on an accessory protein termed rho (ρ); the other is independent of accessory factors. In an ATP-driven reaction to dissociate RNA polymerase from its template, rho raises the frequency of termination to near 100%. This strong terminator is sometimes called

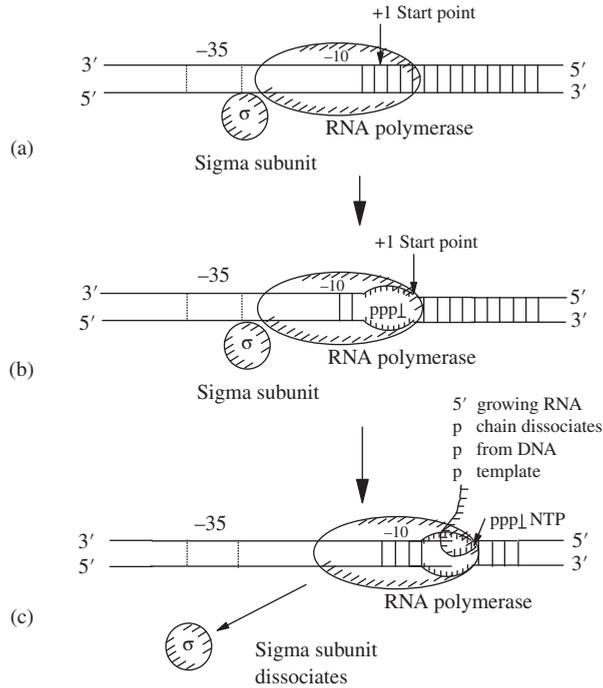


Figure 2.7 Initiation and elongation of RNA chains by *E. coli* RNA polymerase.

rho independent, whereas weak ones are said to be *rho* dependent. Strong *rho*-dependent terminators are distinguished by the presence in the DNA of a GC-rich sequence that shows dyad symmetry, followed by five or six A (adenine) residues. The RNA transcribed from this sequence can form a stem-loop (or hairpin) structure as a result of intramolecular H bonding between the complementary bases. DNA-RNA hybrids between the transcribed DNA and the nascent RNA strand will leave only the oligo-(U) sequence. Oligo-(U)-oligo-(A) hybrids are the least stable base pairing, and thus the RNA is able to dissociate from the DNA. Duplex DNA then forms in the transcription bubble, and RNA polymerase (core enzyme) is released. On the other hand, a weak *rho*-independent terminator requires a similar stem-and-loop structure, but the oligo-(U) sequence is absent.

The transcriptional machinery of eukaryotes is more complex than that of prokaryotes, both in terms of the structure of RNA polymerase and in the DNA sequences involved in transcription control. A sizable proportion of eukaryotic genomes have highly condensed chromatin, which is transcriptionally inert; thus only a small proportion of the genome (maximally $\approx 10\%$) is ever expressed as an RNA sequence. Other specific accessory proteins called transcription factors are required for efficient transcription of all classes of eukaryotic genes. Unlike bacterial *rho* factors, eukaryotic transcription factors do not interact with the RNA polymerase per se; rather, they form stable complexes with chromatin before initiation of transcription and act as positive regulators. The nuclei of eukaryotes contain three distinct forms of RNA polymerase. These are responsible for transcribing different classes of genes. Polymerase I transcribes the tandemly repeated rRNA genes in the nucleolus. Polymerase II, which is nucleoplasmic, transcribes protein coding genes.

Polymerase III transcribes 5 S rRNA, tRNA, and other small RNA genes. The promoters for these different classes of genes show considerable variation.

In prokaryotes, at least three different mechanisms of transcription control exist: DNA binding, which is part of the catabolic repression control system in *E. coli*, exchange of the sigma factor components of RNA polymerase, and attenuation. The simple model for the regulation of the lactose operon and the process by which cyclic adenosine monophosphate (cAMP)–CAP stimulates transcription is well known. The cAMP–CAP complex enhances the affinity of RNA polymerase for the promoter by interacting with an adjacent upstream site and destabilizing the duplex, thereby increasing the efficiency of open promoter formation. The precise mechanism by which CAP stimulates transcription is unknown, inasmuch as different operons have CAP binding sites at different positions relative to the promoter. It is likely that in the lac operon cAMP–CAP interacts directly with RNA polymerase, but in the ara operon the CAP binding site is much too distant for a direct interaction with RNA polymerase to occur. Also, catabolite repression is a common phenomenon, but the molecular details vary, and cAMP is not involved in the mechanics of catabolite repression in bacilli or in streptomycetes.

For some time, DNA binding proteins were considered to be the exclusive route by which transcriptional control of gene expression was effected. However, the mechanism of action of binding proteins has remarkable flexibility. When bound to DNA, a protein can either stimulate or inhibit transcription, and the small-molecule effector can either stimulate or inhibit binding of the protein to DNA. This flexibility can be observed in the regulation of the lac, arg, and trp operons. These repressors are different examples of one particular mode of gene regulation called negative control. There is also a mode of regulation called positive control.

Under negative control, in the absence of the regulatory protein (the repressor), the gene is transcribed; but in the presence of repressor, transcription does not proceed. In the presence of inducer, the repressor is altered and transcription proceeds, being visible in an inducible operon. In positive control, transcription does not occur in the absence of the regulatory protein (R); thus R acts to switch on transcription and must be called an activator protein rather than a repressor. The activator protein alone cannot bind to the operon, and thus it requires an inducer to be bound to the activator protein. The cAMP–CAP protein is an example of a system. Another is the arabinose operon.

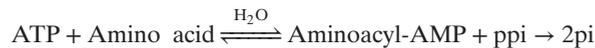
A third mode of transcriptional control, attenuation, operates in amino acid biosynthetic operons. It involves the premature termination of transcription at a site between the operator and structural gene (trpE). The long piece of mRNA that precedes trpE is called the *leader sequence* (trpL, 141 bases). The degree of transcriptional termination (attenuation) varies according to the amount of available tryptophan. Under conditions of inadequate amounts of tryptophan, more mRNA is made and proceeds past the attenuator, transcribing the entire operon. If tryptophan is present in adequate amounts, the ribosome proceeds to the stop codon of trpL, preventing formation of the second loop, which causes termination of transcription. Thus, attenuation is another method of controlling gene expression.

2.1.3.2 Translation and its control The final stage in the expression of genetic information is often loosely called *protein synthesis*. Here the information encoded in the nucleotide sequence of the mRNA is translated into the corresponding amino acid sequence constituting the polypeptide. Translation occurs in ribosomes and requires, also, tRNA molecules and a series of protein factors by which polypeptide chains are produced. This complex process can be analyzed by considering the individual component reactions. Although the process of translation is essentially the same in all organisms, there are substantial differences in the detailed mechanism and components of translation between

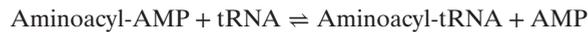
prokaryotes and eukaryotes. The process is generally more complex in eukaryotes. Furthermore, most of the protein synthesis in eukaryotic cells occurs in the cytoplasm, but certain eukaryotic cellular organelles (chloroplasts and mitochondria) have their own translational apparatus for protein synthesis by mitochondrial and chloroplast DNA. Translation does not appear to be a major control mechanism in protein synthesis in prokaryotes, but it probably is more important in eukaryotes. Translation results in the production of a polypeptide, but often a fully functional protein is produced only when the initial polypeptide chain is modified in one or more of a number of ways described as PTMs.

Activation of amino acids The activated (or changed) forms of amino acids which are synthesized to form proteins are the aminoacyl-tRNAs. They are synthesized in two steps by a group of enzymes, aminoacyl-tRNA synthetases. Each of these 20 enzymes is specific for a particular amino acid, but some react with several different tRNA molecules.

1. The amino acid first reacts with ATP to form an enzyme-bound intermediate, aminoacyl adenylate:



2. The aminoacyl unit is transferred to the appropriate tRNA molecule by the synthetase



These two separate steps form the basis of the proofreading process by which the aminoacyl-tRNA synthetases pick up the “correct” amino acid.

The ribosome cycle The process of translating mRNA consists of initiation, elongation, and termination. The ribosomal cycle, is essentially identical in all cases, despite differences in the sizes of ribosomal subunits between prokaryotes and eukaryotes (Figure 2.8).

Initiation Initiation requires a specific interaction between the 16S rRNA of the small ribosomal subunit and the *ribosomal binding site* (RBS) or a *Shine–Delgarno* (SD) *sequence* of the message. A consensus sequence (AGGAGGU), termed a *Shine–Delgarno* (SD) *sequence* and centered about 10 nucleotide residues from the initiation codon (AUG) is complementary to the 3'-hydroxyl end of the 16S RNA and necessary for formation of the initiation complex together with *N*-formyl methionyl-tRNA^{Met} and the initiation factors.

Although regulation of the initiation of translation is a controlling parameter for the synthesis of ribosomal proteins in *E. coli*, the major interest for the biotechnologist is to render the initiation event as efficient as possible. The ribosomal binding sites of different genes may vary by nearly a thousand-fold in relative efficiency, and thus it is important that the translation initiation signal be modified to provide both an efficient SD sequence for the organism targeted and the optimum space between the SD and the initiation code.

Gram-positive cells do not translate well the mRNAs from gram-negative bacteria, but gram-negative bacteria generally translate gram-positive messages. Thus *E. coli* can express *B. subtilis* genes but not vice versa. Gram-positive bacteria appear to require

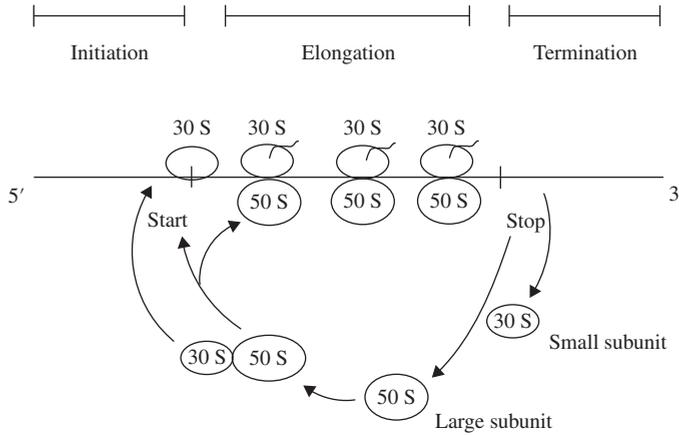
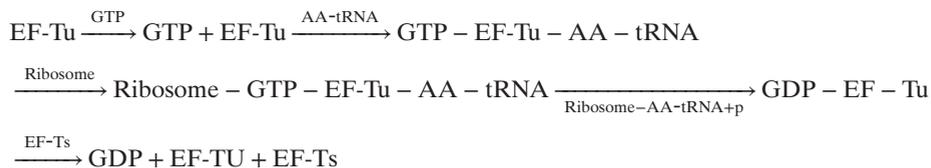


Figure 2.8 The ribosomal cycle showing initiation, elongation, and termination with a 70S prokaryotic ribosome.

greater complementarity between the SD sequence and 3' end of the 16S RNA than is found in gram-negative bacteria. Since most mRNAs in bacteria are also unstable, with half-lives of 2–4 min at 37 °C, in many cases gene productivity could be improved by stabilizing the message.

In the initiation phase, prokaryotes and eukaryotes differ in the ways in which mRNA is bound and the initiation codons selected. In bacteria the initiation factors are known as IFs (IF-1, IF-2, IF-3, etc.); in eukaryotes they are termed eIF-1 and so on.

Elongation Elongation is the stage of translation in which the mRNA is decoded in the 5'-to-3' direction and the polypeptide chain is synthesized starting at the amino terminus. This process is essentially similar in prokaryotic and eukaryotic cells. Elongation is a cyclic process, each turn of the cycle corresponding to the decoding of one triplet codon and to the addition to the growing polypeptide chain of one amino acid (AA) residue. Elongation requires proteins called elongation factors (EFs). In prokaryotes, these are EF-Tu, EF-Ts, and EF-G; in eukaryotes, they are EF-1 (equivalent to EF-Tu) and EF-2 (equivalent to EF-G). EF-Tu and EF-Ts participate in recognition, and one molecule of GTP is hydrolyzed to GDP and inorganic phosphate as follows:



There are normally binding sites, termed the A (aminoacyl) site and the P (peptidyl) site. After recognition, both ribosomal binding sites are occupied by aminoacylated tRNAs. The peptide bond forms between the terminal carboxyl group of the peptide and the α -amino group of the amino acid in the A site. The peptidyl transfer reaction requires neither accessory protein factors nor the expenditure of energy. Peptidyl

transfer is followed by a complex and incompletely understood series of reactions called *translocation*. The free tRNA molecule in the P site is released and the ribosome moves to the next codon of the mRNA, thereby moving peptidyl-tRNA from the A site to the P site and putting in register in the empty A site the next codon to be read. Translocation requires one accessory protein (elongation factors $G = EF-G$) and the hydrolysis of an additional molecule of GTP.

By repetitive recognition, peptidyl transfer, and translocation steps, successive amino acids are added to the peptide chain. The process continues until a *nonsense codon* (UAG, UAA, or UGA) is reached, which causes the release of the completed protein from the 70S ribosome. The process requires the intervention of a protein release factor (RF) and, through the action of IF3, the ribosome dissociates into its 30S and 50S subunits. There are some similarities and differences between control of transcription and translation by binding proteins. In both cases, control depends on the binding of a protein to a specific site on a nucleic acid DNA (in transcriptional control) and mRNA (in translational control). In both cases, the bound protein prevents attachment of a catalyst to its functional site – RNA polymerase to the promoter in transcription control and ribosome to the RBS in translation control. In cases of transcriptional control, the binding protein is an allosteric protein, whose activity in relation to DNA binding is regulated by a small molecule (inducer or corepressor). In contrast, ribosomal proteins have no allosteric properties, and the synthesis of ribosomal proteins is precisely coordinated with the need for them to be assembled into ribosomes. If a site is available on a partially assembled ribosome, the protein binds there; if it is not, the excess protein molecules bind to the mRNA, preventing the further synthesis of the protein.

Posttranslational control After release from the ribosome, polypeptides may undergo a number of types of covalent modification. These modifications, which are necessary to form a fully functional protein, include limited proteolysis (e.g., removal of residues at the N-terminus, specifically, the first methionine or formylmethionine residue) and proteolytic modification involved in zymogen activation and removal of signal peptides (for secreted proteins and membrane proteins), the latter being necessary because the initial N-terminal region (the signal sequence) of virtually all secreted proteins must be removed by a specific peptidase. In addition, polypeptides undergo chemical modification of amino acids by the addition of acetyl, methyl, phosphate, hydroxy, and carboxyl groups, as well as glycosylation and acylation. In fact, more than 140 modified amino acid residues have been isolated from proteins. Chemical modification is not a random, uncontrolled process but occurs at highly specific positions in polypeptide chains. Examples are common among eukaryotes but much less often seen in prokaryotes. The best studied case in prokaryotes is the control of glutamine synthetase activity, which plays an important role in the nitrogen metabolism of many bacteria. Covalent modification of glutamine synthetase through adenylation is stimulated by a high concentration of ammonia and results in a reduction of the enzyme activity. The adenylation form of glutamine synthetase is sensitive to changes in metabolism, whereas the unsubstituted form is considerably less sensitive.

Since biotechnology encompasses the cloning and expression of genes from eukaryote to bacteria, it is important to know how protein synthesis in eukaryotes differs from that in prokaryotes. From our investigation thus far of posttranscriptional modification and PTM, we can deduce that the primary transcript from a eukaryotic chromosome is not mRNA but a high molecular weight *heterogeneous nuclear (hn)* RNA. The hn RNA is processed by the removal of segments (introns) and the “splicing” of the remaining sequences (exons)

to provide the message. Further modification in the form of the addition of a methylated oligonucleotide CAP to the 5' end and a stretch of poly(A) to the 3' end completes the processing. Therefore, eukaryotic genes containing complete introns cannot be expressed in bacteria or in lower eukaryotes such as yeast because the enzymes necessary for processing are absent.

2.2 Concepts of recombinant DNA technology

In the late 1970s, new techniques such as rDNA, cell fusion (monoclonal antibody), protein engineering, plant cell culture, and bioprocess engineering were developed. It was, however, the discovery of rDNA technology, popularly known as *genetic engineering*, which led to the current biotechnology boom. The techniques for the exchange of genetic materials through mutation and genetic recombination that have been described so far have certain limitations. Conventional techniques could not go beyond the boundaries of a genus or even, in many cases, those of a species. Not only does rDNA technology offer the prospect of improving existing processes and products, but it also enables us to develop totally new heterologous products, as well as processes that were not possible using standard mutation techniques. Through rDNA technology it became possible to understand the organization, the expression, and the structure of genes, as well as to diagnose many inherited diseases. This technology is also creating unique industrial microorganisms able to synthesize valuable proteins and is finding a wide spectrum of uses in other manufacturing processes and agriculture. rDNA technology was an outgrowth of basic research on three major scientific developments that occurred roughly at the same time. The first was the discovery and subsequent commercial supply of restriction endonuclease enzymes, which can be used to recognize and cut specific nucleotide sequences within the DNA molecule. The second was the creation and the purification in high yield of artificial cloning vectors (i.e., plasmid DNA in which selectable marker and restriction sites are introduced artificially), and the third was the ability to achieve transformation – the introduction of DNA into *E. coli*, and subsequently to identify the transformants containing recombinant clones.

Using just these three simple procedures, recombinant DNA can be accomplished *in vitro*. Before we discuss some of the key methods of genetic engineering, an overview of the process and some new terminology is appropriate. *Cloning* means to obtain, from among many millions of different DNA fragments, a colony of genetically identical cells that contain the DNA fragment of interest. The principle of the gene cloning technique is that genetic information carrying DNA is isolated from a donor organism and is cleaved by restriction endonuclease enzymes into individual pieces that create cohesive or sticky ends. The fragments of DNA are then joined to a suitable vector DNA using another enzyme, *DNA ligase*. The cohesive ends have complementary sequences and can thus base pair. A heterologous population of molecules results; but some plasmids will contain an inserted fragment, thus producing recombinant DNA molecules (*chimeras*). The ligated plasmid mixture then undergoes transformation into a bacterial cell. From the original mix of ligated plasmid and other DNA, one particular DNA fragment has been cloned. Because each bacterial colony contains a plasmid that includes the foreign insert DNA, it must be identified by one of the methods described later. There are three possible routes in cloning a foreign DNA fragment. These may be referred to as the protein route (shotgun cloning), the complimentary DNA (cDNA) route, and the synthetic DNA route (Figure 2.9).

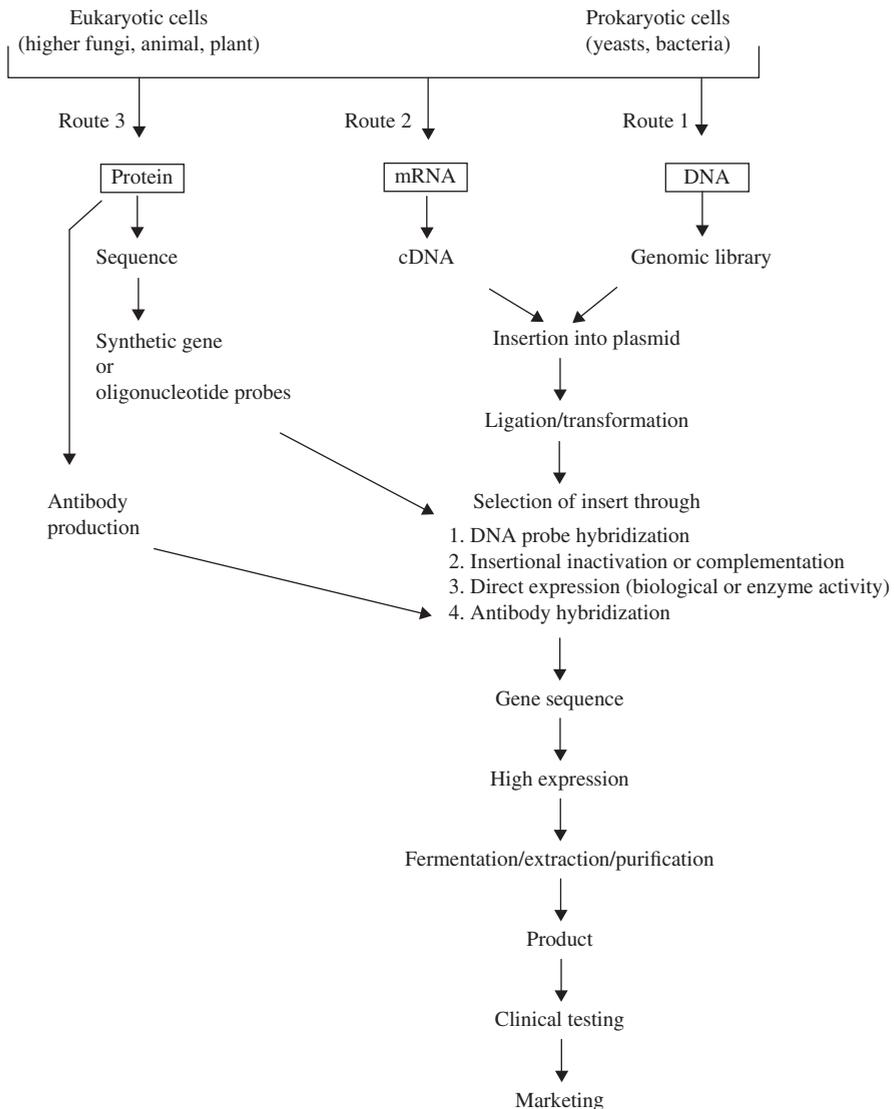


Figure 2.9 Outline of the three general strategies of gene cloning.

2.2.1 Restriction endonucleases

Most bacteria produce one or more restriction enzymes (restriction endonucleases) to protect themselves from infection by foreign DNA such as bacteriophages. Bacteria can recognize foreign DNA and degrade it, while the restriction enzymes do not affect the normal cellular DNA of their own cells because potential cleavage points are usually protected by methylation. These enzymes do not hydrolyze the DNA completely but give rise to a number of gene-sized fragments (4–11 nucleotides in length). If the entering phage

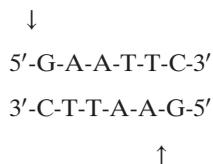
Table 2.2 Some restriction endonucleases and their origins and specificities

Enzyme*	Source	Specificity†
EcoRI	<i>Escherichia coli</i>	-G A-A-T-T-C- -C-T-T-A-A G-
HaeIII	<i>Haemophilus aegyptus</i>	-G-G C-C- -C-C G-G-
BamHI	<i>Bacillus amyloliquefaciens</i>	-G G-A-T-C-C- -C-C-T-A-G G-
BglII	<i>Bacillus globigii</i>	-A G-A-T-C-T- -T-C-T-A-G A-
HindIII	<i>Haemophilus influenza</i>	-A A-G-C-T-T- -T-T-C-G-A A-
HpaI	<i>Haemophilus parainfluenza</i>	-G-T-T A-A-C- -C-A-A T-T-G-
PstI	<i>Providencia stuartii</i>	-C-T-G-C-A G- -G A-C-G-T-C-
Sau3AI	<i>Staphylococcus aureus</i>	- G-A-T-C- -C-T-A-G -

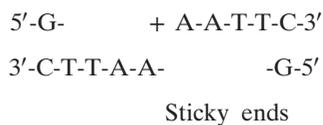
*Nomenclature is based on the organism in which the restriction enzyme was discovered. The first capital letter is the first letter of the genus and next two or three letters are the species of this organism. The Roman numeral denotes the order of discovery.

†HaeIII and HpaI leave "blunt ends," and the others leave "sticky ends."

DNA is cleaved in such a manner, the molecules become susceptible to further degradation by the nonspecific exodeoxynucleases that all bacteria contain. The cuts or nicks produced by the endonuclease may be either double-stranded blunt ends or short, cohesive, single-stranded sticky ends. Thus, not all restriction enzymes leave sticky ends. Enzymes that give sticky ends have found the most application in genetic engineering. The two cohesive ends are complementary and tend to base pair and to join by hydrogen bonding to each other. More than 100 different restriction enzymes have now been identified (Table 2.2). The special utility of restriction enzymes in genetic engineering rests with their remarkable specificity. For example, the restriction enzyme called EcoRI from *E. coli* recognizes the following double-stranded, six-nucleotide sequence and cleaves each strand between the G and A residues:

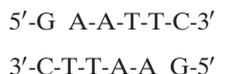


The two ends may then separate to give



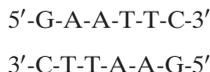
When the sticky ends obtained by EcoRI hydrolysis of different molecules are allowed to anneal, they come together by base pairing through hydrogen bonds that are stable at

reduced temperature. However, this configuration is not covalently closed, which means that the phosphodiester bonds are missing between the G and A residues in both strands as shown schematically here. The gaps can be resealed by the action of DNA ligase.



(Annealed fragment)

↓ DNA ligase



(Covalently closed DNA fragment)

2.2.2 Plasmid vectors

Plasmids are extrachromosomal, self-replicating, double-stranded DNA molecules that are often found in bacteria and some lower eukaryotes. Most are circular, but there are some linear ones. They are not essential for growth, but they confer some unusual properties on the cells that harbor them. The plasmids that confer unusual properties are large (average molecular weight, 65×10^6) and usually exist in one or two copies per bacterial chromosome in the cell. Purifying such large plasmids is difficult and yields are generally poor. These molecules are transmissible; that is, they have the ability to transfer themselves during conjugation (e.g., F factor and R factor discussed earlier).

Other unusual plasmids are *tol* plasmids in *Pseudomonas* sp., and the *nod* plasmids in *Rhizobium*, where they degrade toluene, and *Rhizobium* bacteria, where they fix nitrogen and form symbiotic associations with leguminous plants. However, small plasmids of many other types (<10 kb) also exist; they are not usually transmissible but confer a simple phenotype on the host bacterium. Such plasmids frequently exist in more than 10 copies (usually 20–40) per bacterial chromosome and can readily be purified. An essential prerequisite for a successful practical application of gene manipulation is the availability of a suitable vector, which ensures replication and maintenance of both the vector DNA and the integrated foreign DNA. A useful vector for cloning must have (i) an origin of replication in the plasmid (i.e., a nucleotide sequence that directs and regulates replication so that each cell contains a reasonably consistent number of plasmid copies), (ii) selectable auxotrophic or phenotypic markers to facilitate the identification of inserts apart from the classical antibiotic resistance genes, (iii) marker genes with unique restriction sites for one or more different restriction endonucleases, and (iv) lowest possible molecular weight plasmids, because low molecular weight is normally accompanied by a high copy number, and because this property helps the material to avoid damage by shear forces during preparation.

Cloning vectors serve primarily to amplify foreign DNA in the host cell, that is, to induce a large increase in the ratio of plasmid to chromosomal DNA. With certain plasmids, the copy number per cell can be increased by growth in the presence of antibiotics, which inhibit the replication of the host genome without affecting plasmid replication. In this way, recombinant plasmids often can be produced in as many as 1000 copies per cell. Two different classes of vectors—plasmids and bacteriophages have been developed for cloning DNA, especially in *E. coli*. The best-known cloning vector is probably the bacterial plasmid

pBR322, which was created artificially by using different parts of certain naturally occurring plasmids. The pBR322 plasmid was constructed by using Col EI plasmid (which kills other *E. coli* strains but is resistant to Colicin EI) by joining two fragments of DNA that confer resistance to the antibiotics ampicillin and tetracycline, respectively. The new pBR322 plasmid (4.3 kb) is easy to use because by including either antibiotic in the growth media, only bacteria that contain pBR322 can grow. The pBR322 plasmid has another important feature. It contains within the genes for antibiotic resistance single sites recognized by the restriction enzymes HindIII, BamHI, PstI, and Sall. Therefore, insertion of a DNA fragment into any one of these sites will disrupt the coding sequence, resulting in inactivation of the gene.

The principle of gene cloning using the *E. coli* plasmid pBR322 is outlined in Figure 2.10. For example, insertion of a fragment into the BamHI site inactivates tetracycline resistance and induces tetracycline sensitivity; bacteria transformed by such a plasmid can still be detected, however, because they are resistant to ampicillin. Therefore, colonies that have plasmids with DNA inserts can be detected by testing for ampicillin resistance (Ap^R) and tetracycline sensitivity (Tc^S), respectively. Inserts of variable length, to an upper limit of 15 kb pairs, may be cloned into pBR322 and related plasmid vectors.

The modified new vectors such as the pUC vectors (pUC18 and pUC19) have the genes for ampicillin resistance and for β -galactosidase (*lacZ*) from *E. coli* (Figure 2.11). The *lacZ* gene has a series of unique restriction sites engineered into the plasmid so that the plasmid can be cut only within the *lacZ* gene. If these plasmids are transformed into a *lacZ*⁻ strain of *E. coli*, they will become *lacZ*⁺. If these colonies are grown on X-gal plates (X-gal is histochemical substrate 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), the colonies will then appear blue as a result of the hydrolysis of X-gal. Any plasmid that has a DNA fragment inserted into it will lack a functional *lacZ* gene, thus producing white colonies on X-gal plates. These white colonies containing a cloned DNA insert can be identified from a background of blue colonies. The DNA cloned into a vector can be purified and analyzed by agarose gel electrophoresis.

Bacteriophage lambda has been extensively developed as a cloning vector. *Cosmids* are plasmids that contain the Cos region of phage, which makes it possible for the plasmid to be packaged within bacteriophage particles and thus transferred by infection to *E. coli*. *Cosmids* are an excellent tool for cloning a large DNA fragment (32–47 kb), of the size normally required for the establishment of the so-called genomic libraries. The coliphage M13, which has a completely different genome organization and life cycle, has been developed as a cloning vehicle. M13 is a rod-shaped bacteriophage that contains a single-stranded DNA genome within a coat of viral protein. When M13 infects a susceptible cell, the single-stranded viral DNA (or strand) is converted to a double-stranded replication form, which amplifies up to 300 copies per cell. M13 has been very useful, particularly for DNA sequencing and site-directed mutagenesis for characterizing cloned DNAs.

Expression vectors contain not only the foreign gene but also promoter, operator and terminator sequences, which permit the efficient transcription of the gene into mRNA. Expression vectors also contain the ribosomal binding site, which is necessary for efficient translation. To replicate in the host cell, a vector must contain the appropriate origin of the replication site (*ori*). Vectors containing *ori* of two separate host systems able to replicate in both such vectors are called *shuttle vectors*. Examples are known for *E. coli* and *B. subtilis* as well as *Saccharomyces cerevisiae*. These vectors can clone a desired DNA fragment in bacteria and then, after purification, be transformed into the appropriate eukaryotic cells. Some examples of different vectors and their properties are given in Table 2.3.

Cosmids, which are constructed vector with features from both plasmids and phages include (i) an *E. coli* *ori* sequence, (ii) a selectable marker such as amp^R , (iii) convenient

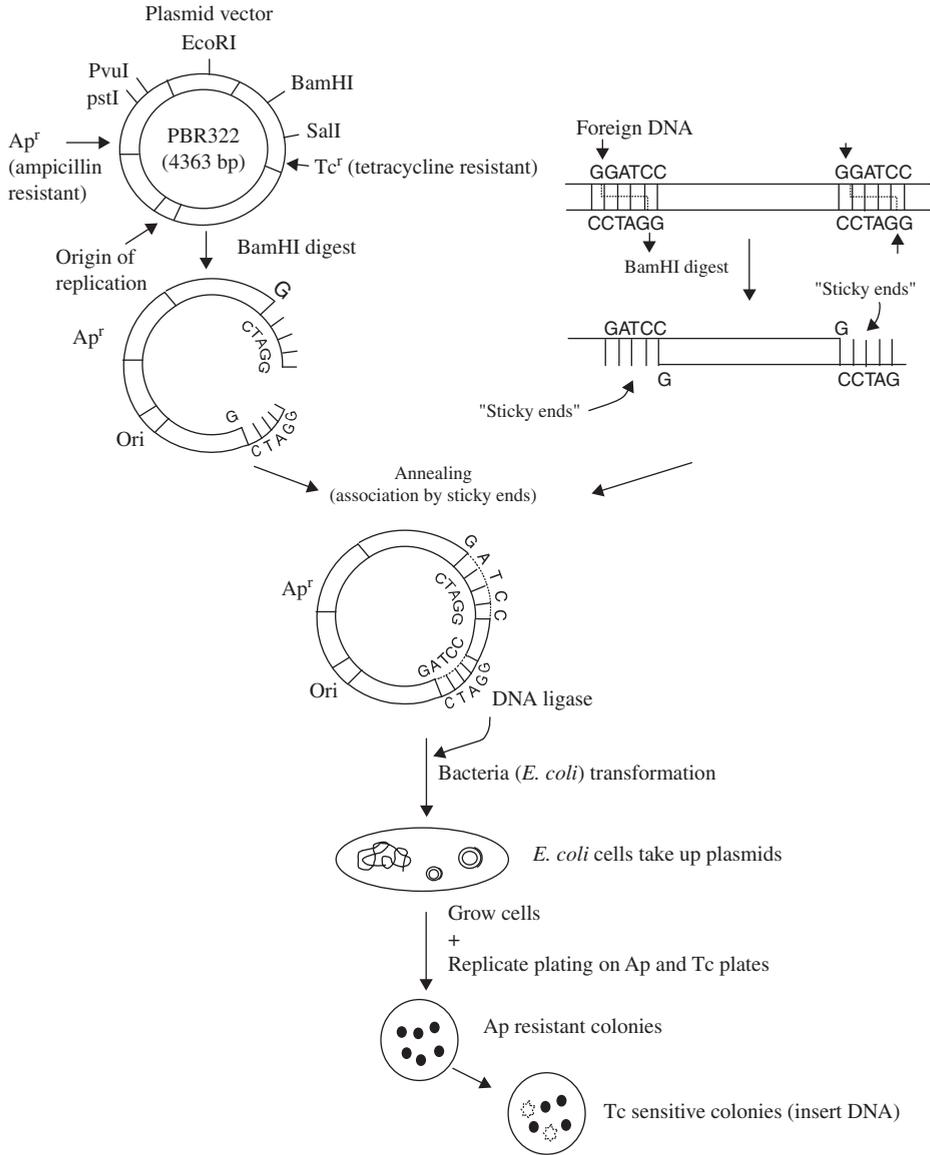


Figure 2.10 Scheme of cloning a foreign chromosomal DNA fragment using a vector (pBR322) as a vehicle and the restriction enzymes BamHI and Sau3A.

restriction sites, and (iv) a phage *cos* site, allowing the DNA to be packaged into a phage head for introduction into *E. coli*. Cosmids as small as 5 kb are available, and 32–47 kb of DNA can be inserted into them. Recombinant cosmids <37 kb or >52 kb cannot be packaged and do not enter *E. coli*, and therefore are not replicated. A cloning vector capable of replicating in two or more types of organism (e.g., *E. coli* and yeast or *E. coli* and lactic

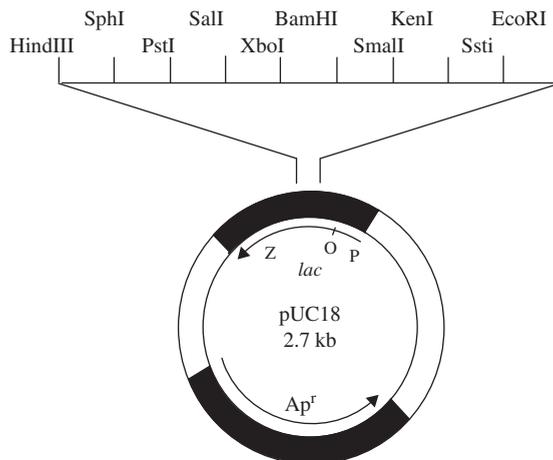


Figure 2.11 Outline structure of the cloning vector pUC18 with some of the restriction enzyme sites.

Table 2.3 Some examples of different vectors and their uses

Vector	Antibiotic marker (resistance)	Host(s)	Use
pBR322	Ampicillin, tetracycline	<i>E. coli</i>	General cloning
pUCI8, 19	Ampicillin	<i>E. coli</i>	General cloning
pHCI	Ampicillin	<i>E. coli</i>	Large-fragment cloning
pBC16	Tetracycline	<i>Bacillus</i>	General cloning
M13 (mp 18)	None	<i>E. coli</i>	DNA sequencing
Cosmids	Ampicillin	<i>E. coli</i>	Large-fragment cloning
YEp13	Ampicillin, tetracycline	<i>E. coli</i> , yeast	Shuttle vector
pHV14	Ampicillin	<i>E. coli</i> , <i>Bacillus</i>	Shuttle vector
pNZ	Ampicillin	<i>E. coli</i> , <i>Lactobacillus</i>	Shuttle vector

acid bacteria) is called a *shuttle vector*. Shuttle vectors may replicate autonomously in both hosts, or integrate into the host genome.

Yeast artificial chromosomes (YACs) vectors function as artificial chromosomes in yeast. Their features include (i) linear structure with a yeast *telomere (TEL)* at each end, (ii) a yeast *centromere sequence (CEN)*, (iii) a marker gene on each arm that is selectable in yeast (e.g., TRP1 and URA3), (iv) a yeast origin of replication known as *autonomous replicating sequence (ARS)*, and (v) unique restriction sites for inserting foreign DNA. Several hundred kb of insert DNA can be cloned in a YAC. YAC clones are made by (i) generating YAC arms by restriction digest, (ii) ligating with insert fragments up to 500 kb in length, (iii) transforming into yeast, and (iv) selecting for markers (e.g. TRP1 and URA3). Bacterial artificial chromosomes (BACs) are also used for cloning fragments up to about 200 kb in *E. coli*. BAC vectors contain (i) the ori of an *E. coli* plasmid called the *F factor*, (ii) multiple cloning sites, (iii) a selectable marker, and (iv) other features, “bells and whistles.” BAC can be handled like regular bacterial plasmids, but the F factor ori keeps copy number at one BAC molecule per cell.

2.2.3 Purpose of gene cloning

There are three possible cloning routes to make a particular product (Figure 2.12). The advantages and disadvantages of the three cloning techniques are shown in Table 2.4. However, the general strategy of gene cloning essentially comprises four stages as explained later. It is easier to clone genes from bacteria and “lower” eukaryotes such as yeast than from higher organisms because the lower organisms have much less chromosomal DNA.

2.2.3.1 Preparation of genome fragments Theoretically, it is now possible to use the technique known as *shotgun cloning* to clone in one organism any desirable gene from another organism. To do this, the entire genome of the first organism is digested with a restriction endonuclease to produce a random mixture of fragments ($\approx 4\text{--}10\text{ kb}$). Some of these restriction enzymes cut two helices a few base pairs apart, generating two fragments with single-strand protrusions called sticky ends because their bases are complementary.

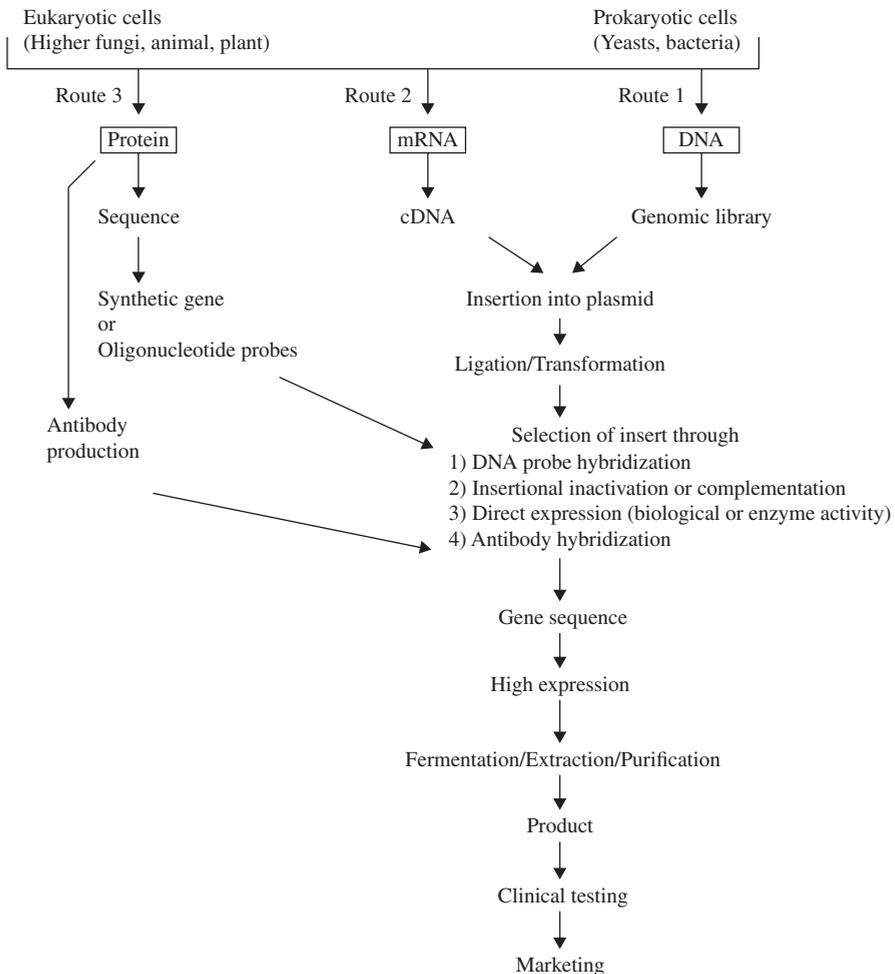


Figure 2.12 Outline of the three general strategies of gene cloning.

Table 2.4 Advantages and disadvantages of the three cloning techniques

Cloning route	Advantages	Disadvantages
Shotgun cloning	Easy to do without knowing general composition of sequence	Need to have a good selection method among many different clones (few thousand) Genes containing introns will not be correctly expressed in <i>E. coli</i> Expression depends on recognition of foreign gene Codon choice may not be optimal
cDNA cloning	No problem with introns, since mRNA will contain molecules Less laborious for selection	Desired mRNA may not always be abundant Technically more difficult to carry out Selection is relatively simpler than shotgun cloning Codon choice may not be optimal for <i>E. coli</i>
Synthetic DNA cloning	Easy to optimize and change sequence of promoters and ribosomal binding sites Makes possible highly specific mutations	Protein sequence required before cloning can begin Codon degeneracy is a problem

Preferably, the initial cloning is carried out with enriched fragments. Enrichment can be done by use of sucrose gradient centrifugation, agarose gel electrophoresis, or column chromatography, or by the specific gene probe route. *Gene probes* are short DNA or RNA fragments that have been tagged in a way that serves to hybridize the specific genome sequence with the respective probe. The strong affinity of biotin-labeled DNA can also be used for purification of the DNA.

Since higher eukaryotic genes contain introns (noncoding regions), which are not processed directly in bacteria, DNA for cloning is generally obtained with a *reverse transcriptase*, which converts mRNA to double-stranded DNA. The normal flow of genetic information is from DNA to RNA, but given a supply of deoxynucleotide triphosphates (dNTP), certain enzymes found in retroviruses will catalyze the reverse process. The enzyme catalyzing the reverse of normal transcription is called *reverse transcriptase*. An alternative way of avoiding the problem of introns, which is possible when the nucleotide or amino sequence is known in advance, is to synthesize an artificial gene. Using an automated DNA synthesis machine, it is possible to synthesize DNA fragments of 20–100 bases, which can be connected together to make longer sequences.

The scheme illustrated in Figure 2.13 consists of the following steps:

1. mRNA is isolated from the target organism and purified by oligo(dT)–cellulose column chromatography; a synthetic oligo(dT) primer is used to bind to the poly(A) tail at the 3' end.
2. Reverse transcriptase is used to initiate the first strand of cDNA from the primer.
3. The second strand of DNA is synthesized by, for example, hybridizing RNA in the RNA–DNA hybrid with RNase H.
4. *E. coli* DNA polymerase I fills in the gaps and the second-strand fragments are ligated by *E. coli* DNA ligase.
5. Methylation and addition of linkers to double-stranded cDNA are completed to enhance cloning efficiency. Double-stranded DNA is now ready to be cloned in an appropriate vector.

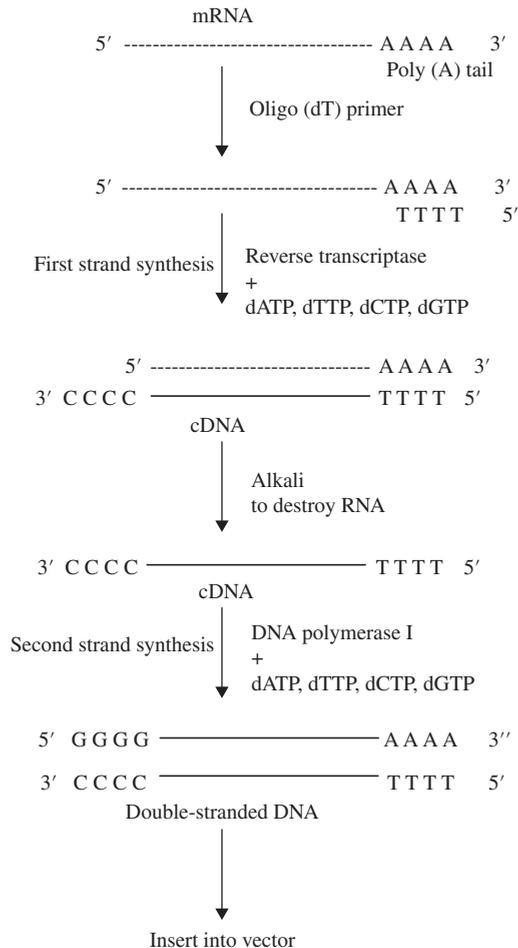


Figure 2.13 Simplified scheme of using reverse transcriptase to convert mRNA into double-stranded DNA for cloning.

2.2.3.2 Insertion into vector The vector is normally cut with the same restriction enzyme used to generate the chromosomal DNA fragments. Linearized vectors are incubated with DNA ligase, which covalently joins the DNA molecules. In a heterogeneous fragment of molecules, some plasmids will contain an inserted fragment, thus producing hybrid recombinant (or chimeric) DNA.

2.2.3.3 Transformation of host cell The resulting recombinants are introduced into the host cell by the process of transformation. In this process, the ligated plasmid mixture is introduced into a suspension of bacteria treated with a cold CaCl_2 solution. After this treatment the cells are mixed with the plasmid DNA that is to be introduced, given a brief heat shock, and allowed to recover. Up to 10^7 transformations per microgram of DNA can be

achieved, which is sufficient for the purpose of gene cloning. More recently, electroporation has been shown to be a very efficient method for physically introducing DNA into bacteria. The DNA enters the cell by a mechanism that is still poorly understood. *E. coli* transcribes and translates most gram-positive and gram-negative genes with the exception of some actinomycete genes. Alternative hosts include *B. subtilis*, *Streptomyces*, *S. cerevisiae*, and pseudomonads. *B. subtilis* has the advantage of being nonpathogenic, with an efficient protein secretion system; but the rDNA in this host is unstable, and proteolytic degradation of the product is excessive. Yeast is well established for large-scale growth, and mammalian proteins have been successfully expressed and some are secreted into the medium. Yeast is able to glycosylate.

A number of methods are available to transfer DNA into plant cells (i) *Agrobacterium*-mediated transformation is the easiest and most simple plant transformation. Plant tissue (often leaves) are cut into small pieces, for example, as 10×10 mm pieces, and soaked for 10 min in a fluid containing suspended *Agrobacterium*. Some cells along the cut will be transformed by the bacterium that inserts its DNA into the cell. By placing on selectable rooting and shooting media, the plants will regrow. Some plants species can be transformed just by dipping the flowers into suspension of *Agrobacterium* and then planting the seeds in a selective medium. Unfortunately, many plants are not transformable by this method. (ii) *Gene gun* (referred to as particle bombardment, microprojectile bombardment, or biolistics) in which particles of gold or tungsten are coated with DNA and then shot into young plant cells or plant embryos. Some genetic material will stay in the cells and transform them. This method also allows transformation of plant plastids. The transformation efficiency is lower than in *Agrobacterium* mediated transformation, but most plants can be transformed with this method. Other techniques use physical or chemical agents to transfer DNA into plant cells. (iii) *Protoplasts* in which plant cells have been stripped of their protective cell walls, that will take up pure DNA when treated with certain membrane-active agents or with electroporation, a rapid pulse of high-voltage direct current. Once inside the cell, the DNA is integrated and the foreign gene will express. These two techniques largely depend upon the development of protoplast systems that retain the capacity to regenerate intact plants. Transgenic corn, rice, and soybean have been produced with these techniques, especially electroporation. Success rates, however, are low, and the techniques not very reproducible. DNA can also be microinjected into target plant cells using very thin glass needles in a method similar to that used with animals. Microinjection, however, has produced only a few transgenic plants. The technique is laborious, technically difficult, and limited to the number of cells actually injected. (iv) In *viral transformation (transduction)*, the desired genetic material can package into a suitable plant virus and allow this modified virus to infect the plant. If the genetic material is DNA, it can recombine with the chromosomes to produce transformant cells. However, genomes of most plant viruses consist of single-stranded RNA, which replicates in the cytoplasm of infected cell.

Transgenic animals are produced by inserting genes into embryos prior to birth. Each transferred gene is assimilated by the genetic material or chromosomes of the embryo and subsequently can be expressed in all tissues of the resulting animal. Transgenic animals are normally produced by two methods: (i) microinjection of cloned gene(s) into the pronucleus of a fertilized ovum, (ii) injection of embryonic stem (ES) cells into embryos, and exposure to retroviruses. The first method is the one that is most widely and successfully used for producing transgenic mice. After microinjection, the recently fertilized single cell embryos are removed from the animal. Micromanipulators on a specially equipped microscope are used to grasp each embryo. A glass pipette drawn or pulled to a fine point immobilizes. The foreign DNA is injected into the embryo's

pronucleus – either of two nuclei (male or female) containing half the chromosomes of a fertilized ovum – with a second finely drawn injection needle. After the injection, the embryos are transferred back into the hormonally prepared or pseudopregnant recipient females or foster mothers. The recipients follow normal pregnancy and deliver full-term young. This method is presently the most efficient for generating transgenic animal lines: about 1–4% of the injected embryos result in a transgenic offspring. The second method involves microinjection of ES cells derived from the inner cell mass of blastocyst-stage embryos (about 7 days postfertilization) into embryos to produce “hybrid” embryos of two or more distinct cell types. The ES cells are able to produce all tissues of an individual. Once isolated, ES cells may be grown in the laboratory for many generations to produce an unlimited number of identical cells capable of developing into fully formed adults. These cells may then be altered genetically before being used to produce embryos. When these transformed cells participate in the formation of sperm and eggs, the offspring that are produced will be transgenic.

2.2.3.4 Selection of recombinant clones Only a few bacteria will take up the recombinant plasmid, and the problem then becomes how to select the bacteria that contain the insert (i.e., foreign) DNA from the vast majority of the bacteria that do not contain insert. Finding a short fragment of DNA within the enormous genome of an eukaryotic cell is like looking for the proverbial needle in a haystack. In shotgun cloning, the insertional inactivation technique can be used. Suppose that DNA fragments were inserted at the BamHI site in plasmid pBR322. Since inserts at this site interrupt the gene (insertional inactivation) from expressing tetracycline resistance, cells containing recombinant plasmids will be ampicillin resistant and tetracycline sensitive, while cells without inserts will be resistant to both ampicillin and tetracycline. To assay a complete gene bank for the presence of gene, as many as thousands of colonies of bacteria must be screened from antibiotic plates by the replica plating technique. Further tests must be performed to make sure that the appropriate clone has been obtained. Some common methods for the identification of the desired clone are listed in the subsections that follow.

Genetic method Complementation of a mutation in the host or expression of a foreign gene can be induced directly by enzyme activity (e.g., β -galactosidase activity on X-gal plates and glucoamylase on starch agar plates).

Immunological detection method Since expression efficiency is often quite low, a very sensitive method for detection of the gene product is necessary. In the immunological detection method, an antibody marked by a radioactive enzyme is used as a probe. When an antibody specific against the desired protein product is available, the method works well. Transformed cells are grown on agar plates and the cells in the duplicate set of colonies on nitrocellulose disc are lysed by 0.5% NaOH, followed by washing and baking at 80°C. The matrix is then exposed to antibodies that have been radioactively labeled *in vitro*. Positively reacting lysates are detected by washing surplus radiolabeled material from the matrix and making an autoradiographic image. Many different variations of this technique have been adopted. The DNA released from the bacteria will denature, and the separated strands will bind loosely to the nitrocellulose. After the filter has been washed to remove the alkali, it is dried in a vacuum oven, and gene proving with antibody is then carried out. An alternative solution for avoiding radioactive labels is to use biotin-labeled nucleotide triphosphates during nick translation. The *nick translation* process is simply a replication of DNA *in vitro* with DNA polymerase I (*Klenow fragment*) and radioactive nucleotide, which becomes

incorporated into the duplicated DNA at a nick (break). Binding the biotin-labeled DNA is detected by means of an avidin/alkaline phosphatase system (see Section 9.2.2.4). The sensitivity of this system has been increased significantly in recent years.

Nucleic acid hybridization *In situ* colony hybridization is a powerful technique for identifying, from among thousands of colonies of bacteria, any colony that contains homologous DNA or RNA sequences. Colony hybridization is usually done by using the replica plating technique to transfer about 200 colonies from agar plates to a nitrocellulose filter. The colonies on the filter are then lysed by sodium dodecyl sulfate (SDS) or lysozyme, and the released DNA is denatured with 0.5 N NaOH. After excess protein has been removed with proteinase K, the single-stranded DNA is fixed to the filter by baking at 80 °C. If the gene is unlikely to be expressed, the filter is then soaked in a buffer containing a radiolabeled (typically with ^{32}P) DNA of the desired gene. This radioactive DNA is known as a probe. The nucleotide sequence of the probe, whether DNA or mRNA, is complementary to a portion of the sequence of the DNA fragment of interest. The excess probe is washed off, leaving the hybridized probe. When the filter is exposed to a sheet of X-ray film, dark spots corresponding to the hybridized colonies can be isolated from the original plates. One method of labeling DNA gene probes *in vitro* is nick translation. After isolation of hybrid DNA molecules, the DNA is treated with a restriction enzyme, and the resulting DNA fragments are separated by agarose gel electrophoresis. The DNA bands are transferred to nylon or filter paper by blotting. The filter containing denatured DNA is exposed to the probe and to X-ray autoradiography as before to identify gel bands containing homologous nucleotide sequences using a gene probe. This method is called the *Southern blotting* technique. The corresponding technique for RNA is called *Northern blotting* and that for protein is called *Western blotting*. Once the fragment size has been obtained, further analyses of the fragment sizes obtained with other restriction enzyme and multiple restriction digestions together will yield a restriction map showing the relative positions of various restriction sites.

2.2.3.5 Maximizing gene expression Even when the gene product is a protein derived from a single gene and has been expressed, the expression efficiency is often quite low. Nevertheless, high levels of expression of the gene must be obtained in a foreign host. Non-protein products resulting from a metabolic pathway are more complicated to deal with. In certain cases, such products have been found to be toxic to host cells. This section discusses the following important factors to maximize gene expression: the number of copies of the plasmid vector per unit cell (copy number) and the strength of promoter, the sequence of the RBS and flanking DNA, codon choice in the cloned gene, genetic stability of the recombinant, and proteolysis.

The limiting factor in expression is the initiation of protein synthesis. Increasing the number of plasmids per cell increases the number of mRNA molecules transcribed from the cloned gene, and this results in increased protein synthesis. Similarly, the stronger the promoter, the more mRNA molecules are synthesized. For efficient transcription, the cloned gene must be provided with a powerful promoter, preferably originating from the host cell. The stability of the mRNA is also related to gene expression, and structural modification of the mRNA could result in greater resistance to nuclease attack; double-stranded RNA is more resistant than the single-stranded forms. Most plasmid vectors of *E. coli* use the *lac*, *try*, or a hybrid *tac* promoter as well as the phage *PL* promoter. In each case the gene is inserted into a site in such a way that transcription gives rise to a fused protein containing a few codons of the natural gene from the promoter. It is known that

transcription of *tac* promoter is 11 times more efficient than the catabolite-derepressed *lac* promoter (UV5) and three times more efficient than *try* promoter. The phage *PL* promoter coupled to a temperature-sensitive CI mutant repressor offers a strong promoter under temperature-sensitive control.

Efficient translation is also important in the maximization of gene expression, as discussed in an earlier section. Promoters will contain information for the correct RBS in the message if the cloned gene is being synthesized as a fused protein, but the binding sites of different genes may vary by nearly a thousand-fold in relative efficiency. The translation initiation signal should be modified to provide an efficient SD sequence for the organism in question and the optimum space between the SD and the initiation codon. The base composition of the intervening sequence is also important in *B. subtilis*.

Another important factor is related to the degeneracy of the genetic code. There are several triplet codes for most amino acids, and different organisms favor different codons in their genes. Therefore, if the genes inserted into cells of another species use rare codons in the host cell, the host's biosynthetic machinery may be starved of charged tRNAs. Such a deficiency could result in premature protein chain termination or a high-error frequency in the amino acid sequence or the protein.

There is little to be gained from high expression of a plasmid-borne gene if the plasmid is lost from the culture. Two different forms of genetic instability can damage recombinant populations. A certain fraction of new progeny cells can be born without plasmids, and if selection pressure is not applied, faster-growing plasmid-free cells may predominate. This instance of complete loss of plasmid is called *segregational instability*. Even with selection pressure, segregational instability reduces the overall growth rate and the productivity of the recombinant population. This occurs through defective partitioning of plasmid molecules into daughter cells at cell division. High copy number plasmids are less susceptible to this type of instability because there is a high probability that at least one plasmid molecule will be transmitted into the daughter cells at each cell division. On the other hand, low copy number plasmids cannot deliver accurate partitioning function (*par* region), which is responsible for distributing daughter plasmids into daughter cells. The plasmid can be stably maintained in the cell culture either by integrating the plasmid DNA at homologous sites in the chromosomal DNA or by including another gene in the plasmid, which in turn will apply a selective pressure in favor of plasmid-containing cells. Some vectors were stabilized by cloning *par* regions from other plasmids into them. For example, an essential gene, such as the valyl-tRNA synthetase (*vals*) gene and a *vals*-deficient host, can be placed in the vector. In this way, some recombinant plasmids have been stabilized for up to 150 generations in continuous culture.

The other type of instability is *structural instability*, which results from the instability of cells with respect to the synthesis of an active cloned gene product. Structural instability can be caused by mutation in the structural cloned gene by deletions, insertions, or rearrangements of DNA; it is difficult to overcome because it is not well understood. Selection pressure does not control this form of instability, but perhaps the most successful route to alleviation is to place the gene under strict transcriptional control using a strong promoter such as a *lac* or *tac* promoter coupled with a regulated copy number. Even if a cloned gene carries its own promoter, this promoter may not function in the new host cell. Generally, it is desirable to use a controllable promoter that can be turned on by some environmental change, such as addition of inducer, depletion of repressor, or temperature shift. In a well-designed expression system, the cloned gene product may constitute up to 70% of the total cellular protein (10–25% is a typical figure). This overproduced product seriously limits the host cells' capacity for growth and even survival. It is therefore common practice to operate a culture first, thus postponing cloned gene expression until a high cell

density has been reached, and then to alter culture conditions to maximize cloned gene expression. *Runaway plasmids* are examples of vectors with a controllable copy number. At low temperatures (<30 °C), the copy number may be as low as 10 plasmids per cell; but when the temperature is raised to 37 °C, the copy number increases to several hundred or even several thousand.

Once expression of a gene on a stable plasmid has been achieved, proteolysis influences the apparent rate of gene expression if the intracellular proteases recognize and degrade small abnormal proteins. Resistance to host proteases is poorly understood, but an APT-dependent ion protease has been implicated in *E. coli*. There are two approaches to avoiding this problem. Protease-deficient host strains (ion-protease-negative strains) have been developed, but the strains lacking proteases are unlikely to be viable. One approach used widely is to protect the desired foreign protein by fusing it to a normal native protein, from which it must then be released. This method was adapted to stabilize several relatively small polypeptides such as chains of somatostatin (14 amino acids) and human insulin A (21 amino acids) and B (30 amino acids).

For effective expression, the structural gene must be bracketed by nucleotide sequences that provide transcription initiation (promoter sequence) and translation start (RBS and start codon) before cessation of gene translation (stop codon) and translation termination after the gene. Expression vectors usually include these control sequences, an origin of replication, and at least one selection marker.

When foreign proteins are expressed at high levels in *E. coli*, the product also tends to accumulate in the cells as dense refractile bodies, called *inclusion bodies* (IBs). These insoluble aggregates are primarily proteins, but some membrane protein aggregates are also included. The product protein is typically inactive, denatured, and cross-linked to other protein molecules by disulfide bonds. The IBs require specific technology for solubilization and renaturation to refold them into active form (see Figure 5.7). It now may be possible to purify IBs proteins by direct addition of affinity resins, without denaturation and refolding steps.

2.2.3.6 Considerations on choice of expression hosts Many different host cells are available for heterologous protein expressions, in which each system has distinct advantages and disadvantages. The expression systems to produce high levels of stable and functional recombinant proteins are an enormous field and the expansion of this field has produced hundreds of therapeutic proteins into clinical applications and food enzymes over the past decades. Despite the significant progress, optimizing protein expression at high levels and understanding PTMs and effect of downstream elements on expression have become the big challenges.

Most common expression systems are microbial (bacterial or yeast), viruses (baculovirus, retrovirus, adenovirus) and higher eukaryotic (insect or mammalian cells), that are listed for their advantages and disadvantages in Table 2.5. The most popular host among bacteria (*E. coli*, *B. subtilis*, *Lactococcus lactis*, *Streptococcus lividans*) is *E. coli* as the expression techniques are well developed and high copy number plasmids such as *lac* promoters or the T7 promoter that are normally regulated by the operator are available. Addition of inducer IPTG (a lactose analog) can activate the *lac* promoter and causes the bacteria to express the protein of interest at high levels. However, its shortcomings are antibiotics-free selection plasmids, extracellular production, and PTMs. Thus, many proteins become insoluble as IBs that are difficult to recover without denaturation and renaturation procedures. Significant progresses have also been made over the past few years in alternative bacterial expression systems such as the *L. lactis* for membrane proteins. Additionally, several *Corynebacterium glutamicum* and *Pseudomonas fluorescens*

Table 2.5 Advantages and disadvantages of different expression hosts

	<i>Escherichia coli</i>	<i>Bacillus subtilis</i>	<i>Sacchromyces cerevisiae</i>	<i>Pichia pastoris</i>	Animal cells
Advantages	Fast growth/ high density cell Well-known genetics Strong promoters	Fast growth/ high density cell Well-known genetics	High cell density Well-known genetics Known strong promoters/ expression vectors	Very high cell density (methanol substrate) High production rate Efficient secretion	Soluble PTM proteins Proper folding Stable/transient infection
Disadvantages	Rare secretion Inclusion body Endotoxin	Plasmid instability/ versatility Secretion of proteases	Slow growth than bacteria Poor expression rate	High heat generation, High oxygen demand Anti-inflammable tools requires	High media costs Slow growth rate Low expression Viral infection

Abbreviation: PTM, post-translational modification.

systems were also developed and offered advantages in some niche areas, providing more choices of bacterial expression systems for recalcitrant proteins.

As *Bacillus* species has a large excretory capacity, *B. subtilis* has been used to improve the quality and quantity of the secreted foreign proteins such as interferon, growth hormone, pepsinogen and epidermal growth factor. However, *B. subtilis* also produces and secretes high levels of extracellular proteases, which degrade the secreted foreign proteins. This species also lacks well-regulated inducible vectors, which limits the wide application of the *B. subtilis* system.

Unicellular eukaryotes such as the yeasts *S. cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*, *Kluyveromyces lactis* and *Schizosaccharomyces pombe* have become important systems in biotechnology for heterologous protein expression for both academic and industrial purposes. Yeasts combine the ability to grow on a simple medium at a very high cell density and secrete heterologous proteins. Moreover, they perform many of eukaryotic PTMs such as protein folding, proteolytic processing, disulfide bond formation and glycosylation. The best known *P. pastoris* and *S. cerevisiae* have been genetically well characterized and are known to perform many PTMs with high cell density. The yeast systems have attained dry-cell-weight densities exceeding 100 g/L and continuous fermentation productivities of 10–12 g of recombinant protein per liter per hour.

P. pastoris is frequently used as an expression system for the production of proteins in high yields. *P. pastoris* has a high growth rate and is able to grow on a simple, inexpensive medium in high density cell. Commercially available plasmids such as the pPICZ α vector have the major advantage of *Pichia* over *E. coli*. *Pichia* is capable of producing disulfide bonds and glycosylations in proteins. *Pichia* has few advantages over *S. cerevisiae* in laboratory and industrial settings: The presence of two strongly inducible promoters (AOXI and AOXII) allows *P. pastoris* to use methanol as its sole carbon and energy source. Large upscale (<130 g/L dry cell weight) can be maximized because of its preference for respiratory growth, which allows it to be cultured at high cell densities compared to the fermentative yeasts such as *S. cerevisiae*. In turn, this allows for large yields of the recombinant

proteins to be produced. In a popular expression vector, the desired protein is produced as a fusion product to the secretion signal of the α -mating factor from *S. cerevisiae*. This causes the protein to be secreted into the growth medium, which greatly facilitates subsequent protein purification. Thus, the major advantages of yeast expression system are (i) high yield, (ii) high productivity, (iii) chemically defined media, (iv) product processing similar to mammalian cells, (v) stable production strains, (vi) durability, and (vii) lower protein production cost. Compared to other expression systems such as S2-cells from *Drosophila melanogaster* or Chinese hamster ovary (CHO) cells, *Pichia* usually gives much better yields. Yeast protein expression system is the most economical eukaryotic expression system for both secretion and intracellular expression. It is ideally suitable for large-scale production of recombinant eukaryotic proteins. However, *Pichia* is unable to produce a number of proteins for which the host lacks the appropriate chaperones. In 2006 a *Pichia* strain was created to produce erythropoietin in its normal glycosylation form by exchanging the enzymes responsible for the fungal type glycosylation, with the mammalian homologs. Thus, the altered glycosylation pattern allowed the protein to be fully functional.

Other yeast expression host, *K. lactis* is best known for its use in commercial production of the milk-clotting enzyme bovine chymosin (Van den Berg et al., 1990). This protein was the first heterologous enzyme originating from a higher eukaryote that was produced at low cost in a microorganism, and the process developed for its industrial-scale production was widely recognized as a major biotechnological achievement. Today, over 40 proteins have been produced with *K. lactis*, illustrating its utility as an alternative yeast expression system. As a host for heterologous protein production, *K. lactis* has a number of advantages over other yeast expression systems. These include easy genetic manipulation, the ability to use both integrative and episomal expression vectors, and the availability of a fully sequenced genome. Growth of *K. lactis* can be performed in standard yeast medium and does not require the explosion-proof fermentation equipment necessary for large-scale growth of methylotrophic yeasts such as *Pichia pastoris*. In addition, enzymes from *K. lactis* have GRAS (generally regarded as safe) FDA status, permitting their use in various food and feed applications. Finally, an easy-to-use reagent kit for *K. lactis* protein expression is now offered by New England Biolabs. A *K. lactis*-based expression system is commercially available that makes use of the strong, inducible LAC4 promoter to drive the expression of heterologous proteins. LAC4 is one of the two LAC genes, not found in *S. cerevisiae*, that are responsible for lactose assimilation. It encodes β -galactosidase and is divergently transcribed and coregulated with the lactose permease gene LAC12.

Aspergillus oryzae is also an excellent host for homologous (fungal) and heterologous protein production. Moreover, unlike any previously described microbial production and secretion system, such as *E. coli*, proper eukaryotic PTMs, including glycosylation and protein folding, are expected to occur in *A. oryzae*. For these reasons, this microorganism is considered one of the most adequate hosts to produce higher eukaryotic proteins. In an attempt to enhance heterologous protein production in *A. oryzae*, the constructed multiple protease gene disruptants led to a significant improvement of recombinant bovine chymosin and human lysozyme yields. In general, the production levels of proteins from higher eukaryotes (animals and plants) by *A. oryzae* are much lower than those of homologous (fungal) proteins. Yet, the fungal secretory pathway and the effects of posttranslational events on protein function are poorly understood. The bottleneck in the production of heterologous proteins is not caused by low expression of the heterologous gene but is due to posttranscriptional processes in the vacuolar protein secretory pathway.

While *Aspergillus nidulans* is the best genetically characterized *Aspergillus* species, it is also deemed to be unsuitable as a recombinant host for biotechnology processes, because it produces sterigmatocystin, which is also a toxin, albeit much less severe than

aflatoxins. *Aspergillus niger* and *A. oryzae* are also recognized potential broad based recombinant hosts for the biopharmaceutical industry for production of recombinant proteins. The special advantages of these organisms, namely, their abilities to grow at high rates and to high densities in commercial fermenters and their natural abilities to secrete high levels of homologous enzymes, are widely recognized. In exploiting these advantages for production of recombinant proteins, researchers have generally used the most effective regulatory, expression and secretion machinery identified in these organisms and hooked it up to the genes encoding the desired recombinant protein, an approach that has met with considerable commercial success. Filamentous fungi also have several disadvantages as hosts for heterologous protein production, perhaps the most serious being their abilities also to produce and secrete homologous proteases, which may degrade the desired recombinant product. In addition to the secreted proteases, filamentous fungi may be subject to different degrees of lysis during the fermentation process, the extent of which will vary with other aspects of fungal physiology including morphology, such that intracellular proteases may be released which can attack the recombinant protein. In addition, there are significant differences in the mechanisms of protein glycosylation observed in fungi and mammalian cells. While considerable research is being invested in modifying the glycosylation machinery in fungi to use them as hosts for production hosts of more human-like glycoproteins, it is widely accepted that yeasts such as *Pichia*, may be better candidate hosts for production of recombinant human therapeutic glycoproteins.

Mass culture of animal cell lines is fundamental to the manufacture of viral vaccines and other recombinant therapeutic products. Recombinant biological products produced in animal cell cultures include enzymes, synthetic hormones, immunobiologicals (monoclonal antibodies, interleukins, lymphokines), and anticancer agents. Although many simpler proteins can be produced using bacterial cultures, more complex proteins that are glycosylated currently must be made in animal cells. An important commercial example of such a complex protein is the hormone erythropoietin.

Most of the therapeutic proteins approved so far have been obtained using transgenic hamster cell lines, namely 49 in CHO cells and 1 in baby hamster kidney (BHK) cells. The main advantage of this expression system is that cells can be adapted to grow in suspension in serum-free media (SFM), protein-free and chemically defined media. This fact increases the biosafety of final products reducing risk of introducing prions of bovine spongiform encephalopathy (BSE) from bovine serum albumin and of infectious variant Creutzfeldt–Jakob disease (vCJD) from human serum albumin. In addition, recombinant products can be secreted into the chemical defined media, which simplifies both upstream and downstream purification process. PTMs in this expression system are almost the same as in human cell lines, although some concerns about comparability in the glycosylation pattern have arisen when comparing different batches of the same manufacturer product and biosimilars. Further development of chemically defined media and fine description of growth conditions would help to overcome this issue.

Cultured insect cells are also used as hosts for recombinant baculovirus infections, as baculovirus does not infect human. However, the production of a recombinant viral vector for gene expression is time-consuming, the cell growth is slow when compared with former expression systems, the cost of growth medium is high and each protein batch preparation has to be obtained from fresh cells since viral infection is lethal. PTMs are also an important limitation of this expression system because of the simple non-sialated N-linked glycosylation, which is translated in a rapid clearance from human sera. Although genetic

engineering has been used to select transgenic insect cell lines (MIMIC™ from Invitrogen and SfSWT-3) expressing galactosyltransferase, *N*-acetylglucosaminyltransferases, sialic acid synthases, and sialyltransferases genes to obtain humanized complex N-linked glycosylation protein patterns, there are still unwanted toxicological issues that need to be overcome. There is only one approved biopharmaceutical product containing recombinant proteins from infected insect cell line Hi Five, Cervarix, consisting of recombinant papillomavirus C-terminal truncated major capsid protein L1 types 16 and 18. Nonetheless, this expression system has been extensively used in structural studies since correctly folded eukaryotic proteins can be obtained in a secreted form in SFM that enormously simplifies protein capture in purification protocols.

Hybridomas, which are fusion cells of murine origin (B-cells and myeloma tumor cells), can express specific monoclonal antibodies against a determined antigen, thus possessing therapeutic potential. Humanized monoclonal antibodies using either recombinant mammalian cells producing chimeric antibodies or genetically modified mice are successfully used to produce human-like antibodies. One such product, Remicade, which binds tumor necrosis factor- α , is a pharmaceutical blockbuster used in the treatment of Crohn's disease. In the recent years, three therapeutic proteins produced in human cell lines have also been approved, namely, Dynepo-erythropoietin, Elaprasedipate-2-sulfatase, and Replagal- α -galactosidase A. As these products are fully glycosylated human proteins, this expression system should be addressed when heavily glycosylation is needed. In general, recombinant biopharmaceuticals obtained from mammalian cells cover a wider spectrum of pathological conditions than those obtained from microbes, and the distribution of applications is less biased than when observing products from *E. coli* or *S. cerevisiae*.

Plant expression systems are also emerging as fast and inexpensive methods for producing vaccines and other heterologous proteins. Plants may surpass mammalian and even many microbial systems in efficiency and cost-benefits for the manufacture recombinant proteins in the future. This is particularly true for the rapid manufacture of large-scale (million- or even billion-dose) vaccine antigens. Whether grown as single cells or tissues in photosynthesis reactors, as whole plants in controlled laboratory situations, or cultivated in fields of transformed food-commodity plants (e.g., rice, potatoes, or tobacco), plants being adapted for recombinant protein manufacture offer high yields and relatively low investment costs. Many cell culture processes are using plant-expressed products such as Cellastim recombinant human serum albumin (rHSA) from Invitria. However, global concerns in scaling up vaccine production for influenza outbreaks paired with biodefense threats have mobilized record-level amounts of US governmental funding toward developing new vaccine techniques and technologies. Those include bioterrorism (anthrax), pandemics (influenza) and infectious diseases that affect the developing world (e.g., malaria). Plant-based expression systems for vaccine production are with regulatory approvals expected over the next several years. Despite the advances, however, several significant factors such as (i) plant expression rate is still low, (ii) difficulty of gaining the acceptance to support adoption of new technology, and (iii) complexities in translating technical analyses into commercial stage as few bioprocessing professionals are yet familiar with these plant technologies are stalling wider bioprocessing industry adoption of new plant expression systems. Bridging knowledge and information gap is required in this field.

Transgenic plant and transgenic animal bioreactors will be discussed in separate chapters (Part III).

2.3 DNA sequencing

There are two methods for sequencing DNA: chemical and enzymatic. Two different methods for rapid and efficient sequencing of DNA are the *Maxam–Gilbert* and *Sanger* techniques, which were invented in the 1970s. Although they differ in chemical details, both rest on the same basic principle: the generation of a series of DNA fragments having a common starting point but variable termini. In the *Maxam–Gilbert* method, the double-stranded DNA is first labeled radioactively at one end of each strand. The DNA is then denatured and a preparation of one of the two types of strands is divided into four aliquots, each of which is treated with different chemicals. Each of the four chemical treatments destroys selectively one or two bases and cleaves the DNA at those points. The fragments resulting from these four parallel treatments are then run on a long polyacrylamide gel. Gel band positions are visualized by exposure to X-ray film. The nucleotide sequence follows directly from reading down the four parallel lanes of the gel. Sequences of about 200 nucleotides may be routinely determined in a single experiment using this method. The *Sanger* DNA sequencing method is usually used for sequencing extensive domains of DNA. The enzymatic method yields more information per experiment. The enzymatic DNA sequencing relies upon the ability to replicate DNA *in vitro* with the Klenow fragment of DNA polymerase I. Sequencing of a purified single-stranded fragment obtained from vector M13 is considered to be easy and is accomplished by generating a series of complementary fragments of DNA by incubating the fragment with DNA polymerase (Klenow), a primer (a short piece of DNA), templates, the four normal deoxynucleotide triphosphates (one of which is radioactive), and one of the four dideoxynucleotide triphosphates (e.g., dideoxythymidine triphosphate). Chance incorporation of dideoxythymidine, which lacks a 3'-hydroxy group, terminates polymerization at that point. In practice, four parallel incubations are performed, identical except that each contains a different dideoxynucleotide triphosphate and thus will terminate at a different base. The mixture is then denatured and electrophoresed to separate the newly synthesized strands by size. The smallest fragments move through the gel fastest. When all the fragments have been separated, the gel is dried and the fragments are detected by exposing a piece of X-ray film to the radioactive DNA fragments. The DNA sequence can easily be read from the developed X-ray film. Currently automated DNA sequences are being developed that can sequence thousands of bases in a day. Also, direct sequencing of plasmid DNA from the transformants utilizing the polymerase chain reaction allows for rapid screening at the sequence level. The high demand for low-cost sequencing has driven the development of high-throughput sequencing (or next-generation sequencing) technologies that parallelize the sequencing process, producing thousands or millions of sequences concurrently. High-throughput sequencing technologies are intended to lower the cost of DNA sequencing beyond what is possible with standard dye-terminator methods. During the new era of predictive and personalized medicine, the cost of full genome sequencing an individual or patient dropped to roughly US\$1000 by 2013. Comparison of next-generation DNA sequencing and Sanger methods are listed in Table 2.6.

2.4 Polymerase chain reaction (PCR)

The *polymerase chain reaction (PCR)* is a new, powerful (*in vitro*) method for amplifying a small amount of DNA (a specific DNA fragment as small as 50–100 bp) several millionfold in a few hours. The PCR, which was developed by Cetus (Emeryville, CA)

Table 2.6 Comparison of next-generation sequencing methods

Method	Single-molecule real-time sequencing (Pacific Bio)	Ion semiconductor (ion torrent sequencing)	Pyrosequencing (454)	Sequencing by synthesis (Illumina)	Sequencing by ligation (SOLiD sequencing)	Chain termination (Sanger sequencing)
Read length, bp	2900 average	200	700	50–250	50+35 or 50+50	400–900
Accuracy, %	87 (read length mode), 99 (accuracy mode)	98	99.9	98	99.9	99.9
Reads per run	35–75 thousand	Up to 5 million	1 million	Up to 3 billion	1.2–1.4 billion	N/A
Time per run	30 min to 2 h	2 h	24 h	1–10 days, depending upon sequencer and specified read length	1–2 weeks	20 min to 3 h
Cost per 1 million bases (in US\$)	2	1	10	0.05–0.15	0.13	2400
Advantages	Longest read length Fast Detects 4 mC, 5 mC, 6 mA	Less expensive equipment Fast	Long read size Fast	Potential for high sequence yield, depending upon sequencer model and desired application	Low cost per base	Long individual reads Useful for many applications
Disadvantages	Low yield at high accuracy Equipment can be very expensive	Homo polymer errors	Runs are expensive Homopolymer errors	Equipment can be very expensive	Slower than other methods	More expensive and impractical for larger sequencing projects

Source: http://en.wikipedia.org/wiki/DNA_sequencing.

research scientists in 1985, is based on the enzymatic amplification of a DNA fragment from oligonucleotide primers that flank the target region in duplex DNA. This technique in a way mimics the *in vivo* DNA replication in that the number of DNA molecules generated by the PCR doubles after each cycle. This method repeats a set of three steps: denaturing the DNA, annealing the primers to their complementary sequences, and extending the annealed primers with a DNA polymerase (Figure 2.14). There are a few alternative methods, including the ligase chain reaction (LCR), nucleic-acid-sequence-based amplication (NASBA) and Qb replicase, but these are not discussed. Typically, PCR work proceeds in three steps – denaturation, annealing, and extension, referred to as a *cycle*. The steps may be briefly described as follows.

In denaturation, a double-stranded DNA sample (the template) is heated at nearly 100 °C, whereupon the two strands separate and remain in solution until the temperature is cooled (annealed) in the presence of two primers. In primer annealing the separated strands are cooled in the presence of two short primers (≈ 20 bases each). Next DNA polymerase mediates the 5'-to-3' extension of the primer–template complex (i.e., the pairs of synthetic oligonucleotides that anneal to sites flanking the region to be amplified). The primers then

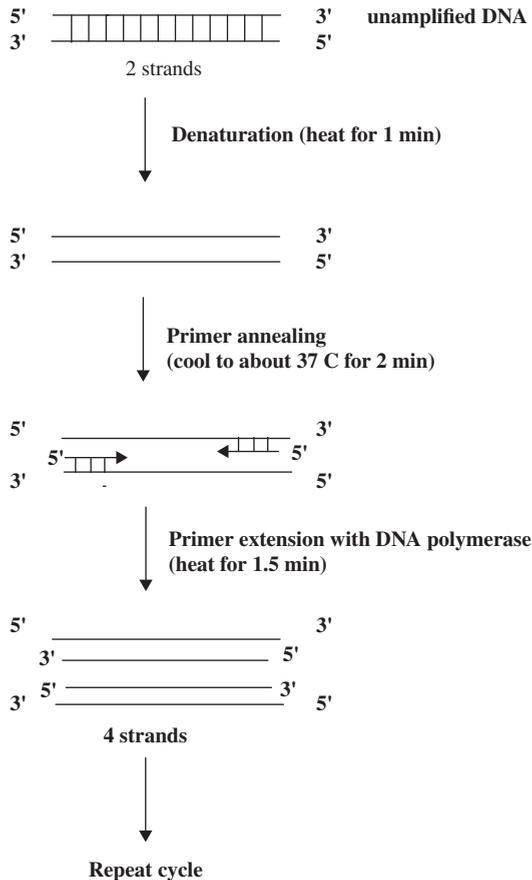


Figure 2.14 Principles of the polymerase chain reaction (PCR).

initiate the synthesis of two new strands, complementary to the two primers in the presence of the DNA polymerase.

Initially, the PCR technique used the Klenow fragment of *E. coli* DNA polymerase I, which has a temperature optimum of 37°C. Since, however, this enzyme is inactivated by the high temperatures required to denature DNA, fresh enzyme had to be added after each cycle, necessitating the tedious procedure of purifying DNA and removing denatured protein from the sample. When the Klenow fragment is replaced by thermostable *Taq* DNA polymerase, which is isolated from the thermophilic bacteria *Thermus aquaticus*, the purification and cleanup step is not required. *Taq* polymerase is stable enough to survive up to 30 cycles, which give about a billionfold amplification. Running more than 30 cycles often leads to accumulation of unspecified product and does not increase the yield of specific amplifications. Thus, the technique does amplify the target DNA faster than the bacteria can multiply themselves. This development has led to the automation of the PCR by a variety of simple temperature-cycling devices.

Pfu DNA polymerase is another enzyme found in the hyperthermophilic archaeon *Pyrococcus furiosus*, where it functions *in vivo* to replicate the organism's DNA. The main difference between *Pfu* and alternative enzymes is *Pfu*'s superior thermostability and "proofreading" properties compared to *Taq* DNA polymerase. Unlike *Taq* DNA polymerase, *Pfu* DNA polymerase possesses 3' to 5' exonuclease proofreading activity, meaning that it works its way along the DNA from the 5' end to the 3' end and corrects nucleotide-misincorporation errors. *Pfu* DNA polymerase-generated PCR fragments will thus have fewer errors than *Taq*-generated PCR inserts. As a result, *Pfu* is more commonly used for molecular cloning of PCR fragments than the historically popular *Taq*. *Pfu* DNA polymerase is hence superior to *Taq* DNA polymerase for techniques that require high-fidelity DNA synthesis, but can also be used in conjunction with *Taq* polymerase to obtain the fidelity of *Pfu* with the speed of *Taq* polymerase activity.

The entire sequence can be repeated by denaturing the preparation and starting again. The specificity of the amplification reaction is increased by allowing the use of higher temperatures for primer annealing and extension. A restriction on the sensitivity of this technique is the replication event error rate of about 2×10^{-4} , which means that an error occurring in each cycle could become significant through amplification. This problem can be eliminated by taking multiple samples. This method is useful not only for amplifying target sequences but also for altering a particular nucleotide sequence. Thus, two current research applications of the PCR are cloning and sequencing. This technique offers a powerful approach to diagnostics, where genetic disease diagnosis has been impossible to carry out using conventional methods, and in forensic analysis, where often only tiny quantities of poor quality materials such as dried blood and semen are available for identifying criminal suspects. Many applications of the PCR in the fields of diagnostics and forensics have been published in connection with the detection of many diseases, as well as bacterial pathogens in water and food.

2.5 Manipulation techniques of DNA

In this section, five essential techniques are described for newcomers.

2.5.1 Isolation and purification of nucleic acids

To separate DNA or RNA from other cellular constituents, basic procedures for manipulating solutions of single- or double-stranded DNA through purification and

concentration steps are useful when proteins or solute molecules need to be removed from aqueous solutions, or when DNA solutions need to be concentrated. As cells contain nucleases that cut nucleic acid polymers into smaller fragments or individual nucleotides, the cell must first be lysed by any method of an osmotic shock, treatment of the cells with detergents such as SDS or treatment of the cells with enzymes such as lysozyme. Yeast cell walls can be lysed by adding lytic enzymes (each company sells own lytic enzyme) or SDS to produce spheroplasts, that can easily be lysed in the presence of the lysing mixture (sorbitol, NaOH, and SDS) and released nucleic acids. The structure of the fungal cell wall is highly complex compared to mammalian cell membranes and bacterial cell walls. Mammalian cell membranes consisting of a lipid bilayer and transmembrane proteins are relatively easy to lyse with proteolytic enzymes such as proteinase K and detergents. In contrast, fungal cell walls consist of thick layers of chitin, (1–3)- β -d-glucan, (1,6) β -glucans, lipids and peptides that have prevented the development of a single universal fungal DNA extraction method. Many fungal DNA isolation methods use a variety of disruption methods to lyse conidia and hyphae using digestion enzymes such as proteinase K, lyticase, zymolyase (Chen et al., 2002), freezing in liquid nitrogen and grinding with mortar and pestle, sonication, and glass bead milling.

The basic protocol using phenol extraction and ethanol (or isopropanol) precipitation is appropriate for purification of DNA from small volumes (<0.4 mL) at concentrations lower than 1 mg/mL. These protocols may also be used for purifying RNA, that are well explained in any book or any company protocol, thus the procedures are not detailed. Cesium chloride centrifugation is an excellent method to remove RNA and proteins in the isolation of DNA. In cesium chloride solutions of appropriate density during centrifugation, the DNA bonds present near the center of the tube, RNA pellets to the bottom and the proteins float near the top. Single-stranded DNA is denser than double-helical DNA. The purification of nucleic acid is based on principles, which depend on differences in (i) molecular size, (ii) secondary structure, and (iii) base composition. Gel filtration on columns of dextran derivatives may be used for the separation of nucleic acid. Gel filtration using polyethyl amide may also be used. This is based on the differential affinity of single-stranded and double-stranded nucleic acids to various materials. Nucleic acid with varying base composition can be separated by using counter current distribution. Variation in base composition also varies the density of nucleic acid.

More recently, each company sells column-based nucleic acid purification kits, which are a solid-phase extraction method to quickly purify nucleic acids. This method relies on the fact that the nucleic acid may bind (adsorption) to the solid phase (silica or other) depending on the pH and the salt content of the buffer. The sample is added to the column and the nucleic acid binds due to the lower pH (relative to the silanol groups on the column) and salt concentration of the binding solution, which may contain buffer, a denaturing agent (such as guanidine hydrochloride), Triton X-100, isopropanol, and a pH indicator.

2.5.2 Agarose gel electrophoresis

Polyacrylamide gel electrophoresis is usually used to separate proteins by charge and or size, but the size of DNA and RNA fragments or separation of nucleic acid molecules are estimated by applying an electric field to move the negatively charged molecules through an agarose matrix using *agarose gel electrophoresis*. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. Gel electrophoresis uses a gel as an anticonvective and sieving medium during electrophoresis and the movement of a charged particle in an electrical field. DNA gel electrophoresis is usually performed for analytical purposes after isolation of crude

DNA from the lysed cells, amplification of DNA via PCR, but may be used as a preparative technique prior to use of other methods such as mass spectrometry, restriction fragment length polymorphism (RFLP), PCR, cloning, DNA sequencing, or Southern blotting for further characterization.

In agarose gel electrophoresis, the sample containing molecules are dispensed into a well in the agarose gel. The gel is placed in an electrophoresis chamber, which is then connected to a power source. When the electric current is applied, the larger molecules move more slowly through the gel while the smaller molecules move faster. The different sized molecules form distinct bands on the gel. Agarose is composed of long unbranched chains of uncharged carbohydrate without cross links resulting in a gel with large pores allowing for the separation of macromolecules and macromolecular complexes.

Several samples can be loaded into adjacent wells in the gel, which will run parallel in individual lanes. Bands in different lanes that end up at the same distance from the top contain molecules that passed through the gel with the same speed, which usually means they are approximately the same size. Molecular size markers containing a mixture of molecules of known sizes are run parallel to the unknown samples to determine their size. Electrophoresis is performed in buffer solutions to reduce the pH changes due to the electric field, which is important because the charge of DNA and RNA depends on pH, but running for too long can exhaust the buffering capacity of the solution. The separation of DNA fragments ranging from 50 base pair to several megabases (millions of bases) can be carried out using specialized apparatus, but for fragments larger than 40 kb, a special apparatus such as pulse-field gel electrophoresis is required. The distance between DNA bands of a given length is determined by the percent agarose in the gel. The disadvantage of higher concentrations is the long run times (sometimes days). Most agarose gels are made with between 0.7% (good separation or resolution of large 5–10 kb DNA fragments) and 2% (good resolution for small 0.2–1 kb fragments) agarose dissolved in electrophoresis buffer. After the electrophoresis is complete, the molecules in the gel can be stained to make them visible using ethidium bromide which, when intercalated into DNA, fluoresce under ultraviolet light. As EtBr is a known mutagen, safer alternatives such as *GelRed* from Biotium, which binds to the minor groove and *SYBR Green I* from Invitrogen is another dsDNA stain. It is more expensive, but 25 times more sensitive, and possibly safer than EtBr. *SYBR Safe* is a variant of SYBR Green that has been shown to have low enough levels of mutagenicity and toxicity to be deemed nonhazardous waste.

After electrophoresis the gel is illuminated with an ultraviolet lamp (usually by placing it on a light box, while using protective gear to limit exposure to ultraviolet radiation). The illuminator apparatus mostly also contains imaging apparatus that takes an image of the gel after illumination with UV radiation. As even short exposure of nucleic acids to UV light causes significant damage to the sample, UV damage to the sample will reduce the efficiency of subsequent manipulation of the sample, such as ligation and cloning. If the DNA is to be used after separation on the agarose gel, it is best to avoid exposure to UV light by using a blue light excitation stain using the SYBR Green or GelGreen stains. The detailed procedures on the DNA separation by agarose gel electrophoresis can be understood by watching the video at the given website address: (<http://www.jove.com/video/3923/agarose-gel-electrophoresis-for-the-separation-of-dna-fragments>).

2.5.3 Blotting and hybridization

A primary limitation of all blot hybridizations is the efficiency of hybridization between the nucleic acids on the membrane, and the labeled nucleic acids in the hybridization solution. The *Western blot* (the protein immunoblot) is a widely accepted analytical technique used

to detect specific proteins in the given sample, which uses gel electrophoresis to separate native proteins by 3D structure or denatured proteins by the length of the polypeptide. The proteins are then transferred to a membrane [typically nitrocellulose or polyvinylidene difluoride (PVDF)], where they are stained with antibodies specific to the target protein. Antibodies either in monoclonal and polyclonal antibodies can be used to detect against tens of thousands of different proteins. Other related techniques known include the immunostaining and enzyme-linked immunosorbent assay (ELISA) to detect proteins in tissues and cells.

The three techniques commonly used in molecular techniques, *Southern blot* (www.dnatube.com/video/1512/Southern-blot), *Northern blot* (www.dnatube.com/video/2999/Northern-Blot), and *Western blot* (www.dnatube.com/video/1511/Western-blot) can be viewed as videos.

DNA sequencing (www.youtube.com/watch?v=3JkL_cIRRnw) and DNA microarray (www.youtube.com/watch?v=VNsthMNjKhM), which were previously described, are also well explained in the video.

2.6 Gene cloning and production of recombinant proteins

To know the principles and techniques involved in manipulation of a prokaryotic enzyme, β -galactosidase and eukaryotic enzyme, rennet (chymosin), we will exercise the cloning and expression of two enzymes in *E. coli* and *Saccharomyces*.

2.6.1 Cloning and expression of bacterial β -galactosidase in *E. coli*

The enzyme β -galactosidase (*lactase*, EC 3.2.1.23) catalyzes the hydrolysis of lactose to glucose and galactose and is industrially important enzyme because it can be used to avoid the problems of lactose intolerance by individuals who are deficient in lactase, eliminate lactose crystallization in sweetened, condensed and frozen dairy products such as ice cream and condensed milk and solve problems associated with whey utilization and disposal. New applications for β -galactosidase, such as in the production of biologically active *galacto-oligosaccharides* (*prebiotics*) are already commercialized. Commercial β -galactosidases are produced from yeasts such as *K. lactis* and *Kluyveromyces marxianus* (formerly known as *Kluyveromyces fragilis*) and molds such as *A. niger* and *A. oryzae* for the treatment of milk to produce low lactose milk, sweet whey and neutral pH dairy products, but *Aspergillus* β -galactosidase is used mainly for acidic whey.

Many general purpose vectors such as *pUC19* usually include a system for detecting the presence of a cloned DNA fragment, based on the loss of an easily scored phenotype. The most widely used is the gene coding for *E. coli* β -galactosidase, whose integrity can easily be detected by the ability of the enzyme it encodes to hydrolyze the soluble, colorless substrate *X-gal* (*5-bromo-4-chloro-3-indolyl-beta-d-galactoside*) into an insoluble, blue product (*5,5'*-dibromo-4,4'-dichloro indigo). Cloning a fragment of DNA within the vector-based gene encoding the β -galactosidase prevents the production of an active enzyme. If *X-gal* is included in the selective agar plates, transformant colonies are generally blue in the case of a vector with no inserted DNA and white in the case of a vector containing a fragment of cloned DNA (Figure 2.15).

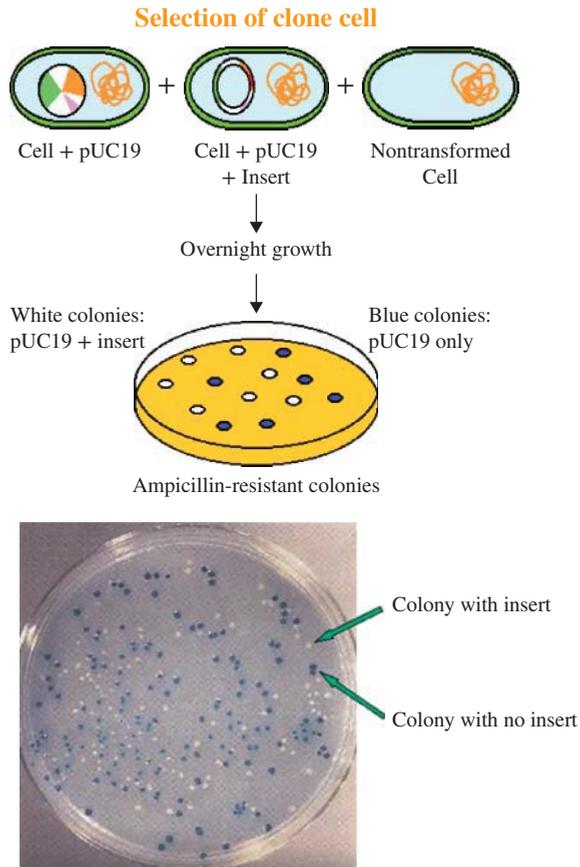


Figure 2.15 Selection of positive β -galactosidase clone which shows white colonies on agar plates containing ampicillin and X-gal. Animation on “Construction of a plasmid vector and cloning of β -galactosidase” and quiz (http://highered.mcgraw-hill.com/sites/0072556781/student_view0/chapter14/animation_quiz_2.html) can be viewed. (See insert for color representation of this figure.)

A typical *E. coli* cloning vector, pUC19 (2,686-bp) has several features:

1. High copy number in *E. coli*, with nearly a hundred copies per cell, provides a good yield of cloned DNA.
2. Its selectable marker is *amp*^R.
3. It has a cluster of unique restriction sites, called the polylinker (multiple cloning site).
4. The polylinker is part of the *lacZ* (β -galactosidase) gene. The pUC19 plasmid will complement a *lacZ*⁻ *E. coli*, allowing it to become *lacZ*⁺. When DNA is cloned into the polylinker, *lacZ* is disrupted, preventing complementation from occurring.
5. X-gal, a chromogenic analog of lactose, turns blue when β -galactosidase is present, and remains white in its absence, so blue-white screening can indicate which colonies contain recombinant plasmids.

DNA can be inserted into a cloning vector by restriction digestion and then ligation.

1. Cut pUC19 (the vector plasmid) with a restriction enzyme that has a unique site in the polylinker.
2. Cut the DNA to be cloned (insert DNA) with the same enzyme.
3. Mix insert DNA with pUC19 DNA and allow random joining of fragments to occur.
4. Resulting plasmids are transformed into *E. coli* either through chemical treatment of the cells or by electroporation. The cells are grown on media plates containing ampicillin and X-gal (Figure 2.15).
5. Ampicillin-resistant colonies result from pUC19 sequences. Blue colonies contain only the vector with its ends rejoined, while white colonies often contain pUC19 with its *lacZ* gene inactivated by insert DNA.
6. If the 5'-phosphates of vector DNA are removed by alkaline phosphatase, DNA ligase will not rejoin their ends, and fewer blue colonies will result.
7. Many plasmid cloning vectors are available, with features including different arrays of unique restriction sites in the polylinker, and phage promoters (e.g., T7, T3, SP6) that can be used to control transcription of the cloned DNA.
8. Plasmid-cloning vectors are available for many prokaryotic and eukaryotic organisms. In some cases, the plasmids are unable to replicate, but are maintained because they integrate into the genome.
9. Size of the insert DNA is limited in plasmid cloning vectors, and plasmids carrying more than 5–10 kb are often unstable.

2.6.2 Cloning, expression, and production of bovine chymosin (rennet) in yeast *K. lactis*

Rennet (chymosin; EC 3.4.23.4) is a complex of enzymes produced in any mammalian stomach and is often used in the production of cheese. Rennet contains many enzymes, including a proteolytic enzyme (protease) that coagulates the milk, causing it to separate into solids (curds) and liquid (whey). They are also very important in the stomach of young mammals as they digest their mothers' milk. The active enzyme in rennet is called *chymosin* or *rennin* (EC 3.4.23.4) but there are also other important enzymes in it, for example, pepsin and lipase. There are non-animal sources for rennet that are suitable for consumption by vegetarians. *Rennet (chymosin)* enzyme secreted by cells lining the stomach in mammals is responsible for clotting milk. It acts on a soluble milk protein (*kappa-casein*), which it converts to the insoluble form casein. This ensures that milk remains in the stomach long enough to be acted on by protein-digesting enzymes. Because of the above imperfections and expensive production costs of microbial and animal rennet, many producers sought further replacements of rennet by *fermentation produced chymosin (FPC)*. With the development of genetic engineering, it became possible to insert animal genes into certain bacteria, fungi or yeasts to make them produce chymosin during fermentation. The genetically modified yeasts or fungi are killed after fermentation and chymosin isolated from the fermentation broth, so that the FPC used by cheese producers does not contain any genetically modified component or ingredient. FPC contains the identical chymosin as the animal source, but produced in a more efficient way. FPC products have been on the market since 1990 and have been considered in the past 20 years the ideal milk-clotting enzyme. By 2008, approximately 80–90% of commercially made cheeses in the United States and Britain were made using FPC. The recombinant *E. coli* chymosin was first successfully produced, but it is no

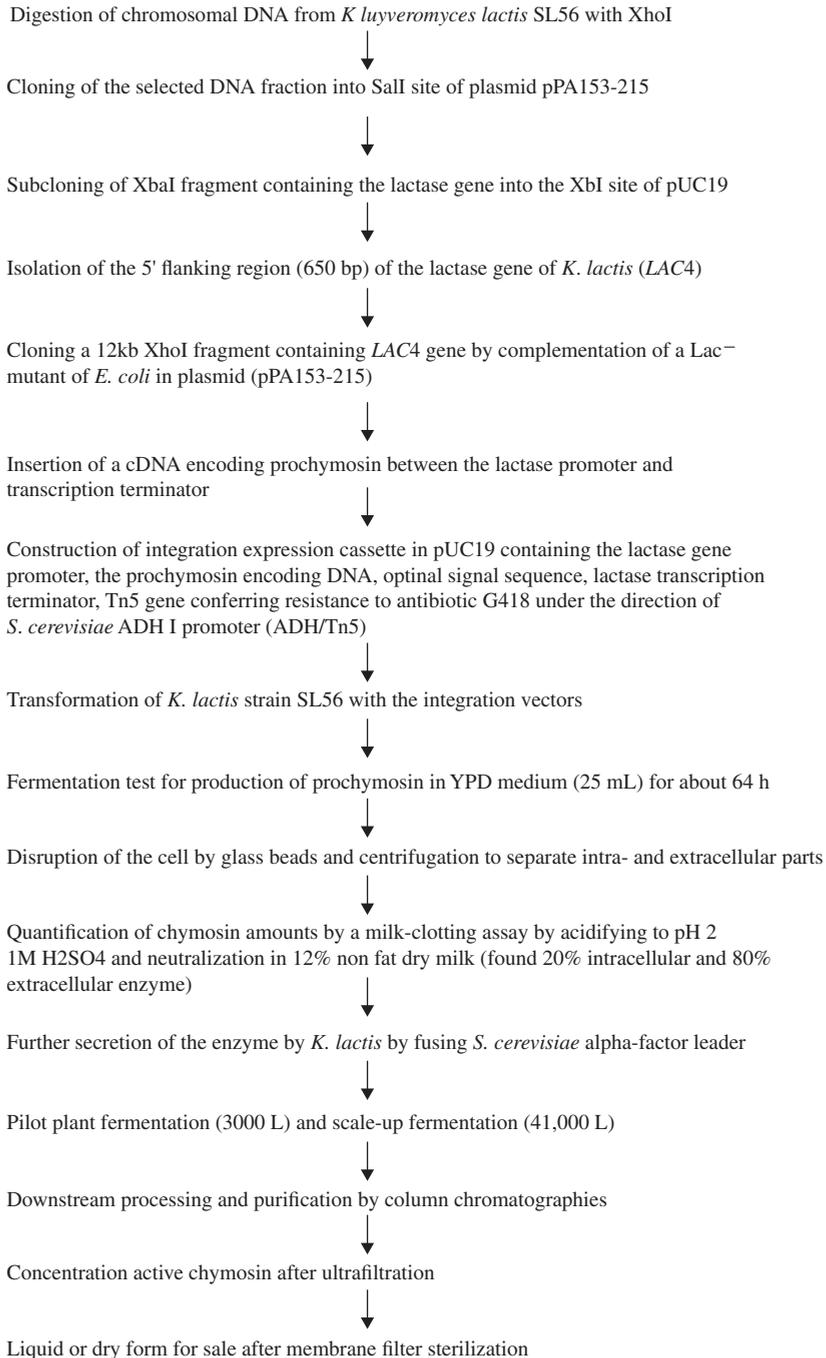


Figure 2.16 Production of recombinant chymosin (rennet) by *Kluyveromyces lactis*.

longer commercially available. The problem with *E. coli* is that recombinant proteins are frequently synthesized as intracellular *IBs*, increasing process costs considerably as shown in Figure 1.20. Another issue with *E. coli* is that it is not generally recognized as safe for human consumption. Today, the most widely used FPC is produced either by the fungus *A. niger* and commercialized under the trademark CHY-MAX[®] by the Danish company Chr. Hansen, or by *K. lactis* and commercialized under the trademark MAXIREN[®] by the Dutch company DSM. The chymosin gene was inserted into the *K. lactis* chromosome and the yeast is grown by fed-batch fermentation. After fermentation, the yeast is killed by addition of benzoic acid and the chymosin is isolated by filtration. Cheeses produced with FPC can be certified Kosher and Halal, and are suitable for vegetarians if there was no animal-based alimentation used during the chymosin production in the fermenter.

The Pfizer Corporation developed CHY-MAX in the late 1980s and Chr. Hansen began marketing the enzyme in 1989. They added CHY-MAX-M developed using a camel gene. It is considered a second generation FPC, as coagulating milk five times faster than first generation FPCs and 25 times faster than microbial rennets developed from *Rhizomucor miehei*. Although the production flow diagram of recombinant chymosin process was already described briefly in Figure 1.20, commercial production process of chymosin (MAXIREN[®], Gist-Brocades) using *K. lactis* is much more complex and is shown in Figure 2.16. The detailed process can be found in the literature (van den Berg et al., 1990).

Summary

In order to understand genetic engineering, one must remember how a fragment of DNA, representing a genetic code is involved in proteins synthesis; that is, mRNA transcription from this DNA fragment followed by translation involving rRNA and tRNA that carries the amino acid. It is now relatively straightforward to isolate and clone the gene for any well-characterized protein by means of genetic engineering or recombinant DNA technology. Terminology was given, along with a general outline of gene cloning techniques and explanations of gene expression and plasmid stability. General cloning techniques along with advantages and disadvantages as the protein route (shotgun cloning), the cDNA route, and the synthetic DNA route were discussed. Important factors to maximize gene expression are found to be the number of copies of the plasmid vector per unit cell (copy number) and the strength of promoter, the sequence of the ribosomal binding site (RBS) and flanking DNA, codon choice in the cloned gene, genetic stability of the recombinant, and proteolysis. Detailed cloning techniques, maximizing gene expression techniques, and advantages and disadvantages of different expression hosts were discussed.

Besides the *Maxam–Gilbert* and the *Sanger* methods, next-generation sequencing methods were compared. The polymerase chain reaction technique, which is based on the enzymatic amplification of a DNA fragment from oligonucleotide primers, is useful not only for amplifying target sequences but also for altering a particular nucleotide sequence. Two current research applications of the PCR are cloning and sequencing, but many applications in the fields of diagnostics, forensics, and the detection of disease and bacterial pathogens are forthcoming.

To know the principles and techniques involved in manipulation of a prokaryotic enzyme, β -galactosidase and eukaryotic enzyme, rennet (chymosin), the cloning and expression methods of two enzymes in *E. coli* and *Saccharomyces* were exercised.

Bibliography

- Bartlett, J. M. S. and D. Stirling, Eds. 2003. PCR Protocols: Methods in Molecular Biology, Vol. 226, 2 ed., Humana Press, New York.
- Chen, S. C., C. L. Halliday, and W. Meyer. 2002. A review of nucleic acid-based diagnostic tests for systemic mycoses with an emphasis on polymerase chain reaction-based assays. *Med. Mycol.* 40: 333–357.
- Cregg, J. M., J. L. Cereghino, J. Shi, and R. Higgins. 2000. Recombinant protein expression in *Pichia pastoris*. *Mol. Biotechnol.* 16: 23–52.
- Defrancesco, L. 2012. Life Technologies promises \$1,000 genome. *Nature Biotechnol.* 30: 126.
- Dolled-Filhart, M. P., M. Lee Jr., C. W. Ou-yang, R. R. Haraksingh, and J. C. H. Lin. 2013. Computational and bioinformatics frameworks for next-generation whole exome and genome sequencing. *Scientific World Journal* 2013: 1–10. <http://dx.doi.org/10.1155/2013/730210>
- Ferrer-Miralles, N., J. Domingo-Espín, J. L. Corchero, E. Vázquez, and A. Villaverde. 2009. Microbial factories for recombinant pharmaceuticals. *Microb. Cell Fact.* 8: 17–25.
- Gagarinova, A. and A. Emili. 2012. Review article: Genome-scale genetic manipulation methods for exploring bacterial molecular biology. *Mol. BioSyst.* 8: 1626–1638.
- Grange, J. M., A. Fox, and N. L. Morgan, Eds. 2010. Genetic Manipulation: Techniques and Applications, p. 401, John Wiley & Sons Ltd., New York.
- Krijger, J. J., J. Baumann, M. Wagner, K. Schulze, C. Reinsch, T. Klose, O. F. Onuma, C. Simon, S. E. Behrens, and K. D. Breunig. 2012. A novel, lactase-based selection and strain improvement strategy for recombinant protein expression in *Kluyveromyces lactis*. *Microb. Cell Fact.* 11: 112–124.
- Krainer, F. W., C. Dietzsch, T. Hajek, C. Herwig, O. Spadiut, and A. Glieder. 2012. Recombinant protein expression in *Pichia pastoris* strains with an engineered methanol utilization pathway. *Microb. Cell Fact.* 11: 22–36.
- Langer, E. 2011. New plant expression systems drive vaccine innovation and opportunity. *BioProcess Intern.* 9: 16–21.
- Lee, P. Y., J. Costumbrado, C. Y. Hsu, and Y. H. Kim. 2012. Agarose gel electrophoresis for the separation of DNA fragments. *J. Vis. Exp.* 62:e3923. doi: 10.3791/3923.
- Lubertozzi, D. and J. D. Keasling. 2009. Developing *Aspergillus* as a host for heterologous expression. *Biotechnol. Adv.* 27: 53–75.
- Menzella, H. G. 2011. Comparison of two codon optimization strategies to enhance recombinant protein production in *Escherichia coli*. *Microb. Cell Fact.* 10: 15–23.
- Sambrook, J., and D. W. Russel. 2001. Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Simon, R. M. et al. 2012. Design and Analysis of DNA Microarray Investigations. Statistics for Biology and Health, p. 209, Springer Publications, New York.
- van den Berg, J. A., K. J. van der Laken, A. J. J. van Ooyen, T. C. H. M. Renniers, K. Rietveld, A. Shaap, A. J. Brake, R. J. Bishop, K. Schultz, D. Moyer, M. Richman, and J. R. Shuster. 1990. *Kluyveromyces* as a host for heterologous gene expression: Expression and secretion of prochymosin. *Biotechnology* 8: 135–139.

- Yoon, J., T. Aishan, J. I. Maruyama, and K. Kitamoto. 2010. Enhanced production and secretion of heterologous proteins by the filamentous fungus *Aspergillus oryzae* via disruption of vacuolar protein sorting receptor gene *Aovps10*. *Appl. Environ. Microbiol.* 76: 5718–5727.
- Wang, Y., S. Zhao, L. Bai, J. Fan, and E. Liu. 2013. Review Article. Expression systems and species used for transgenic animal bioreactors. *BioMed Res. Intern.* <http://dx.doi.org/10.1155/2013/580463>.
- Ward, O. P. 2012. Production of recombinant proteins by filamentous fungi. *Biotechnol. Adv.* 30: 1119–1139.

Part I

Questions and Answers

1. What are the three broad categories of food biotechnology?

Answer: Animal foods, plant foods, microbial foods. See Figure 1.1.

2. What are some important achievements in (a) transgenic animals/animal cell cultures, (b) plants/plant cell cultures, and (c) microorganisms?

Answer:

- (a) Transgenic animals: Cloned pet cats, ornamental fish, cloned horses and at least one rodeo bull. Two pharmaceutical products from the milk of GM animals. The first drug manufactured from the milk of a genetically modified goat was ATryn (brand name of the anticoagulant antithrombin) by GTC Biotherapeutics in 2006. It is produced from A goat that produces spider's web protein, stronger and more flexible than steel (BioSteel) was successfully produced by a Quebec-based Canadian company, Nixia. Faster-growing GM salmon developed by a Canadian company is also awaiting regulatory approval. The genetically modified pig (trade named "Enviro-pig") designed to reduce phosphorus pollution of water and farmers feed costs. Animal cell cultures include enzymes, synthetic hormones, immunobiologicals (monoclonal antibodies, interleukins, lymphokines) and anticancer agents.
- (b) Transgenic plants: pharmacologically active proteins, including mammalian antibodies, blood product substitutes, vaccines, hormones, cytokines, a variety of other therapeutic agents, and enzymes. Some of the metabolites produced by plant cell culture are flavors, natural sweeteners, industrial feedstocks, perfumes, and commercial insecticides. Another useful metabolite produced by plants includes shikokin, which is a chemical used as both a dye and a pharmaceutical.
- (c) Transgenic microorganisms: All the biopharmaceutical products are mostly manufactured commercially through various fermentation routes on using genetically engineered microorganisms such as *E. coli*, yeast, and fungi. Some commercial products are human insulin, streptokinase, erythropoietin, hepatitis B vaccine, human growth hormone, interleukin, granulocyte-colony stimulating factor (GCSF), granulocyte-macrophage colony stimulating factor (GMCSF), α -interferon, and γ -interferon, and many recombinant enzymes.

3. What are the two big groups into which cells are classified?

Answer: Cells can be classified as eukaryotic or prokaryotic. While prokaryotic cells are those without a delimited nucleus, eukaryotic cells are those with nucleus delimited by membrane.

4. What are the main respective constituents of cell walls in bacteria, protists, fungi, and plants?

Answer: In bacteria the cell wall is made of peptidoglycans; among protists algae have cell walls made of cellulose; in fungi, the cell wall is made of chitin; in plants, the cell wall is made of cellulose too.

5. Concerning the presence of the nucleus, what is the difference between animal and bacterial cells?

Answer: Animal cells have an interior membrane that delimits a cell nucleus and thus they are eukaryotic cells; in these cells the genetic material is located within the nucleus. Bacterial cells do not have organized cellular nuclei and so they are prokaryotic cells and their genetic material is found dispersed in the cytosol.

6. List the similarities and differences among bacterial, animal, and plant cells.

Answer: See Table 1.1.

7. List some bacteria of biotechnologically important among 19 bacterial groups.

Answer: See Table 1.3.

8. What are extremophiles? List their uses.

Answer: (a) An extremophile is an organism that thrives in physically or geochemically extreme conditions such as hydrothermal vents that are detrimental to most life on Earth. Many extremophiles are unicellular organisms known as archaea and there are three major types of archaeobacteria: methanogens, halophiles and thermoacidophiles. These microorganisms are considered the oldest life forms on earth that thrive at unusual conditions, such as environments without oxygen. (b) The unique enzymes used by these organisms, called *extremozymes*, enable these organisms to function in such forbidding environments. These creatures hold great promise for genetically based medications and industrial chemicals and processes.

9. What are some industrial processes that use bacteria?

Answer: Bacteria are used by industry in various ways. There are vaccines made of attenuated pathogenic bacteria or of antigens present in bacteria. One of the most ancient uses of bacteria is the fermentation of milk to produce yogurt, cheese, and so on. Some methods of antibiotic production involve bacteria. The recombinant DNA technology (genetic engineering) allows the industrial production and commercialization of human proteins, such as insulin for diabetics by mutant bacteria.

10. List three different cultures uses as an industrial process. Why is continuous culture not widely used as an industrial process?

Answer: Batch, fed-batch (semi-continuous), and continuous (chemostat); Continuous culture is not using, mainly because of the problems of chance contamination, and the danger of strain degeneration by spontaneous mutation, which produces a new strain of low product formation.

11. What is the difference between growth rate and specific growth rate of bacteria?

Answer: The growth rate of a microbial population is a measure of the increase in biomass over time and it is determined from the exponential phase. Specific growth rate is defined as the increase in cell mass per unit time, for example, grams cells (g) per gram cells (g) per hour. The specific growth rate is commonly given by the symbol, μ , and the most common units are in reciprocal hours (h^{-1}); however, it can also be expressed in reciprocal seconds (s^{-1}) or minutes (min^{-1}) or any other units of time. Specific growth rate constant is a way of measuring how fast the cells are dividing in a culture. It is defined on the basis of doubling rate.

12. What is the difference between solid-state fermentation and submerged fermentation? What are advantages and disadvantages of SSF?

Answer: In contrast to submerged (liquid state) fermentation (SmF) in a sterile large reactor containing liquid suspension culture, solid-state fermentation (SSF) is the cultivation of normally fungi on solid, normally organic materials such as cereal grains, wheat bran, legumes, and lignocellulosics under controlled conditions in the absence of free water. Examples of products of SSF include industrial enzymes, fuels and nutrient-enriched animal feeds. Traditionally, SSF has been used in Oriental foods to produce *Koji* using rice in order to manufacture alcoholic beverages such as *Sake* or *Koji* using soybean to produce soy sauce or *temph*, *sufu*, and other similar products. SSF systems offers numerous advantages over submerged fermentation system, including high volumetric productivity, relatively higher concentration of the products, less effluent generation, requirement for simple fermentation equipments. However, SSF has several problems encountered in the control of different parameters such as pH, temperature, aeration and oxygen transfer, moisture and agitation. SSF lacks the robust control mechanisms that are usually associated with SmF.

13. What is the difference between (a) auxotrophic mutation, (b) genetic recombination, and (c) recombinant DNA?

Answer: (a) Auxotrophic mutant strains are blocked at some point in a pathway vital for growth, and unless the specific nutrients or products of the pathway are supplied in the media, the auxotrophs do not survive. Auxotrophs are primarily isolated by plating the mutagenized population on a complete medium that has all the nutrients needed for growth. The clones are then replica-plated to minimal medium lacking some specific nutrients, and auxotrophs that fail to grow on minimal media are identified. (b) In addition to the manipulation of microorganisms by mutation, the techniques of genetic recombination (mechanism of gene alteration and strain modification) can be employed to get new strains containing novel combinations of mutations and superior microbial strains. Genetic recombination methods include those techniques that combine two DNA molecules having similar sequences (homologs). Through the special event of crossing-over, they are reunited to give a new series of nucleotide sequences along the DNA that are stable, expressible genetic traits which is called genetic recombination. This definition includes the techniques of protoplast fusion, transformation, and conjugation. (c) Most recently, recombinant DNA technology has been employed to assemble new combinations of DNA *in vitro*, which are then reinserted into the genome of the microbe, creating new varieties of microbe not attainable through traditional mutation and rationalized selection approaches. This approach overlaps the other methods to some extent in that it involves transformation

of microbes with laboratory-engineered specific recombinant molecules via plasmid or phage vectors.

14. How could auxotrophic mutant for lysine production by *Corynebacterium* have been accomplished in the past? How metabolic pathway engineering could recently design to overproduce lysine?

Answer: (a) A mutant of *C. glutamicum* lacks *homoserine dehydrogenase*, so that the inhibition of end product, threonine on lysine synthesis by *asparto (aspartate) kinase* does not occur. As the auxotrophic mutant does not synthesize threonine or methionine, these amino acids must be added in the growth medium. By auxotrophic mutant, L-lysine is overproduced in the range of 30–35g/L but using fed-batch fermentation of *C. glutamicum* mutant strains, impressive L-lysine-HCl concentrations of up to 170 g/L have been reported. Metabolic engineering of *C. glutamicum* has significantly increased L-lysine production from 35–50 to 80 g/L. Metabolic engineering of *E. coli* has also overproduced up to 50 g/L.

15. What is the difference between metabolic engineering, synthetic biology, and systems biology?

Answer: See Figure 1.8.

16. How was synthetic biology successful in producing the antimalarial drug *artemisinin* by microorganism?

Answer: Antimalarial drug, Artemisinin, a sesquiter lactone endoperoxide extracted from *Artemisia annua* L (a sweet wormwood) is highly effective against multi drug-resistant malaria parasite *Plasmodium falciparum*. Modified *Saccharomyces cerevisiae* could produce high titers (up to 100 mg/L) of artemisinic acid using an engineered mevalonate pathway, amorphaadiene synthase, and a novel cytochrome P450 monooxygenase (CYP71AV1) from *A. annua* that performs a three-step oxidation of amorpha-4,11-diene to precursor, artemisinic acid.

17. What types of tools are required for the studies of metabolic engineering?

Answer: (a) molecular biological tools such as genomic, proteomics, transcriptomics, interactomics, and bioinformatics, and (b) metabolic pathway analysis tools such as identification of the metabolic network structure (or pathway topology), and quantification of the fluxes through the branches of the metabolic network, and identification of the control structures within the metabolic network.

18. What is the scale-up process? Why cannot scale up be done directly from a laboratory to a commercial scale? What are the methods normally used in scaling up?

Answer: (a) Scale up is simply the conversion of a small-scale process developed at a laboratory to an industrial scale in either the volume or the linear dimensions of a fermentor. (b) However, it is not possible to apply fermentation conditions of laboratory directly on a commercial scale. This is because success in scale up is evaluated on the basis of maximal yield in terms of minimal operating cost and time; (c) The most commonly used method for scale up is a constant oxygen transfer rate, value $K_L a$ is because $K_L a$ relates well with process results such as yield and titer of the products. However, the $K_L a$ value cannot be used in highly viscous non-Newtonian fermentation solutions, which have been found in many antibiotic fermentations, or under conditions of high speed stirring. Another simple method is based on the power consumed per unit volume (P/V) in aerated bioreactors. The desired prerequisite for using P/V for scale up is a geometric similarity that is rarely attained. Generally, a 1:10 ratio is used in scaling fermentation vessels.

19. What is the difference between upstream and downstream in fermentation?

Answer: Upstream involves all the steps related with media sterilization, inoculum development. Downstream involves all work done from separation of biomass, disruption cells, purification, drying, and final product preparation. There is an increasing use of non-denaturing methods such as various chromatography and electrophoresis techniques for biological materials.

20. Name the types of bioreactors.

Answer: 1. Stirred tank fermentor, 2. Air lift fermentor, 3. Fluidized bed- bioreactor, 4. Packed bed bioreactor, and 5. Bubble column fermentor.

21. What are the advantages of immobilized enzymes and cells?

Answer: See Table 1.10.

22. What is meant by Newtonian and non-Newtonian fluids?

Answer: The fluids that obey the Newton's law of viscosity are called Newtonians fluids and those which do not obey are called non-Newtonian fluids.

23. What is the difference between heat transfer and mass transfer?

Answer: Heat transfer deals with the movement of heat and temperature gradients. The three types of heat transfer are conduction, convection, and radiation. Mass transfer deals with concentrations of a particular substance. Types of mass transfer include diffusion and convection.

24. What is the most limiting factor in mass transfer of aerobic fermentation? What are the relationship between temperature and pressure in oxygen solubility?

Answer: (a) The maximum value of the concentration gradient is limited due to the low solubility of oxygen (0.01 g/L: see Table 1.17). Therefore, the maximum mass transfer rate from the gas to the liquid in the bioreactor can be estimated by oxygen transfer rate $OTR = K_L a * (C_{in} - C_{out})$, where C is the concentration of oxygen in the liquid coming in and out. (b) Temperature will decrease oxygen solubility by Henry's law, and pressure will increase oxygen solubility.

25. What types of data are required from laboratory and pilot experiments for effective scale up into production?

Answer: See Table 1.10.

26. What is the supervisory control and data acquisition (SCADA) control system?

Answer: SCADA is a kind of software application program used for process control and gather real-time data from remote locations for exercising this control on equipments and conditions. The SCADA System consists of hardware and software components. The hardware collects and feeds data into a computer with SCADA software installed. The data is then processed by the computer before presenting it in a timely manner. The function of SCADA is recording and logging all events in a file that is stored in a hard disk or sending them to a printer. If conditions become hazardous, SCADA sounds warning alarm.

27. Why scale down of fermentation is necessary? What problems of scale down?

Answer: (a) Fermentation processes often involve several scales of operation, encompassing inoculum development, seed expansion, and production fermentation. The differences in volumes between the steps in a single fermentation process can be 10–100× for the pilot scale, and 1000–100,000× for the production scale. This may

cause the fermentation processes to be challenging to scale down and the specific process parameters, vessel geometries, and operational control strategies must be evaluated for each step. Some general guidelines to consider in developing a representative scale-down model follow. Scale it down to a laboratory scale bioreactor is often necessary to obtain more consistent process performance during the scale up. Creation and qualification of scale-down models are essential for performing several critical activities that support process validation and commercial manufacturing. (b) A key parameter that frequently limits a scale-down aerobic fermentation model is the volumetric oxygen mass transfer coefficient (kLa), which is proportional to the rate of oxygen transfer from rising gas bubbles to the cells. This approach can be coupled with a small-scale experimental design to more accurately predict and optimize larger-scale fermentation conditions prior to the actual process transfer (growth and production temperature profile, feed rate, air/ O_2 control scheme, induction cell density, etc.).

28. Do you think the universal scale-up criterion has been formulated so far? How are industrial applications currently done?

Answer: No universal scale-up process has been formulated; individual approaches for different bioprocesses have been practiced.

29. What are the studies that can be useful to understand the complex cellular response on large-scale reactor conditions?

Answer: Systems biology (Read textbook).

30. What is the purpose of molecular thermodynamics? In which biotechnology fields can molecular thermodynamics be useful?

Answer: It is to study the microscopic and molecular origin of the mechanisms on which it is based on and it is to develop a basic knowledge in statistical thermodynamics to understand the forces that drive molecules and be able to predict their combined behavior in physical, chemical and biological systems; applications can be many (See Table 1.27).

31. Describe the working principle of ion-exchange chromatography, hydrophobic interaction chromatography and affinity chromatography used in large-scale downstream processing.

Answer: See Table 1.23.

32. What controls protein folding?

Answer: The linear arrangement of the constituent amino acid subunits controls protein folding. All 20 amino acids have the same base, but all have different R groups that have different classes of bonding. Some R groups are hydrophobic, some are hydrophilic, some are acidic, some are thiols, and some are basic. So, all different types of folding of the tertiary structure of polypeptides are possible and due to the combinatorial arrangement of the twenty amino acids hundreds of thousands of different protein foldings are possible.

33. Explain the role of chaperone proteins in protein folding. Name some known chaperons.

Answer:

- (a) Chaperones are the class of protein molecules that assists in proper folding of proteins. They are also called heat-shock proteins because they protect the

molecule (mRNA) from heat which would degrade the molecule (mRNA) and ruin the process (such as transcription).

(b) For types of chaperons, see Table 1.26.

34. How can a disease that is caused by a prion protein instead of a virus or bacterium be contagious?

Answer: Incorrectly folded prion proteins must be able to deform their correctly folded analogues and to change their spatial structure. They transfer their own incorrect shape to the healthy proteins. When incorrectly folded, the protein has many regions containing beta sheets, structures and has a tendency to self-assemble into larger aggregates. These amyloids cannot be broken down and thus form deposits in the brain's tissue. Read more at <http://phys.org/news/2013-08-deadly-molecular-mechanism-prion-protein.html#jCp>.

35. What is the difference between genomics, proteomics, and metabolomics?

Answer: (a) Genomics is the study of the genomes of organisms, such as the study of all the genes of and Genomics has use in studying the human genome, bacteriophages and cyanobacteria. (b) Proteomics is the large-scale study of proteins, particularly their structures and functions; the study of all the proteins made by the organism and the conditions under which the organism makes them (growth phase, always, shock, etc). Proteomics can create potential new drugs for the treatment of diseases, now understood by Genomics. (c) Metabolomics is the study of small molecules (metabolites) that are left behind as a result of cellular processes.

36. What is bioinformatics?

Answer: Bioinformatics is the application of computer technology to the management of biological information to understand and organize the information associated with these molecules, on a large-scale. Computers are used to gather, store, analyze, and integrate biological and genetic information which can then be applied to biotechnology fields.

37. What is metagenomics? List its uses?

Answer: (a) Metagenomics is the genomic analysis of unculturable microorganisms by direct extraction and cloning of DNA from an assemblage of microorganisms to discover new genes and make functional predictions. Pyrosequencing systems have become the standard for ribosomal RNA identification (i.e., 16S, 18S, etc.) and whole genome surveys having enabled an unprecedented view of microbial diversity in environments such as the human gut and mouth, soil, coral reefs, deep sea thermal vents, and drinking water. (b) Metagenomics has broad implications for human health and disease (e.g., human microbiome, animal production, and environmental health and has opened up a creative wealth of data, tools, technologies and applications that allow us to access the majority of organisms that we still cannot access in pure culture (an estimated 99% of microbial life). Novel genes and gene products discovered through metagenomics include the first bacteriorhodopsin of bacterial origin; novel small molecules with antimicrobial activity; and new members of families of known proteins, such as an $\text{Na}^+(\text{Li}^+)/\text{H}^+$ antiporter, RecA, DNA polymerase, and antibiotic resistance determinants.

38. What is pyrosequencing?

Answer: Pyrosequencing is a method of DNA sequencing (determining the order of nucleotides in DNA) based on the "sequencing-by-synthesis" principle. It differs

from Sanger sequencing in that it relies on the detection of pyrophosphate release on nucleotide incorporation, rather than chain termination with dideoxynucleotides. DNA pyrosequencing has been successfully applied in a variety of applications including genotyping, single nucleotide polymorphism (SNP) detection, and microorganism identification.

39. What is DNA microarray technology? List its uses.

Answer: (a) Microarray is a multiplex technology used in molecular biology and in medicine. It consists of any arrayed series of thousands of microscopic spots of DNA. (b) It is useful for gene expression profiling, single nucleotide polymorphism assays and array-based comparative genomic hybridization.

40. What is biosensor? What are its uses?

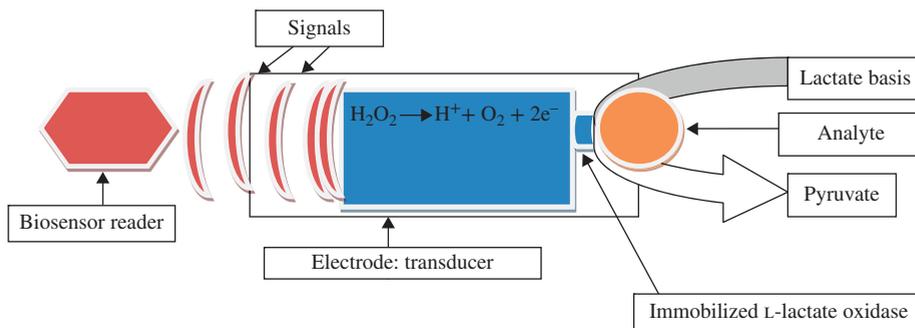
Answer: (a) Biosensor is an analytical device in which a biologically derived sensing element is in intimate contact with a physiochemical transducer to give an electrical signal (See Figure 1.27). Three basic components are biological element, transducer and electronic component. (i) The sensitive biological element (e.g., tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, and nucleic acids) immobilized on the surface of the silicas, quartz, metals, carbons, semiconductors and polymers. (ii) The detector element (optical, piezoelectric electrochemical, thermometric or magnetic.) and (iii) biosensor reader device with the associated electronics or signal processors that are primarily responsible for the display of the results. Transducers and electronics can be combined. (b) The most widespread example of a commercial biosensor is the blood glucose biosensor, which uses an enzyme to break blood glucose down and other applications as shown in Table 1.29.

41. Dairy industries are searching for a system for automated analysis of lactic acid in the manufacturing of fermented milk products. From the biosensor and nanobiotechnology knowledge, consider possible enzyme-coenzyme reaction schemes for continuous monitoring of lactic acid. You are asked to (a) design a biosensor for lactic acid by drawing a figure and (b) discuss the components required for your design.

Answer:

- (a) The lactic acid can be detected using a biosensor in the form of screen printed electrodes (biochips) with immobilized L-lactate oxidase (LOD). L-Lactate is oxidized to pyruvate according to the reaction: H_2O_2 produced in reaction when is oxidized produces current which is proportional to L-lactate concentration on the electrode surface. The concentration of L-lactate in the sample is calculated by comparison of current produced with standard solution of known concentration. It can also be detected by using the screen printed electrodes with L- or D-lactate dehydrogenase. The concentration of NADH is propositional to the concentration of L- or D-lactic acid and it calculated. In this case, a spectrometric biosensor can be used. In designing this biosensor, one must immobilize the L-lactate dehydrogenase on the electrode, that connected it to amperometric transducer. The sample containing lactic acid be added to the enzyme immobilized electrode, that will oxidize L-lactate into pyruvate. The oxidation of H_2O_2 will produce the current which will be the measurement of the concentration L-lactate in the sample. The current which produced will be detected by amperometric transducer. The following is the figure which shows how my biosensor can be designed.

Amperometric biosensor for detection of lactic acid.



(b) Discuss the components required for your design: The above biosensor is mainly made of biological component, transducer and electronic component: (i) The biological element in this biosensor is an enzyme called lactate oxidase, that immobilized on the electrode. This will recognize and bind the lactic acid and oxidize it into pyruvate and H_2O_2 will be produced according to the reaction. H_2O_2 produced in reaction will be oxidized and produces current that is proportional to L-lactate concentration on the electrode surface. The concentration of L-lactate in the sample will be calculated by comparison of current produced with standard solution of known concentration. (ii) The transducer or the detector element will transforms the signal resulting from the interaction of the analyte (lactate) with the biological element (lactate oxidase) into electrical signal (current) that will be more easily measured and quantified; biosensor reader device with the associated electronics or signal processors are primarily responsible for the display of the results in a user-friendly way.

42. What is electronic nose sensing? What are its uses?

Answer: (a) Arrays of many different detector molecules have been applied in the so-called electronic nose devices, where the pattern of response from the detectors is used to fingerprint a substance. An electronic nose sensing is a device intended to detect odors or flavors. In all industries, odor assessment is usually performed by human sensory analysis, by chemosensors, or by gas chromatography. However, the correlation between analytical results and actual odor perception is not direct due to potential interactions between several odorous components. Over the past decade, “electronic sensing” or “e-sensing” technologies have undergone important developments from a technical and commercial point of view. The expression “electronic sensing” refers to the capability of reproducing human senses using sensor arrays and pattern recognition systems. (b) Electronic nose instruments are used by research and development laboratories, quality control laboratories, and process and production departments for various purposes, and future applications in the fields of health and security. Detailed information can be found in references.

43. What is nanobiotechnology? What is nanosensor and its uses?

Answer: (a) Nanobiotechnology is the application of nanotechnology to life sciences including the application of nanoscale tools to biological systems and the use of biological templates to create nanoscale products. Nanotechnology focuses on the understanding and control of matter at approximately 1–100 nm (a nanometer is a billionth

of a meter). (b) It has become one of the most promising scientific fields of research in recent decades and has the potential to provide new solutions on medical and food industries, for example, for disease-treatment delivery methods and biosensors for pathogen detection. For the applications, see Table 1.30.

44. What are quorum sensing and quorum quenching? What are the uses?

Answer: (a) Quorum sensing (QS) is a kind of microbial language that sends out molecules that announce their presence to each other. It is a system of stimulus and response correlated to population density to coordinate gene expression according to the density of their local population. (b) Quorum quenching (QQ) is an attempt to disrupt the bacteria from sensing the tiny, molecular howls they send out to each other. Three strategies can be applied for inhibition of QS. The first method is to block signal production. The second strategy is to inactivate signal molecules. That can be achieved in two ways: either use the antagonist of autoinducers or degrade signals that are already created. The last method is to target the signal receptor. (c) QQ can generate a new type of antibiotic drug for the infectious pathogens, and inhibit QS system, to prevent food pathogens and food spoilages.

45. What is the difference between microencapsulation and nanoencapsulation?

Answer: (a) Micro- or nanoencapsulation is a process in which tiny particles or droplets are surrounded by a coating to give small capsules with many useful properties.

A microcapsule is a small sphere with a uniform wall around it. The material inside the microcapsule is referred to as the core, internal phase, or fill, whereas the wall is sometimes called a shell, coating, or membrane. Most microcapsules have diameters between a few micrometers and a few millimeters. (b) Recent advances in miniaturization have designed materials with minute sizes in the nanometer regime below 100 nm, which takes advantage of their dramatically increased surface area to volume ratio.

46. What are uses of microencapsulation and nanoencapsulation?

Answer: Technologies of micro- and nanoencapsulation are currently providing solutions to complex issues such as sustained release and targeted delivery. Cell microencapsulation technology involves immobilization of the cells within a polymeric semipermeable membrane that permits the bidirectional diffusion of molecules such as the influx of oxygen, nutrients, and growth factors, essential for cell metabolism and the outward diffusion of waste products and therapeutic proteins. The areas of applications are broad including food industry, pharmaceuticals, cosmetics, electronics, photography, agriculture, chemical industry, textile industry, graphic industry, and biotechnology. For the applications of microencapsulation, see Table 1.32. For the possible applications of nanoencapsulation, see Table 1.33.

47. What are the safety issues of nanoparticles?

Answer: The application of nanoparticles in food and food contact substances concerns the potential short- and long-term effect the substances have on human health. Some nanoparticles can move from the respiratory system to other organs. Research is continuing to understand how these unique properties may lead to specific health effects. The FDA may require food companies to provide data establishing the safety of any packaging using nanotechnology. The FDA currently considers safety issues on a case-by-case basis.

48. What are the basic steps involved in recombinant DNA technology?

(a) Include in your discussion the role played by plasmids, viruses, and restriction endonucleases. (b) Name and describe five commercial recombinant food/ medical applications of genetically engineered products and give the organism used to produce that product.

Answer:

(a) First, the gene you want is cleaved from an organism's DNA with restriction endonucleases (enzymes). That same enzyme cuts open a plasmid and the sticky ends of the gene you just cut from the organism is placed in the plasmid and the ends are sealed with DNA ligase. This plasmid is now recombinant DNA, and is placed with bacteria so it can transform into them. Another option is inserting the plasmid into viruses, so the viruses can then attack an organism's cells, insert its DNA into the organism's DNA, and hopefully the gene was inserted in the correct place.

(b) Food: recombinant rennet (chymosin) into yeast, *Kluyveromyces lactis* and fungus, *Aspergillus niger*; recombinant lipase (Lipolase) in recombinant human insulin.

49. What are restriction enzymes? How do these enzymes participate in the recombinant DNA technology?

Answer: Restriction enzymes, or restriction endonucleases, are enzymes specialized in the cutting of DNA fragments each acting upon specific sites of the DNA molecule. Restriction enzymes are used in the recombinant DNA technology to obtain with precision pieces of DNA molecules to be later inserted into other DNA molecules cut by the same enzymes.

50. What are DNA ligases? How do these enzymes participate in the recombinant DNA technology?

Answer: DNA ligases are enzymes specialized in tying the complementary DNA chains that form the DNA double helix. These enzymes are used in the recombinant DNA technology to insert pieces of DNA cut by restriction enzymes into other DNA molecules submitted to the action of the same endonucleases.

51. What are plasmids? What is the importance of plasmids for the recombinant DNA technology?

Answer: Plasmids are DNA molecules, usually circular, that are independent of the chromosomal DNA. In genetic engineering, plasmids are called vectors, and are used to isolate and multiply a specific gene. They can be transferred into other organisms to produce recombinant bacteria for producing utile proteins and chemicals on an industrial scale. They may contain genes responsible for bacterial resistance to some antibiotics and for proteins that cause virulence (pathogenic hostility).

52. What are essential techniques in working with DNA?

Answer: (a) Hybridization: After denaturing double-stranded DNA, each strand can reanneal with the other strand. However, each strand can also hybridize with any added DNA or RNA (usually radioactively labeled), which also contains the correct complimentary base sequence, that is called the *hybridization probe*. It can either be a synthetic DNA (made by organic chemistry) or an isolated cloned DNA, usually radioactively labeled. To keep the original two DNA strands from reannealing,

these are often attached to a solid support like a nitrocellulose membrane after denaturation. (b) Agarose gel electrophoresis: DNA, being negatively charged moves toward the + pole (anode) of an electrical field. Since all DNA has a uniform charge to mass ratio (-1 charge per base), DNA's of all sizes move about equally through water. By using a gel, which slows down larger molecules by sieving action, DNA fragments can be separated by size and calculate the size of each DNA as compared with the standard DNA masses. DNAs are detected by UV fluorescence when bound to the chemical, ethidium bromide or SYBR-based dyes. Most DNA fragments of interest are those generated by cutting a plasmid or viral DNA with restriction enzymes. By analyzing the number of fragments produced when a specific DNA is cut with each of several restriction enzymes, alone and in pairs, one can deduce the restriction map of the DNA. (c) Southern blotting: This uses gel electrophoresis followed by hybridization to detect only the subset of DNA's in a complex mixture or pattern, which hybridize to a particular nucleic acid sequence (probe). (d) Screening a clone library: uses hybridization to detect only those bacterial colonies or plaques that contain a recombinant DNA insert which is complementary (hybridizes to) to the probe. (e) *In situ* hybridization uses a labeled complementary DNA or RNA strand (i.e., probe) to localize a specific DNA or RNA sequence in a portion or section of tissue or cell (*in situ*). (f) DNA sequencing: A ddNTP is used to produce a ladder of DNA fragments that terminate only at that specific nucleotide (nucleotide "N"). By running four different ladders next to one another a pattern is generated from which the DNA sequence can be read. (g) Polymerase chain reaction (PCR): based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, it needs a primer to which it can add the first nucleotide. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies (amplicons). (h) *In vitro* mutagenesis: a mutation is produced in a segment of cloned DNA and the DNA is then inserted into a cell or organism, and the effects of the mutation are studied. One method of *in vitro* mutagenesis is oligonucleotide-directed mutagenesis, where a specific point in a sequenced gene is pinpointed for mutation. An oligonucleotide, a short stretch of synthetic DNA of the desired sequence, is made chemically. For example, the oligonucleotide might have adenine in one specific location instead of guanine. This oligonucleotide is hybridized to the complementary strand.

53. You are assigned to clone (a) a *Bifidobacterium* β -galactosidase (lactase) and (b) an egg lysozyme into *E. coli*. What basic steps are used to produce a genetically engineered *E. coli* to produce these prokaryotic and eukaryotic enzymes? How can the bacteria that make the product of a particular cloned gene be identified after cloning? What special problems must be considered if you clone eukaryotic genes into prokaryotic cell, *E. coli*? If the expression level of a cloned enzyme in prokaryotic cell is very low, how can you improve gene expression on this enzyme?

Answer: Read Part I (Section 2.20).

54. What are the advantages and disadvantages of different expression hosts?

Answer: Read Table 2.5.

Part II

Applications of Biotechnology to Food Products

3

Yeast-Based Processes and Products

3.1 Food yeasts and derivatives

3.1.1 Introduction

Yeasts, which are widely distributed in nature and on the skin of certain fruits, are single-cell organisms classified as fungi. The presently recognized number of fungal species exceeds 50,000, but only about 39 genera with 350 species are accepted as yeasts. Thus, yeasts represent only a small fraction of the fungal kingdom. The use of yeasts as a primary foodstuff in bread making and in the brewing of beer has been recognized since about 4000 BC. Yeasts have been used for their fermentative capabilities to break down starch or sugar to alcohol and carbon dioxide, which are important to the brewing, wine, distilling, and baking industries. Only *Saccharomyces*, *Candida*, and *Kluyveromyces* are commercially cultivated; but two genera, *Candida* and *Kluyveromyces*, have shown substantial economic importance in the production of foods, flavors or alcohol from substrates such as sulfite liquor and whey. Yeasts are classified as either active or inactive. *Active yeasts* are those used for fermentation. *Inactive yeasts*, also called dried yeasts, are nonfermentative substances used predominantly as nutritional and flavor components.

3.1.2 Industrial processes

The industrial processes commonly carried out by the specific types of yeast can be divided into four major classes: (i) the production of yeasts as a source of baker's yeast or single-cell protein (SCP), (ii) the production of nutritional, flavor, and bulking aids provided by inactive yeast, (iii) the production of alcohol beverages by brewer's and wine yeasts, and (iv) the production of bread or baked goods by baker's yeast. More detailed information on food yeasts and derivatives can be found in the references (Johnson, 1983; Nagodawithana, 1991; Guan et al., 2013).

3.1.2.1 Baker's yeast Baker's yeast is produced at a rate of 1.8 million tons annually and is one of the largest products of the fermentation industry. Baker's yeast is available in four

active forms, compressed and cream yeasts, active dry yeast, and instant active dry yeast, which differ in activity and stability. *Compressed yeast*, the most widely used form of yeast, contains 70% water and 27% (w/v) dry matter; it is marketed as cake or cake-crumbled products. Compressed yeast is a top-fermenting yeast, *Saccharomyces cerevisiae*, manufactured from sugar-containing raw materials. Cake yeast is produced from a blend of wet yeast and emulsifiers that is extruded, then cut into block form in a different sizes. Cake-crumbled yeast is wet yeast crumbled into irregular pieces of about 1 cm × 5–10 cm before being packed into 25–50 lb bags. The pieces are wrapped in waxed paper and stored under refrigeration to ensure stability during storage for about 4 weeks. Despite these precautionary measures, a loss of fermentation activity of 3–5% per week at 5–8°C is unavoidable. Compressed yeast exhibits a high level of viability for several months under frozen conditions, but there is discoloration and softening of yeast after thawing. *Cream yeast*, which contains around 85% water and is delivered directly in chilled stainless steel containers for bakeries, is now favored by many bakeries.

New forms of *active dry yeast* are now available which can be added directly to the dough ingredients with no hydration step before addition. The production procedure for active dry yeast is not significantly different from that used in the manufacture of compressed yeast. The yeast cream is pressed and the pressed cake is then extruded through a perforated plate to provide strands of about 3 mm in diameter. These strands break down into smaller strands (0.3–1 mm) and are dried over a 6-h period at temperatures of 25–45°C using a continuous belt dryer. The dehydrated product, which has an equilibrated moisture of 7.5–8.3%, is then ground and packaged in small pouches under nitrogen. Unground, granular yeast, with its good stability, is sold air-packed in cartons and drums for use in large bakery operations. Active dry yeast has better stability than compressed yeast, lasting for up to 3 months without refrigeration when stored in the presence of air. Its storage stability can be extended for up to a year by packaging under nitrogen or vacuum at room temperature. This dried yeast costs more than compressed and cream yeasts, hence is uneconomical for commercial bread production, where supplies of fresh yeast are readily available. Active dry yeast is widely used in areas of the country where the supply of fresh yeast is limited and ambient temperatures cause delivery and storage problems for fresh yeast. Before it can be added to the dough mix, the yeast must be rehydrated for 10–15 minutes, with a ratio of water to dry yeast of 4:1. One pound of compressed yeast can be replaced by about 0.4–0.5 lb of active dry yeast.

Instant active dry yeast has baking activities comparable to those of compressed yeast, though it is a dried form, like traditional active dry yeast. Special strains of the yeast *S. cerevisiae* are chosen for their ability to retain maximum activity through the specialized drying process. The protocol for this yeast production is similar to that of active dry yeast, except for the drying process. In place of a continuous belt dryer, dehydration is accomplished with an air-lift fluidized-bed drier. The wet strands break into particles that are fine particles of active dry yeast, and thus the particles dry very rapidly. The yeast is normally pretreated with emulsifier (e.g., sorbitan monostearate) to permit lower moisture content and to facilitate wettability under instant usage conditions. Because of its porosity and fine particle size, this yeast is highly unstable when exposed to air at room temperature. Thus, it requires packaging under nitrogen or vacuum. Rehydration is so rapid that the yeast can be mixed in with dry ingredients.

3.1.2.2 Single-cell protein (SCP) The yeast used in SCP can be primary grown or spent yeast from distillery or brewery fermentations. Primary yeasts are organisms that are aerobically grown on a variety of substrates especially for use in the human food and feed industries. SCP is further discussed in Sections 4.5 and 5.5.

3.1.2.3 Inactive yeast and its derivatives *Inactive dry yeasts*, which are used primarily for their nutritional and flavor-enhancing properties, include dried forms of brewer's yeasts and the primary grown yeasts. Brewer's yeast is produced at a rate of half pound of surplus yeast solids per barrel as a by-product of beer manufacture. The United States alone produces about 75 million pounds of spent brewer's yeast solids annually. There are many uses for surplus brewer's yeast, but brewer's yeast is by no means the only yeast source available. Primary yeasts can be grown in carbohydrates such as sugar and oil refinery wastes, in sulfite liquor wastes from the pulp and paper industry, and in other by-products of food wastes. The primary yeasts may be treated and processed the same as the brewer's yeasts, but the end products will have different characteristics and flavors. Baker's yeast cake may be resuspended in water and dried on steam rollers to yield thin flat flakes of inactive yeast cells. Brewer's yeast can also be treated this way, but it must first be washed with a mild alkaline solution (sodium carbonate) to remove the bitter hop residues. This is the brewer's yeast we buy in health food stores. Either as powder or as flakes, these products find a broad spectrum of further uses. Dried yeast from *S. cerevisiae* and *Klugveromyces fragilis*, as well as torula yeast from *Candida utilis* can be used in human food, provided the total folic acid content of the yeast does not exceed 0.4 mg/g yeast and the intake of nucleic acids will not exceed 2 g/day in adult diets. (Higher levels of consumption of nucleic acids may lead to gout or arthritis.)

3.1.2.4 Yeast derivatives Liquid or dried yeast products can be further processed to produce autolysates and extracts, enzymes, and other biochemicals. Yeast autolysates or extracts are produced by optimizing the autolysis process to achieve maximum possible solubilization of the yeast cell contents. Many different methods and patents deal with the manufacture of yeast extract. In general, yeast autolysis takes place when cells are heated to between 40 and 55 °C, which kills cells but promotes the activity of intracellular hydrolytic enzymes such as proteases, carbohydrases, and nucleases. Cell death brings about disorderliness, resulting in the indiscriminate action of the hydrolytic enzymes on the cell constituents. The intracellular proteolytic enzymes degrade proteins to peptides and amino acids, and the nucleases act on nucleic acid to produce nucleotides and nucleosides. The two most important such products are 5'-GMP (*guanosine monophosphate*) and 5'-IMP (*inosine monophosphate*) that are produced by autolysis and fermentation methods. These naturally occurring ribonucleotides behave synergistically with glutamate in yeast extracts, which lead to powerful flavor-enhancing properties, the so-called the *umami* effect. The highly organized cell membrane begins to degenerate and lose its integrity, thus releasing the solubles into the aqueous environment. Following removal of the insoluble residue by filtration steps, the clear extract is evaporated to a paste ($\approx 70\%$ solids) or spray-dried to a powder (95% solids). This autolysis may sometimes be accelerated by the addition of proteases or gluconases. Most of the low sodium extracts are produced by this procedure. High content of glutamate ($\approx 6\%$) and other nucleotides and amino acids makes yeast extracts useful flavor potentiators. Their natural origin and their long history as wholesome ingredients often render them more acceptable than their counter-parts such as hydrolyzed vegetable protein (HVP) and monosodium glutamate (MSG). The low sodium extracts have a significant advantage in this expanding market and are used mainly as inexpensive substitutes for meat extracts in savory applications such as sauces, soups, gravy mixes, ready meals and snacks, and pet foods. The other extraction methods, such as plasmolysis and hydrolysis, are also used often. The *plasmolysis* process uses chemicals such as salt to create osmotic pressure or ethyl acetate to alter cell permeability to enhance the extraction. These products have limited use in the food industry because of the high level of salt present in the final product. *Hydrolsates* are prepared by treating a slurry

of inactive dry yeast (65–80%) with HCl in varied concentration, followed by cooking, cooling, neutralizing with NaOH, filtration, and concentration to paste or spray-drying to powder. This method is the most efficient process in terms of yield, but high salt content and the destruction of certain amino acids and vitamins have made the powder less appealing to most yeast extract manufacturers. Also, soy bean and other protein hydrolysates are cheaper than yeast hydrolysates.

The discovery of the flavor-enhancing properties of 5'-IMP and 5'-GMP has prompted scientists to search for inexpensive sources for the commercial production of such products. Since the nucleic acid content in yeast is high – in the range of 9–12% (dry weight basis) – hydrolysis of RNA using 5'-phosphodiesterase (exonuclease from *Penicillium citrinum*) could produce four nucleotides (AMP, GMP, CMP, UMP), among which only two (5'-IMP after conversion of AMP and 5'-GMP) are important to the food industry for the production of 5'-nucleotide-rich extracts. After CMP/UMP has been removed by absorption, 5'-IMP is produced by the deamination of 5'-adenylic acid with adenylic deaminase from *Aspergillus oryzae* (Figure 3.1). These flavor enhancers, which are now commercially available either as crude extracts or in the purified form, are particularly effective in improving savory flavors and can be used as MSG replacers. The use in the recipe of low levels of flavor-enhancing nucleotides also enhances the perceived flavor of salt, thus reducing the amount of salt used in a recipe. New yeast extracts can also be made by digestion of heat-treated yeast with commercial enzymes, fermentation with lactic acid bacteria (LAB), and then pasteurization before clarification and spray dried. The extract lacks yeastiness and other unwanted flavors, and can find use in dairy spreads, soups, and meat products. Further recent developments have resulted in the creation of *processed* or *reaction yeast extracts*. Flavors of identifiable origin such as meat and cheese can be blended to make processed yeast extracts. This simple blending can also be extended to control cooking stages in which the base is reacted with sugars and sulfur-containing amino acids (cystein, thiamine, methionine) and animal fats. Thus, flavors with the distinct character of cooked beef, chicken or pork can be produced by reaction. Yeasts contain a relatively high content of protein, vitamins, and minerals and they can be incorporated

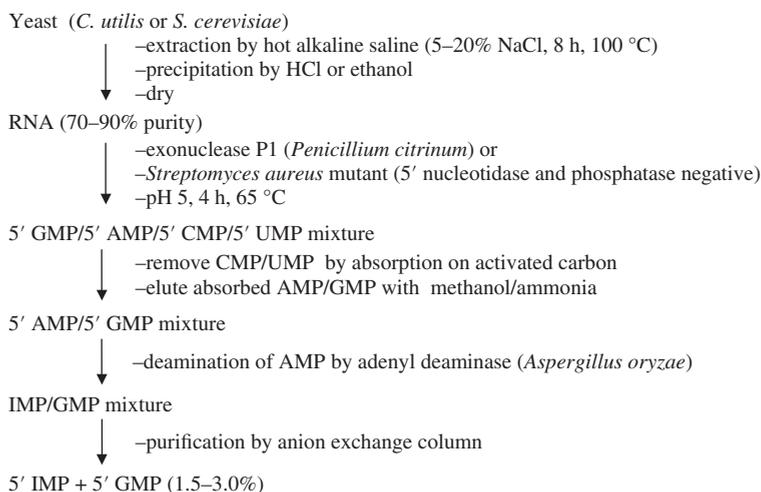


Figure 3.1 Production of 5'-ribonucleotides by enzymatic hydrolysis of RNA (50% produced by this method).

into many foods as ingredients for nutritional fortification. Many yeasts and derivatives are available for a range of functions aside from flavor enhancement and nutritional fortification. Yeast products impart unique texturizing effects, serving as stabilizers and thickeners in foods.

Other products such as enzymes – for example, invertase, which hydrolyzes sucrose to glucose and fructose for chocolate enrobing and β -galactosidase (lactase), which converts lactose to glucose and galactose – are well known. Natural color (*astaxanthin*) produced by red yeasts such as *Phaffia rhodoryma* has received much attention. The astaxanthin pigment provides for the red color of salmon, trout, and other marine invertebrates and has shown to be a potentially important source of fish feed to restore the red color in the flesh of pen-reared salmonids. The cost of astaxanthin production, high commercial price and lack of a leading fermentation production systems, combined with the shortfalls of chemical synthesis mean that research into alternative fermentation production methods has been carried out. Currently, the primary natural source for astaxanthin is the microalgae *Haematococcus pluvialis* and commercially more than 40 g of astaxanthin can be obtained from 1 kg of dry biomass (www.algatech.com). Metabolic engineering offers the opportunity to produce astaxanthin. The metabolic engineering of *Escherichia coli* recently allowed production of astaxanthin at >90% of the total carotenoids, providing the first engineered production system capable of efficient astaxanthin production. Astaxanthin biosynthesis proceeds from beta-carotene via either zeaxanthin or canthaxanthin. The production of astaxanthin by metabolic engineering alone will not provide a suitable alternative to current commercial methods.

Glycan, the crude cell wall fraction of brewer's or baker's yeast, is recovered by centrifugation, pasteurization, and spray-drying after preparation of yeast extracts. After several passages of the glycan through a homogenizer, the resulting high viscosity product provides a fatlike mouthfeel. Thus, it can be used as an emulsifier, stabilizer, thickener or texturizer and for developing low-fat, low-calorie food formulations. The best known products are low-fat, low-calorie salad dressings, frozen desserts, cheese analogs, and ice cream. Most glycans consist of glucan and mannan units (2:1). The β -glycan ($\beta(1 \rightarrow 3)$ glycosidic bonds), which is made up of glucose polymers, is predominantly a polysaccharide and can be hydrolyzed by enzymes to produce oligosaccharides (bifidus growth factor). *Yeast protein* can also be isolated from mechanically disrupted cells of *S. cerevisiae* for use as nutrient supplements or functional proteins in food.

3.1.2.5 Molds in fermentation The three main types of cheese that rely on molds are blue cheese, soft ripened cheese (such as camembert and brie) and rind-washed cheese (such as limburger). Blue cheese is treated with a mold, usually *Penicillium roqueforti*, while it is still in the loosely pressed curd form. As the cheese matures, the mold grows, creating blue veins within it which gives the cheese its characteristic flavor, methylketone. Soft ripened cheese such as brie and camembert are made by allowing *P. camemberti* to grow on the outside of the cheese, which causes them to age from the outside in. The mold forms a soft white crust, and the interior becomes runny with a strong flavor. Also inoculations of sausages with molds were done with the indigenous biota of the slaughters. Different molds (such as *P. chrysogenum* and *P. nalgiovense*) can be used to ripen surfaces of sausages. The mold cultures develop the aroma and improve the texture of the sausages; some mold strains can produce mycotoxins, and thus selection and developments of strains need careful consideration. In particular, molds and yeasts frequently develop on the surface of ferments, particularly on certain solid ferments, such as vegetables or miso. Molds such as genera *Aspergillus*, *Penicillium*, and a few *Rhizopus* and *Mucor* have also been used for beneficial purposes in production of food and food additives in Chapter 5.

Summary

As one of the oldest microorganisms known to man, yeast clearly has great possibilities in the food industry. Spent brewer's yeast, which used to be a waste product, is really an important raw material for a whole range of modern yeast technology. Research is still going on to find ways to recover other vital ingredients from yeasts. Potassium chloride coupled with yeast and yeast extracts holds much hope as a salt replacement. Yeast extracts high in ribonucleotides can also be used as *MSG replacers*. Closely related products with no added salt and with enhanced flavor stabilization properties have already been made, and the food industry can look forward to further innovations in the near future. As a result of recombinant DNA technology, many yeast-derived products such as rennin, insulin, interferon and hepatitis B vaccine are already in the marketplace. A large number of genetically engineered yeast products are currently undergoing human clinical trials and are being evaluated for final approval. Mold is also an important food fermentation strain involving in cheeses and food additives (enzymes, organic acids, etc.), that are described in Chapter 5.

3.2 Alcoholic beverages

3.2.1 Introduction

Alcoholic beverages are in essence flavored solutions of ethanol derived from numerous substrates, such as grains (as in beer), grapes and other fruit (as in wine), or any carbohydrate source (as in distilled spirits). Alcoholic fermentation is certainly the oldest biochemical process executed by man, probably dating back some 3000 years. Fermentation was first used to preserve fruit juices, but later it was adapted to produce alcoholic beverages from fermentation of grain and subsequent distillation. Little was known of the mechanisms involved in the brewing process by living yeast cells, however, until Pasteur's work in the late nineteenth century. Hansen at the Carlsberg Institute in Denmark has been one of the major discoverers of wild yeast and initiated the use of pure cultures in beer production. Fermentation is carried out by species of *Saccharomyces*, but in some cases yeast is present naturally, as in grapes used in wine making. *Saccharomyces* species are not capable of hydrolyzing polysaccharide materials; sugars must be produced from starches in cereals by the malting process. The production of ethanol from glucose occurs by the Embden–Meyerhof (EM) pathway. The yeasts used in the manufacture of alcoholic beverages are strains of *S. cerevisiae* or *S. carlsbergensis*.

3.2.2 Production and sales of major alcoholic beverages

The world beer production in 1990 was approximately 1141×10^2 megaliters (ML) and the United States is the largest producer, making 239×10^2 ML, with retail sales in excess of US\$20 billion per year. Germany is also a major producer, with 104×10^2 ML. However, China became the largest producer in 2011 and China produced 10.7% more beer in 2011 than in 2010, and kept the title of the world's largest beer producer for the tenth year in a row (Table 3.1). In terms of per capita consumption in 1990, Germany ranked first with 144.6 L, while the United States ranked eleventh (90.8 L). However, Czech Republic became the top country in 2011. Most of the top countries in terms of per capita consumption were European. Marketing practices account for the major cause of these differences; the larger brewers in the United States market their products on a national or regional basis, but most of the Czech Republic or German breweries sell on a local basis

Table 3.1 World beer production (2011) in different countries

		2011				2010			
2011 ranking	2010 ranking	Country	Production volume (kL)	Change from 2010 (%)	Production share in the global market		Production volume (kL)	Change from 2009 (%)	
						Incremental (%)	Cumulative (%)		
1	1	China	48,988,000	10.7	25.4	25.4	44,252,936	4.9	
2	2	United States	22,545,817	-1.5	11.7	37.1	22,898,177	-0.8	
3	3	Brazil	13,200,000	3.4	6.8	44.0	12,769,662	18.2	
4	4	Russia	9,810,000	-4.2	5.1	49.1	10,240,000	-6.2	
5	5	Germany	9,554,500	-0.1	5.0	54.0	9,568,300	-2.4	
6	6	Mexico	8,150,000	2.0	4.2	58.2	7,988,900	-3.0	
7	7	Japan	5,629,566	-3.8	2.9	61.2	5,850,450	-2.4	
8	8	United Kingdom	4,569,400	1.5	2.4	63.5	4,499,700	-3.2	
9	9	Poland	3,785,000	5.1	2.0	65.5	3,600,000	11.8	
10	10	Spain	3,360,000	0.7	1.7	67.2	3,337,500	-1.3	
11	11	South Africa	3,087,000	4.3	1.6	68.8	2,960,000	2.8	
12	12	Ukraine	3,051,000	-1.6	1.6	70.4	3,100,000	1.6	
13	13	Vietnam	2,780,000	4.9	1.4	71.9	2,650,000	15.2	
14	14	The Netherlands	2,360,000	-1.4	1.2	73.1	2,393,600	-5.7	
15	16	Venezuela	2,350,000	17.5	1.2	74.3	2,000,000	-13.6	
16	15	Colombia	2,100,000	2.4	1.1	75.4	2,050,000	1.8	
17	17	Thailand	2,060,000	3.3	1.1	76.5	1,995,000	2.6	
18	21	Nigeria	1,959,600	11.3	1.0	77.5	1,760,000	10.0	
19	18	Canada	1,951,500	-0.7	1.0	78.5	1,964,700	-12.3	
20	20	Belgium	1,857,083	2.5	1.0	79.5	1,812,266	0.6	
21	27	India	1,850,000	18.6	1.0	80.4	1,560,000	0.6	
22	19	Republic of Korea	1,849,700	1.8	1.0	81.4	1,817,300	1.0	
23	24	Czech Republic	1,741,584	1.2	0.9	82.3	1,723,147	-4.5	
24	23	Australia	1,738,000	-0.2	0.9	83.2	1,742,000	0.6	
25	27	France	1,710,000	9.6	0.9	84.1	1,560,000	9.0	
		Total	192,712,284	3.7	100	100	185,769,669	2.2	

Source: Kirin Holdings Company (<http://www.kirinholdings.co.jp/english/news/2012>). Reproduced with permission.

only. The estimated world production of wine was 27,106 (ML) in 2009 and 26,384 in 2010 (Table 3.2). About 80% was produced in Europe and about 14% in North and South America. France (45.7 L) and Italy (42.2 L) were top in the lists of the biggest wine consuming nations (Table 3.3), but US consumption reached 311.3 m cases (3.7 billion bottles) and China's wine consumption is predicted to rise to 1.9–2 L per capita by 2015.

3.2.3 Production processes

3.2.3.1 Beer Beer brewing is the largest biotechnological industry in the world. Beer can legally be defined as a malt beverage resulting from an alcoholic fermentation of the aqueous extract of malted barley flavored with hops, with or without other cereal grains. Most beer is made from malt, hops, yeasts, water, and malt adjuncts. Adjuncts are nonmalted carbohydrate-containing materials that beneficially supplement or attenuate malt barley. The quantity of dry adjuncts such as corn, rice, sorghum, and wheat may vary from 10% to

Table 3.2 World production of wine and annual per capita consumption in 2010

	Production (L)	Consumption (L)
World, total	26,384,872	–
France	4,626,900	45.7
Italy	4,580,000	42.2
Spain	3,609,700	26.2
United States	2,653,187	9.4
Argentina	1,625,000	23.7
Australia	1,073,000	24.9
Australia	1,073,000	24.9
Germany	932,000	25.5
Portugal	587,000	41.8
Russia	540,000	8.2
Romania	495,740	22.9
China	425,000	0.7

Source: Author's compiled data.

Table 3.3 Per capita consumption of beer in different countries (2011)

Rank	Country	Liters per capita
1	Czechoslovakia	132.0
2	Germany	107.0
3	Austria	106.0
4	Ireland	104.0
5	Australia	98.1
8	Poland	84.0
9	Venezuela	83.0
10	Finland	83.0
11	Slovenia	83.0
12	United States	78.0
13	Belgium	78.0
14	Croatia	78.0
15	Romania	77.0
16	Panama	75.0
17	Netherlands	74.0
18	United Kingdom	74.0
19	Bulgaria	73.0
20	New Zealand	71.0
21	Hungary	70.0
22	Spain	70.0
23	Canada	68.0
24	Denmark	67.0
25	Latvia	67.0
26	Russia	66.0
27	Brazil	65.0

Source: Kirin Holdings Company (<http://www.kirinholdings.co.jp/english/news/2012>). Reproduced with permission.

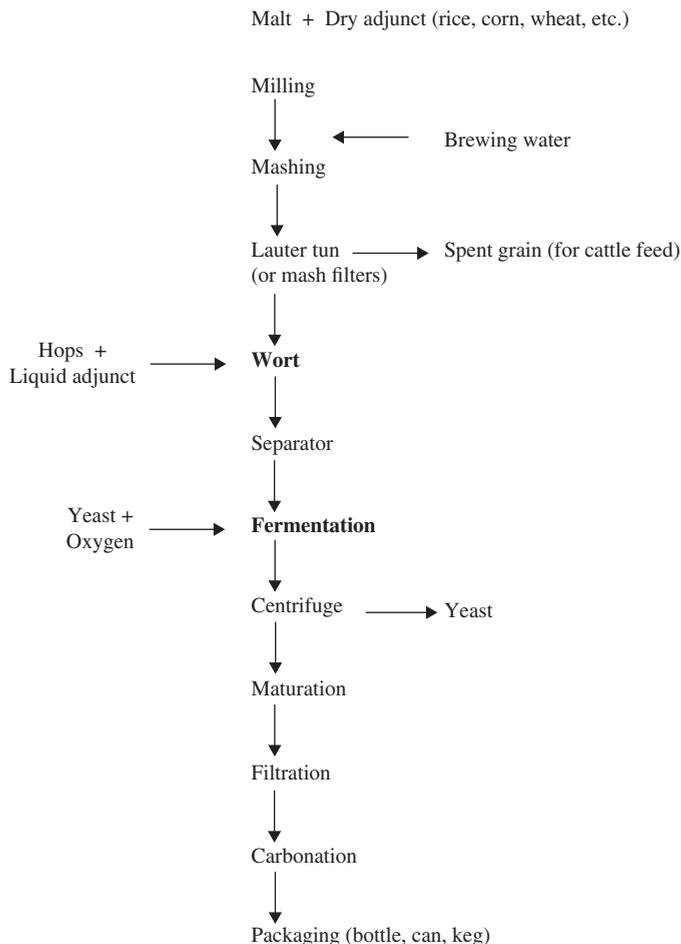


Figure 3.2 Schematic outline of the brewing process.

50% of the total. The choice depends on the individual brewer and on the availability of the cereals to regulate the composition of the resulting wort and to save costs. The starch of these adjuncts is in its native form and is not susceptible to enzymatic hydrolysis during mashing. Thus, these adjuncts are normally boiled in a cooker mash to solubilize and gelatinize the starch granules. Today liquid adjuncts, which are clean, noncrystallizing mixtures of fermentable sugars and dextrans, are available to achieve a better runoff wort and to control over the kettle operation. The significant difference between the beer, whiskey, and wine industries is the composition of the raw material. Beer production involves three distinct but interrelated malt steps (Figure 3.2). The first step in brewing is the malting process, which is the production of a soluble malt extract, so-called *wort*.

Wort production The first step in wort production is mashing, which is simply the mixing of warm water with ground malt, with a suitable standing period (5–7 days). At this time

dry malt adjuncts may be added. Malt, which is a sugar-containing material, is prepared from barley grains by soaking or steeping the grain in water at 10–15.6 °C and allowing it to germinate at 15.6–21 °C for 3–4 days or more (7 days). This malting step activates the enzyme systems (amylases and proteases). The α - and β -amylases break down the barley's endosperm to fermentable sugars, while proteases produce amino acids that will be used by the yeast. The germination is then arrested by *kilning*, which is basically the use of heat to dry the green malt from a moisture content of about 45–4%; this stops the enzymatic and chemical reactions. The heating process produces some of the characteristic colors and flavors of beer. The dried malt can then be stored until needed. Drying is normally carried out on so-called kilning floors made from perforated sheets (arranged in layers to a height of 60–120 cm) by the direct mixing of gases with the dried air.

The aqueous extract called wort is separated from the grain and filtered. The spent grains can be sold as cattle feed. The clarified wort is then boiled with hops in a kettle for 1–1.5 hours to give flavor to the beer. The boiling of the wort with hops concentrates the liquid, sterilizes it, inactivates enzymes, extracts the hop solubles, precipitates protein, and caramelizes sugars. Liquid adjuncts, such as sugar solutions or corn syrup, may be added at this time to increase the amount of fermentable sugar. Hops are dried blossoms of the female hop plant (*Humulus lupulus*) and contain a group of compounds, called *humulones* or α -acids (Figure 3.3), which are very insoluble in water but undergo an isomerization during the brewing processing to form *isohumulones* or *iso- α -acids*, which account for most of the bitterness of beer. The hop industry has sought to maximize the α -acid content of its hops, and brewers use α -acid content as a criterion for purchase. Humulones can be isomerized in aqueous sodium or potassium carbonate, and under these conditions the formation of degradation products (e.g., humulinic acid) is minimized. Hop extracts also contain a small amount of odorous volatile oil compounds such as terpenes, humaladienone, capryophyllene, epoxide, linalool, geraniol, and ketones, some of which survive into the finished beer, resulting in the hoppy aroma of some beers. After boiling, the aqueous wort is separated from the trub (precipitates and spent hops) by one of many methods such as filtration or whirlpool separation. The wort is cooled in a heat exchanger to the temperature desired for fermentation. The pH is adjusted to 5.2 by addition of lactic acid or phosphoric acid. Immediately after cooling, the wort is aerated to provide oxygen for the yeast. Yeast (about a pound of liquid) from a preceding brew is pitched in per barrel of wort, which will give a count of about 12×10^6 cells per milliliter.

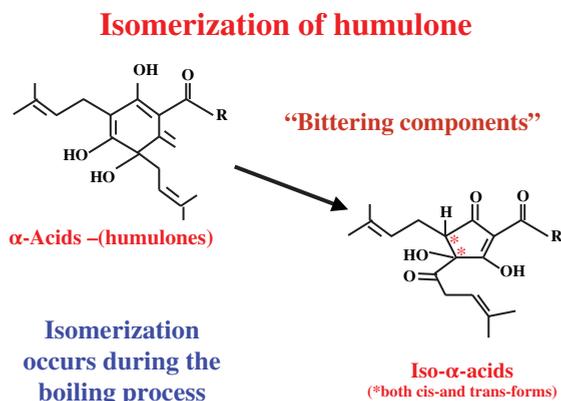


Figure 3.3 Structure of humulone and isomerization.

Fermentation Yeasts are added to the wort, which contains oxygen, fermentable sugars, and various nutrients. The yeast quickly consumes the oxygen and minor nutrients, and then metabolizes sugars and amino acids. Although wort fermentations in the production of beer are largely anaerobic, some oxygen must be made available to the yeast because the new inoculum contains no reserves of necessary lipids such as sterols and unsaturated fatty acids, which are essential membrane components. Two types of yeast are used in the brewing industry. The first is a bottom-fermenting (settle-out) yeast, *Saccharomyces uvarum* (*S. carlsbergensis*). Fermentation takes 8–10 days at 7–15 °C and yields a product called *lager*. *S. uvarum* has a marked ability to flocculate when fermentable sugars are exhausted. Some brewers hasten this settling by using centrifuges to collect their yeast. On the other hand, *ale* beer is produced using the top-fermenting yeast *S. cerevisiae*. Top-fermenting yeasts tend to be somewhat less flocculent, and cells adsorb to CO₂ bubbles, which cause the yeast to rise to the top of its fermenting tank, where it may be collected by skimming. Fermentation takes place at slightly higher temperatures (15–22 °C) for 3–5 days. The yeasts ferment the sugars (primary fermentation) mainly via the EM pathway to yield equimolar amounts of ethanol and carbon dioxide. Other products such as diacetyl, diketones, isobutyraldehyde, and hydrogen sulfide are also produced; these add to the overall flavor of the beer. The carbon dioxide that is given off is usually collected and cleaned for reinjection at a later stage. Part of the leftover yeast can be easily recycled from 5 to 100 times before viability and contamination become a problem.

Finishing/packaging The fermented beer is separated from the yeast sediment into wooden or stainless steel casks for maturation. During the next 2–6 weeks, at temperatures close to 0 °C, the beer undergoes a secondary fermentation, using up the remaining added sugars; the precipitation of yeasts, resins, proteins, and other undesirable substances occurs, as well. Caramel may be added at this stage to control the color of the finished products. Similarly, isomerized hop extracts may be added to control bitterness. Lager, which is also held at low temperatures, is stored in lagering tanks for up to 9 months. During this time the remaining yeast, tannins, and proteins settle out to yield a haze-free beer with a long shelf life. The cooled, aged beer can then be clarified by filtration and pasteurized at 60 °C for 6–8 minutes or at 71–74 °C for 15 seconds. The beer is rapidly cooled, filtered, and packaged in cans or bottles or kegs (for use in restaurants and taverns). If such packaged beer has not been sterile-filtered, the pasteurization may be done just before filling (bulk pasteurization) for a minute, or after filling in long tunnels with hot-water sprays (tunnel pasteurization) for about an hour. Because the delicate flavor of beer is adversely affected by oxygen, great care must be taken to minimize oxygen pickup. Modern fillers evacuate the bottle before it is filled and replace the air with CO₂. Another shortcoming of traditional beer manufacturing that has largely been eliminated is the tendency of the product to become cloudy during chilling. The source of the cloudiness, a reversibly insoluble protein–polyphenol complex, can be removed by the enzyme papain; or, the polyphenols may be removed by absorption on silica gel.

New developments: light (“lite”) and ice beers Beers that are lower in calories than conventional beers represent a significant share of the beer volume. In 1994 they accounted for about 34% of the US market (65 million hL) and 14% (2.7 million hL) in Canada, but in 2006 market share reached 51% in America. These light beers usually have reduced dextrin (starch) content. Traditional brewer’s yeasts cannot ferment dextrins, and thus brewing masters find means to decrease the complex carbohydrate content of wort. There are several methods for producing light beers: (i) dilution of regular strength beer

with water, (ii) addition of the combination of fungal α -amylase or glucoamylase and bacterial pullulanase to wort during mashing or fermentation, (iii) use of a liquid adjunct such as glucose, fructose or sucrose, (iv) use of malt enzyme preparations during mashing or fermentation, and (v) use of amylolytic brewer's yeast. The most common technique employed by brewers is the addition of glucoamylase to produce a reduced carbohydrate beer without quality defects. However, the glucoamylase used for this purpose is a thermostable enzyme derived from *Aspergillus niger* or *A. oryzae*. Thus, residual enzyme activity in the final beer after mild pasteurization treatment can lead to product defects such as sweetness and flavor instability. It is beneficial to genetically modify yeast to produce extracellular thermolabile glucoamylase, which will result in a special brewer's yeast capable of fermenting wort dextrins. The yeast enzyme is favored for the construction of amylolytic strains because it is more thermolabile than fungal glucoamylase. Unlike the glucoamylases of other fungi, however, the yeast enzyme possesses no debranching activity (α -1,6) with starch or dextrins. *Saccharomyces diastaticus* is closely allied to *S. cerevisiae* except that the former produces extracellular glucoamylase. Three unlinked glucoamylase genes (*STA 1, 2, 3*) are known in *S. diastaticus*, and these genes were cloned into *S. cerevisiae* by complementing a *STA*⁻ strain to *STA*⁺. A dextrin utilizing a transformant of the lager brewer's strain, *S. cerevisiae* (pLHCD6) was developed, and *S. diastaticus* carrying genes responsible for glucoamylase synthesis were also fused with *S. uvarvm* lager strain. Fusion products were capable of utilizing melibiose and dextrin as carbon sources. *Schwanniomyces castelli* and *Schwanniomyces occidentalis* produce significant amounts of α -amylase, glucoamylase, and debranching enzyme. Thus, a two-stage fermentation system was developed by producing extracellular amylases (glucoamylase and α -amylase) from *S. castelli* and adding them to wort previously inoculated with *S. uvarvm*.

S. diastaticus strains are desirable in the manufacture of light beer because they possess the capacity to ferment to a high degree, but beer produced by these strains had a characteristic *phenolic off-flavor* (POF) that was due to the presence of *4-vinylguaiacol* (4-VG). These strains possess the POF genes, which can produce the enzyme capable of decarboxylating ferulic acid. Hybrids were constructed that exhibited glucoamylase activity but lacked the ability to produce ferulic acid. Fermentations with these hybrids resulted in superattenuated worts, and palatable beers.

Some strains of *Saccharomyces* spp. and other yeast strains secrete a proteinaceous toxin, so-called *killer* or *zymocidal factor* that affects organisms from laboratory haploid strains to brewing yeast strains. The killer factor is lethal only toward yeast and not bacteria or cells of higher organisms. An infection of as little as 0.1% of the cell population can completely eliminate all the brewing yeast from the fermentor. One of the most common methods of preventing a wild yeast that possesses zymocidal activity from becoming established is to maintain vigorous hygiene standards. Employing the technique of rare mating, brewing strains have been modified to acquire resistance to killing by a zymocidal yeast. Details on molecular biology and genetic engineering of yeasts are described (Feldmann, 2011).

In Canada a new type of beer called ice beer – named for the subfreezing temperature at which it is brewed – was introduced by both Labatt Brewing Co. Ltd. and Molson Companies Ltd. in Ontario in spring of 1993. During the brewing process, it is super-chilled to below freezing temperatures, leading to the formation of ice crystals. The beer is described as having a “crisp, clean taste” and 5.6% alcohol by volume. Ice beer has grown to represent 6% of the North American beer market and made inroads in Japan.

3.2.3.2 Wine Wine is the fermented product of the fruit of several species of *Vitis*, mainly *Vitis vinifera*. Wine grape juice is a good medium for wine production by yeasts because

of its high nutrient concentration, a natural acidity (which inhibits undesirable microbial growth), high sugar content, and pleasant aromas and flavors. Wine can also originate from other fruits, including raspberries and kiwi, and from flowers, honey, and maple syrup. There are two types of wine standard in the United States, and in most other countries: those based on taxes (mainly on alcohol content) and those based on compositions other than alcohol (e.g., volatile acidity, acetate, sulfur oxide). The distinctive character of various wines depends on the composition of the grapes, the nature of the fermentation process, and the processing and aging treatments applied.

The fermentation of grapes into wine is a simpler process than that for beer or distilled spirits. This is because grapes contain fermentable sugars, mainly glucose, fructose, arabinose, and rhamnose. Thus, steps for the conversion of polysaccharides into fermentable sugars, such as malting and mashing in beer, are not needed. Today, most grape varieties and clones from original grapes are modified to improve their resistance to viruses, and to give fuller clusters and high yield. Since the level of maturity influences the balance of ethanol, acids, and different flavors, the exact time for harvesting is determined by the wine maker. In some regions, the grapes are not washed to maintain the indigenous wild yeasts at the surface of the skin, but most wineries now use starter yeasts to initiate fermentation.

Crushing and pressing The first step in wine making is to harvest the grapes and to extract the juice by crushing the ripened grapes using the Rauth Crusher–Stemmer. The grapes are harvested when ripe enough to produce 11–13% alcohol; a sugar content of 20.5–23.55% in must produces the best wines, depending on the type desired. Sulfur dioxide is added to the crushed grapes immediately to control enzymatic oxidation, mainly polyphenol oxidase. The pressing process and the outflow of juice may be substantially facilitated by adding pectinolytic enzymes when the grapes contain high contents of mucilaginous materials. It is at this stage that the production process differs for red and white wines.

White wines use only the juice of white grapes after pressing, and the juice is then clarified to remove any suspended solids, called the marc or pomace. Wineries often use the William press, horizontal basket presses, revolving screen presses, or continuous-screw presses. For *red wine*, the skin is kept in the liquid to transmit its color or to enhance the taste of the product. The diffusion of tannins from the skins increases the astringency of wine. Red grapes that have colorless pulp can be crushed and pressed to obtain a nearly white must. This is more successful with red grapes of low color and sugar than with the highly colored ripe red grapes. After the appropriate pretreatment or clarification using pressing aids such as bentonite, silica gel, and cellulose fibers, the pressing musts pass into the fermentation vats. The wine containers can be made of wood, metal or glass-reinforced plastic (GFP).

Fermentation Before the fermentation step, the liquid is aerated to allow the growth of the yeast, to make sure there is enough oxygen to produce a good fermentation: the musts are first aerated to promote yeast growth and then are made anaerobic for the production of ethanol and carbon dioxide. The CO₂ generated forms a gas cover on top of the liquid, protecting it from the microorganisms and keeping it from air that would limit the fermentation by favoring growth of the yeast. For red wine production, the skins are left at the surface to contribute to the flavor and color. The skin has to be immersed daily to prevent overheating and consequent spoilage (acetification). This step may last several weeks, depending on the species of yeast, the type of wine, and the other conditions, such as temperature. For red wines, fermentation takes 3–5 days at 25–30 °C, while white wines ferment in 7–14 days at 20 °C. The reduction of the density of the liquid due to the transformation of sugars into lower density alcohol and gas tells the wine maker

that the wine has reached a certain degree of alcohol. Once the fermentation has been completed, sulfite is added, and lees and skins are removed from the wine by racking or centrifuging to eliminate the risks of hydrogen sulfide formation. The time of racking varies according to whether a malolactic fermentation is desired. Update review in 2005 (http://www.lallemmandwine.com/IMG/pdf_LALLEMAND_MLF_IN_WINE.pdf) gave a good explanation of malolactic fermentation and modern commercial wine production.

Aging In red wines, a *secondary fermentation*, the *malolactic fermentation*, converts malic acid into lactic acid, reducing the total acidity and raising the pH. The lower acidity improves the taste of high acid wines and gives the beverages a special and desirable flavor. In the United States, where native flora do not produce this second fermentation, a suitable bacterial culture such as *Leuconostoc oenos* (reclassified as *Oenococcus oeni*) is introduced. Most wine makers do not especially favor malolactic fermentation for white wines, though studies of its use with high acid white wines continue. Aging allows the release of amino acids into the wine by autolyzing yeast. The amino acids then aid in the growth of LAB, which bring about malolactic fermentation. During storage, the excess tannin-like compounds are gradually oxidized or combined with protein precipitates and a clarification occurs. The wine is often clarified by addition of fine agents such as bentonite, egg white or gelatin to remove other suspended or precipitable materials. Finally, filtration through a pad filter with or without a filter aid completes the clarification. Following filtration into bottles and corking, the wine is allowed to age for one to several years. Wines are sometimes heated before bottling to prevent microbial contamination. During aging the color slowly changes from red to light brown, the flavor becomes more complex, with the development of a bouquet, and the texture rounder and fuller. The wines may be converted into sparkling wines by one of two methods: direct injection of excess carbon dioxide or secondary fermentation under anaerobic conditions, with added sugar. A schematic outline of the wine-making process is presented in Figure 3.4.

New developments in wine making Ontario, Canada's main ice wine region, expects to harvest 5500 tons this season, a 50% increase on last year. Ice wine is a type of dessert wine produced from grapes that have been frozen while still on the vine. The sugars and other dissolved solids do not freeze, but the water does, allowing a more concentrated grape must to be pressed from the frozen grapes, resulting in a smaller amount of more concentrated, very sweet wine. With ice wines, the freezing happens before the fermentation, not afterwards.

Significant advances have been made in nearly all areas of viticulture and enology, and today the state of the art of wine making has reached a high level. The study of wine yeasts and their genetics had been neglected, but some work in the application of genetics to wine yeasts was initiated since 1970s. The most desirable characteristics of wine yeast for improvement by rDNA technology are shown in Table 3.4.

Wine yeast strain selection is based on fermentation performance more than on sensory characteristics of the wine, with emphasis on increased tolerance of the yeast to alcohol and of bacteria to low pH. Hardly any wine yeast in commercial use has all these characteristics. Some degree of variation can be achieved by altering the fermentation conditions such as temperature, but the major source of variation is the genetic constitution of the wine yeasts. Thus it is possible to change the properties or characteristics of yeast by altering the genetic material of the organism. Several genetic techniques (e.g., hybridization, mutagenesis, cell fusion, transformation, genetic engineering) may be used to modify the properties of wine yeasts. A first attempt at genetic selection in yeast was achieved with *Saccharomyces ellipsoideus*, a yeast strain used in wine making. The genetic criteria selected, in the hope of

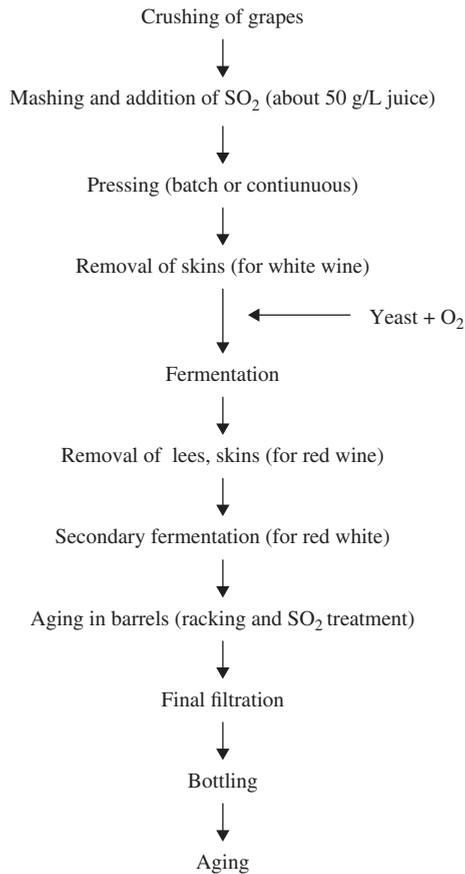


Figure 3.4 Schematic outline of wine making.

Table 3.4 Some targeted desirable characteristics of a wine yeast that may be improved by recombinant DNA technology

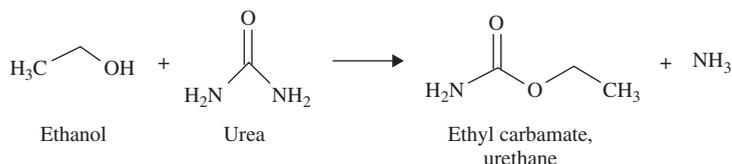
1. Alcohol-tolerant wine yeast
2. Manipulability of wine yeasts having a killer character (to obtain yeasts that would be immune to their own toxin and that of related killer yeasts and would prevent the proliferation of wild yeasts)
3. Possibility of constructing wine yeasts that can carry out malolactic fermentation by cloning malolactic genes into *S. cerevisiae*
4. Sedimentation characteristics that allow for easier separation of yeast from wine
5. Genetic manipulation of flavor development by yeast

obtaining improved wine yeast, were energy of fermentation, color stability, resistance to SO₂, inhibitory effect on acetic acid enzymes, and development characteristics.

Leuconostoc, *Lactobacillus*, and *Pediococcus* are the primary genera of lactic bacteria involved in the malolactic reaction. To provide malolactic capability in wine yeast, the required *Lactobacillus delbrueckii* gene from *Luc-1* was successfully introduced into wine yeast using rDNA techniques, with the result that primary and secondary fermentations are controlled by a single yeast. The yeast killer toxin gene was integrated into a genome of wine yeasts by rDNA technology, the objective being to obtain commercial wine yeasts resistant to killer toxins. This modified material was found to have a wide spectrum of killing and to offer advantages over other strains of yeast in wine fermentation.

3.2.3.3 Health aspects of beer and wine Both beer and wine contain significant amounts of polyphenols, antioxidant phytochemicals that occur naturally in plants and these have been shown to reduce “bad” LDL cholesterol, reduce the risk of heart disease and certain cancers and prevent free radicals from causing cell damage. Studies have shown that similar levels of polyphenols are found in both red wine and beer, which is about four or five times the amount found in white wine. Drinking alcohol decreases the risk for some diseases, but increases others. At a moderate level, the benefits simply outweigh the harms.

The presence of ethyl carbamate, a suspected carcinogen, is subjected to regulation in many countries. The compound is produced from the degradation of the amino acid arginine, which is present in both grape must and beer that released through the autolysis of dead yeast cells. While the use of urea as a source of yeast assimilable nitrogen (no longer legal in most countries) was the most common cause of ethyl carbamate in wine, *O. oeni* and *Lactobacillus buchneri* have been known to produce both carbamyl phosphate and citrulline which can be precursors to ethyl carbamate formation. *Lactobacillus hilgardii* has also been suspected of contributing to ethyl carbamate production. All yeast-fermented alcoholic beverages contain traces of ethyl carbamate (15 ppb to 12 ppm). Other foods and beverages prepared by means of fermentation also contain ethyl carbamate. It has been shown that ethyl carbamate forms from the reaction of alcohol (ethanol) with urea:



This reaction occurs much faster at higher temperatures, and therefore higher concentrations of ethyl carbamate are found in beverages that are heated during processing, such as brandy, whiskey, and other distilled beverages. Additionally, heating after bottling either during shipping or in preparation will cause ethyl carbamate levels to rise further.

Biogenic amines including tyramine, histamine, and phenylethylamine produced mostly from fermented foods have been known to cause nausea, headaches, and respiratory disorders. Biogenic amines have been implicated as a potential cause of red wine headaches. In wine, histamine, cadaverine, phenylethylamine, putrescine and tyramine have all been detected. These amines are created by the degradation of amino acids found in grape must and leftover from the breakdown of dead yeast cells after fermentation. Most LAB have the potential to create biogenic amines, even some strains of *O. oeni*, but high levels of biogenic amines are most often associated with species from the *Lactobacillus* and *Pediococcus*

genera. In the European Union, the concentration of biogenic amines in wine is beginning to be monitored while the United States currently does not have any regulations.

3.2.3.4 Distilled beverage spirits As with beer, distilled beverages have legal definitions that specify the raw materials and some of the processes that must be used. These definitions ensure that all products of the same name have the same basic character. In general, distilled beverages are the result of both a fermentation and a distillation step. Depending on the intended end product, the raw materials that are used will vary. *Brandy* is a distilled wine, rum is made from sugarcane or molasses, *rye whisky* from rye, *Scotch whisky* from malted barley, and *bourbon whisky* from corn (>51%), rye, and malt. On the other hand, *vodka* can be made from a wide range of grains or from potatoes. Brandy, rum, and gin are substantial items in world markets, but whiskies are by far the leading distilled spirits. Canada, Scotland, and the United States account for most of the sales. The Irish use the spelling *whiskey*, the Scotch and Canadians use *whisky*, and US citizens use both, though US regulations use the former spelling. Canadian whisky manufactured according to the laws of Canada contains no distilled spirits less than 3 years old. Since these products are blended, they are not straight whiskies. Although the major cereal grains such as corn, rye, and barley malt are used, their formulation in the mashing step remains a distiller's trade secret. The characteristic unique smoky flavor of Scotch whisky is due to the aroma of burning peat and a high standard of quality, but not much is known about government regulations and required processing methods. The grain whiskies used in Scotch brands are generally aged in matured oak casks of 190 L capacity, whereas US and Canadian barrels are slightly bigger (200 L). Unlike Scotch, Irish whiskey does not have a smoky taste. In the United States, a detailed statement of the production process and any improvements or changes must be filed with and approved by the federal government before being placed into operation. Consequently, the distiller is restrained within narrow limits of trade secrets and does not enjoy the degree of latitude available to the Canadian and Scotch distillers.

The steps for the production of distilled spirits are similar to those for beer. The first step in most typical processes is the milling of the grains, which increases the surface area, hence enhances enzymatic activity. The mashing process involves cooking the milled grains in water to hydrate and swell the starch granules, resulting in a gel for whisky production. Batch cooking at atmospheric pressure is commonly used, though some batch pressure cooking is practiced. Amylases are included with the grain to partially break down the gel matrix (liquefaction) in the cooker. After cooking at 120–152 °C and cooling to 67 °C, more amylases from malt and increasingly from microorganisms such as *Bacillus* and *Aspergillus* are added to the mash to convert the grain sugars.

When saccharification is complete, the resulting sugars (largely maltose) are inoculated with a yeast culture, *S. cerevisiae*. The set mash is inoculated with 2–3 vol% of ripe yeast prepared separately. However, *Schizosaccharomyces pombe* is used for certain rums. The mash is usually acidified to the optimum pH (3.9–4.1) by a 4–8 h fermentation at 41–54 °C with *L. delbrueckii*. Before inoculation with yeast, the sour mass is pasteurized to 71–87 °C to curtail bacterial activity. Temperatures of 21–24 °C are chosen for yeast development to ensure that the fermentation does not go above 32 °C, which would result in loss of yield and development of off-flavor by bacterial contamination. The sugar in the mash is usually depleted within 48 h, but to optimize alcohol yields and to obtain characteristic flavors, most fermentations require 3 days, and the end product is known as beer.

Alcohol recovery from beer is accomplished by using a multilayer continuous still. The discharge from the base of the whiskey column is called stillage and contains substances derived from grain and from the mashing and fermentation processes. The by-products, known as distillers' dried grains and distillers' solubles, are used by the feed industry

to fortify cattle, poultry, and swine formula feeds. The final alcohol content should be approximately 40 vol%. If vodka is to be the end product, the distilled alcohol is treated with activated charcoal to remove any unwanted colors and flavors. The colorless, tasteless liquid is called *neutral spirits* and is bottled as vodka.

In all other forms of whisky and brandy, maturation in a cask made of a particular wood is required. The cask helps in developing the characteristic flavors of whisky by imparting new flavors to the spirits, while altering (i.e., mellowing) the existing flavors. Each distilled beverage has its own requirements for type of cask and minimum time in the casks. The changes occurring during the maturation of whisky in the barrel are likely to include the extraction of complex wood constituents by the liquid, the oxidation of components originally in the liquid and of material extracted from the wood, and reactions among the various organic materials, leading to the formation of new secondary products (known as *congeners*).

3.2.3.5 Sake Although *sake* is sometimes called rice wine, its production more closely resembles that of beer with a saccharification step. Sake production is based on the simultaneous occurrence of the saccharification of the sugars in rice with fermentation, and small quantities of rice and the yeast culture are added during the fermentation. In sake production, the inhibition of the yeast by elevated concentrations of substrate is avoided by bringing about the action of two different microorganisms in succession; the result is a beverage with high alcohol content (25% v/v). The first step is the polishing of the rice. Once the proteins, lipid, and ash have been removed from the grains, the rice is soaked in warm water (20–25 °C). This water is then removed, and the rice is steamed for 30–60 min. The steaming has the effect of sterilizing the rice, denaturing its proteins, enlarging water absorption capacity, and converting starch to the α form. *Koji* is a culture of *A. oryzae* that has been grown on steamed rice; it contains about 50 enzymes, but those of importance are α -amylases. The glucoamylases in sake production contain acid and alkaline proteases.

The third step in sake production is the preparation of *moto*, which is the yeast starter culture. The modern way of preparing *moto* is by artificially acidifying the culture with LAB at the start of the fermentation, followed by inoculation with yeast cultures. The yeast used is *Saccharomyces sake*, but *S. cerevisiae*, which is tolerant of ethanol, allows the production of a beverage with a higher content of alcohol. The first fermentation, called the *moromi*, is achieved in an open vessel. Successive additions of fresh substrates (steamed rice and koji) result in a high alcohol content. The conversion of starch to fermentable sugars by *A. oryzae* and the fermentation of those sugars to alcohol by *S. cerevisiae* take place at the same time. The fermentation lasts for 25 days, with temperatures ranging from 7 °C at the start to a maximum of 18 °C. After the sake has been filtered under pressure, the alcohol extract is followed by a maturation period of 5–10 days. Both *A. oryzae* and *S. sake* provide enzymes for the maturation process. The sake is then filtered through activated charcoal. At this point another maturation period may be allowed before pasteurization and bottling. Pasteurization is necessary to inactivate the ethanol-tolerant LAB, which often spoil the sake. Usually sake is bottled in opaque materials to avoid photooxidation reactions.

Figure 3.5 summarizes the steps of sake manufacturing. In the process depicted, mutants of *S. cerevisiae* to be used as sake yeast were selected from a haploid wild type using an analog, 5,5,5-trifluoro-D-leucine. The mutants had overcome inhibition of α -isopropylmalate synthetase by L-leucine and produced much higher concentrations of isoamyl alcohol and isoamyl acetate than the wild type. The use of a suitable mutant sake yeast can thus produce excellent sake flavor without the usual requirements for highly polished rice and a low fermentation temperature.

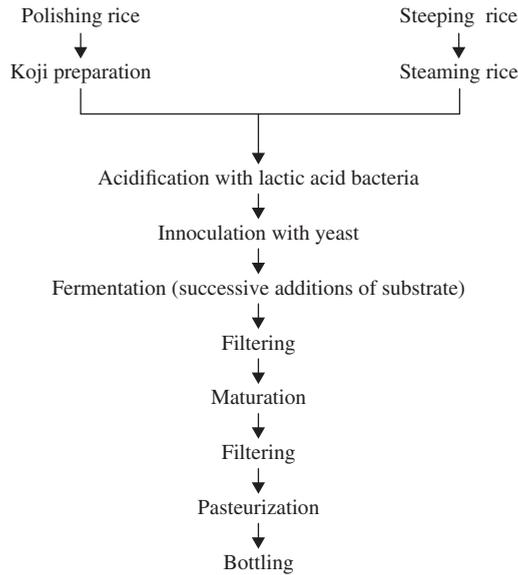


Figure 3.5 Schematic operation of sake production.

Summary

There are virtually thousands of varieties of alcoholic beverages produced around the world. Four of the major beverages – beer, wine, distilled spirits, and sake – were discussed. Most, if not all, other alcoholic beverages are local variations on these four. The production of alcoholic beverages is the oldest biotechnological process known to man. In fact, modern brewing has changed little for more than 50 years. Continuing development of this industry has been slow, mainly because of the difficulty of improving the yeasts, but steady nonetheless.

Recombinant DNA technology holds great promise of imparting new, desirable characteristics to yeasts, while not compromising the traits already possessed. This technology is hard to apply to commercial yeast strains, however, because these yeasts rarely, if ever, contain a dominant selectable marker. Some results have been obtained with protoplast fusion, but instability of the hybrids in the absence of selection is a universal problem. The importance of alcohol in today's societies is unquestionable, and for this reason the alcoholic beverage industry will continue to flourish for decades to come.

3.3 Industrial alcohols

3.3.1 Introduction

Industrial alcohol includes power or fuel alcohol that is used in combination with gasoline or other motor fuels. The use of industrial alcohol started in the 1800s with the growth of the synthetic chemical industry, and it expanded very rapidly in the United States in the early

1900s. For many years, however, all ethanol except that used for beverages was obtained by chemical means instead of through the catalytic hydration of ethylene. In the light of present-day energy shortages, ethanol from fermentation has gained renewed importance. In countries with a large agricultural surplus, such as Brazil, South Africa, Canada, and the United States, intensive studies are being conducted on the production of ethanol from carbohydrate materials (sucrose, starch and plant biomass, etc.). Currently, about 80% of fuel-grade alcohol in the United States comes from fermentation, and the current ethanol production level is the equivalent of about 65,000 barrels per day of imported oil. The choice of the raw material is very critical because it makes up 55–75% of the final alcohol selling price. The microorganisms used to produce alcohol biochemically are directly cultivated in the fermentation substrate or in another suitable medium. At the present time, industrial alcohol is mainly recovered by a thermal separating process, so-called *distillation*. The United States became the world's largest producer of ethanol fuel in 2005. Brazil and US production accounted for 87.1% of global production in 2011. In the United States, ethanol fuel is mainly used as an oxygenate in gasoline in the form of low-level blends up to 10%, and to an increasing extent, as E85 fuel for flex-fuel vehicles.

The ethanol market share in the US gasoline supply grew by volume from just over 1% in 2000 to more than 3% in 2006 to 10% in 2011. Domestic production capacity increased 15 times after 1990, from 900 million US gallons to 1.63 billion US gallons in 2000, to 13.5 billion US gallons in 2010. The Renewable Fuels Association reported 209 ethanol distilleries in operation located in 29 states in 2011, and 140 under construction or expansion as of December 2011, that upon completion, would bring US total installed capacity to 15.0 billion US gallons. Most expansion projects are aimed to update the refinery's technology to improve ethanol production, energy efficiency, and the quality of the livestock feed they produce.

3.3.2 Raw materials and microorganisms

Raw materials may be classified into three principal types: (i) the saccharine materials such as sugarcane, sugar beets, molasses, and fruit juice, (ii) starch materials such as cereals, potatoes, Jerusalem artichokes, and manioca, and (iii) cellulosic materials such as wood and sulfite liquor. The efficiency of energy conversion by ethanol fermentation varies considerably depending on the starting material. Saccharine materials such as molasses usually require little or no special preliminary treatment other than dilution and can be fermented directly after certain customary adjustments have been made in the mash. Basically, all raw materials that contain fermentable sugars or constituents such as starch and cellulose can be converted into sugars and ethanol by fermentation. Based on modern commercial and industrial energy demand, the two most important end products of carbohydrate conversion are *ethanol* and *methane*. However, ethanol production is a far more attractive proposition, and the economics of ethanol fermentation are particularly important owing to the potential fuel and chemical uses of this alcohol. Because of plans to phase out the use of lead in gasoline in the interest of pollution control, in 1990 the level of gasoline consumption in the United States alone ($\approx 100 \times 10^9$ gallons annually) means a significant boost in use of fuel-grade ethanol. Ethanol in a 10% blend is known to increase the octane rating by about three points; or, the ethanol can be used at the 2.5% level as a cosolvent with methanol.

One of the most important commercial issues relating to fuel-grade alcohol production is the availability and cost of the feedstocks. Table 3.5 shows the potential alcohol yield from some raw materials. From the sugar source, only 50% by weight is usually transformed to ethanol, and the rest is used as a carbon source. Thus, 1 g of sugar converted with 90%

Table 3.5 Theoretical ethanol yield from different biomass feedstocks

Feedstock	Theoretical ethanol yield (gallons/dry ton of feedstock)
Corn Grain	124.4
Corn Stover	113.0
Rice Straw	109.9
Cotton Gin Trash	56.8
Forest Thinnings	81.5
Hardwood Sawdust	100.8
Bagasse	111.5
Mixed Paper	116.2
Switchgrass*	96.7

*74 Switchgrass Alamo Whole Plant.

Source: US Department of Energy Bioenergy Technologies Office (http://www.afdc.energy.gov/fuels/ethanol_feedstocks.html).

efficiency will give about 0.45 g of alcohol. To achieve an optimum fermentation process, the liquid sugar is adjusted to a desirable sugar concentration, temperature, and pH by addition of acid, mixed with the starter culture. Approximately 2.5 L of molasses (3.5 kg) is required to produce 1 L of 95% ethanol.

Ethanol can also be produced from fruits and fruit juice sugars, and from the waste of fruit canneries. The fermentation of lactose in whey, a by-product of cheese making, has also been used with *Torula cremoris* and *Candida pseudotropicalis* in small industrial plants for the production of ethanol. *S. cerevisiae* cannot utilize lactose directly, whereas the yeast *Kluyveromyces lactis* can utilize lactose but cannot perform an efficient alcohol fermentation. To develop an efficient lactose fermenting yeast, the β -galactosidase gene from *K. lactis*, along with the cloned lactose permease gene, was introduced into *S. cerevisiae*, leading to the fermentation of lactose. Starch and cellulosic materials must be hydrolyzed to fermentable sugars before they can be utilized by yeast.

Corn is the main feedstock used for producing ethanol fuel in the United States. Most of the controversies surrounding US ethanol fuel production and use are related to corn ethanol's energy balance and its social and environmental impacts, as bioethanol is competing with food.

Cellulosic sources have the potential to produce a renewable, cleaner-burning, and carbon-neutral alternative to gasoline. Significant research efforts have been invested in enzymatic, thermochemical, acid hydrolysis, hybrid hydrolysis/enzymatic, and other research approaches targeting more efficient and lower-cost conversion of cellulose to ethanol. Ethanol yields (L)/ton of cellulosic biomass are between 140 (cane bagasse) to 228 (corn stover) and production costs are about 0.396 (US\$/L) in Table 3.5. Cellulosic ethanol currently costs about double the price as making the fuel from corn, that is about US\$2.25 per US gallons (US\$0.59 per liter), primarily due to the current poor conversion efficiency of cellulose. However, biological advances in the fungi and other organisms used to break down woody plant bits into fuel will make the process cheaper. Switchgrass is an alternative that can sequester carbon dioxide in the ground because they have extensive root systems that remain buried after the crop is harvested.

Table 3.6 Fermentation ethanol from feedstocks

Starch feedstock	Microorganism	Net feedstock costs* (US\$/gallons)
Maltose (starch)	<i>Saccharomyces cerevisiae</i>	–
Wheat (Europe)	<i>Saccharomyces cerevisiae</i>	1.53
Wheat	<i>Saccharomyces cerevisiae</i>	1.23
Corn (wet-milling)	<i>Saccharomyces cerevisiae</i>	0.38
Corn (wet-milling, without by-products)	<i>Saccharomyces cerevisiae</i>	1.00
Cassava	<i>Saccharomyces cerevisiae</i>	0.69
Sugar cane	<i>Saccharomyces cerevisiae</i>	0.81
Insulin	<i>Kluyveromyces marxianus</i>	0.64
Jerusalem artichokes	<i>Torulopsis colliculosa</i>	–
Without by-products	<i>Saccharomyces cerevisiae</i>	–
	<i>S. diastaticus</i> , <i>K. cicerisporus</i>	–
Sorghum	<i>Zymomonas mobilis</i>	–
Cellulose	<i>Saccharomyces cerevisiae</i>	2.25 [†]

*1982 and 2010 price.

†2010 price.

At the present time, it is possible to convert starch enzymatically into mono- and disaccharides (sugars), which are then fermented by yeast or *Zymomonas* bacteria to ethanol. The microorganisms involved and the costs of fermentation for ethanol from starch materials are summarized in Table 3.6.

Other potential raw materials for alcohol production such as sulfite waste, whey, new crops, and cellulosic materials are of relatively little interest at the present time. Sulfite wastes and whey are produced in small quantities and are too dilute to transport. On a worldwide scale, lignocellulosic materials are the most abundant source, having been estimated in 1991 at 150×10^9 tons per year. However, one of the key problems in lignocellulosic conversion is the difficulty of hydrolyzing cellulose to glucose. The pretreatment costs for ethanol production from cellulosic materials would account for more than 50% of the total process capital cost. The main disadvantage of these wastes is that the costs of collecting and transporting sufficient quantities to operate a processing facility large enough to be economically viable can be prohibitive, even in less-developed countries. Despite this disadvantage, there have been several studies of the simultaneous saccharification and fermentation (SSF) process, which utilizes the cellulase enzyme, primarily from *Trichoderma* spp. and yeasts or bacterial species, for bioconversion of pretreated cellulose into ethanol. Various approaches entailing immobilization and recycling systems or an enriched vapor stream have also been used to increase the volumetric productivity of ethanol from cellulose. To develop a successful SSF process, the compatibility of the saccharification and fermentation system with respect to temperature, pH, and substrate concentration must be developed. The optimum temperature of *Trichoderma reesei* cellulase is about 50°C; for the anaerobic fermentation by *S. cerevisiae* or *Zymomonas mobilis*, it is 30°C. Thus, a compromise between two temperatures must be considered. The use of thermotolerant yeasts or bacteria such as *Clostridium thermosaccharolyticum*, *C. thermocellum*, and

C. thermohydrosulfuricum has also been tried in an SSF process. The thermophilic bacteria, however, suffer ethanol toxicity and instability of strains that have high ethanol yields. Nonetheless, they may compete with the yeast if more efficient technologies to recover ethanol from dilute solutions are applied. Because fuel alcohol is not destined for human consumption, gene-cloning techniques are most likely to have advantages for constructing ethanol strains that can increase the range of fermentable substrates by incorporating some amylolytic capability into industrial *S. cerevisiae*. Bacteria, *Z. mobilis* are able to produce ethanol faster than comparable yeast concentrations. *Z. mobilis* rapidly ferments glucose, fructose and sucrose, and can yield up to 12% ethanol. Cloning of amylase or cellulase genes into *Z. mobilis* has been tried, but genetic transfer systems for this ethanologen are relatively poorly developed.

Owing to the complex nature of the carbohydrates present in lignocellulosic biomass, a significant amount of xylose and arabinose (five-carbon sugars derived from the hemicellulose portion of the lignocellulose) is also present in the hydrolysate. In the hydrolysate of corn stover, approximately 30% of the total fermentable sugars is xylose. As a result, the ability of the fermenting microorganisms to use the whole range of sugars available from the hydrolysate is vital to increase the economic competitiveness of cellulosic ethanol and potentially biobased proteins. In recent years, metabolic engineering for microorganisms used in fuel ethanol production has shown significant progress. Besides *S. cerevisiae*, microorganisms such as *Z. mobilis* and *E. coli* have been targeted through metabolic engineering for cellulosic ethanol production. Recently, engineered yeasts have been described efficiently fermenting xylose, and arabinose, and even both together. Other new yeast strain, *Clavispora* NRRL Y-50464 could break down and ferment the sugars in corn cobs left behind after xylose has been extracted. This strain can tolerate cob-derived compounds that interfere with yeast growth and fermentation rates at 37 °C than normal 30 °C This takes place without the addition of a costly enzyme, a breakthrough that could help make cellulosic ethanol production a cost-effective proposition, thus SSF, a one-step process in cellulosic ethanol production that combines releasing and fermenting feedstock sugars may be possible in the future (<http://phys.org/news/2013-05-yeast-strain-cellulosic-ethanol-production.html>). Other thermophilic bacterium, *Thermoanaerobacterium saccharolyticum* growing at 50 °C has also been metabolically engineered. However, most of all yeast cells are especially attractive for cellulosic ethanol processes because they have been used in biotechnology for hundreds of years, are tolerant to high ethanol and inhibitor concentrations and can grow at low pH values to reduce bacterial contamination. Cellulosic ethanol commercialization still faces a number of challenges. Capital and operating costs are expected to remain higher than for corn ethanol producers, and even they are currently struggling with low margins. Cyanobacteria have the potential to produce cellulose, glucose and sucrose, the latter two easily converted into ethanol. This offers the potential to create ethanol without plant matter. Cyanobacteria, enzymes and microbes, combined with ingredients from plants and human waste and grasses offer the potential to replace all food ingredients now converted into ethanol at a fraction of the financial and economic cost. Unlike lignocellulosic biomass, which is a major sugar in seaweed, an abundant source of galactose is available, but ethanol yield and productivity from galactose by *S. cerevisiae* are significantly lower than those from glucose; thus, inverse metabolic engineering has also been tried to improve the yield.

Other simple gasification processes that do not rely on chemical decomposition of the cellulose chain (cellulolysis) have been proposed. Instead of breaking the cellulose into sugar molecules, the carbon in the raw material is converted into synthesis gas, using what

amounts to partial combustion. The carbon monoxide, carbon dioxide and hydrogen may then be fed into a special kind of fermenter and *Clostridium ljungdahlii* bacteria are growing in carbon monoxide, carbon dioxide and hydrogen, which will produce ethanol and water. The process can thus be broken into three steps: (i) Gasification-complex carbon-based molecules are broken apart to access the carbon as carbon monoxide, carbon dioxide and hydrogen, (ii) fermentation-convert the carbon monoxide, carbon dioxide and hydrogen into ethanol using the *C. ljungdahlii* organism, and (iii) distillation-ethanol is separated from water.

3.3.3 Production processes

Three-quarters of ethanol production uses the classical batch method, which is a slow process, followed by an efficient multistep distillation. Figure 3.6 gives the schemes of a classical process. In this process, the fermentation of the wort (molasses) or mashes takes place in a cylindroconical Nathan vessel with yeast that has been cultivated in a separate process step. The continuous multiplication of the working yeast takes place in a prefermentation tank in sterilized molasses and is distributed over the main fermentation tank. Between fermentation and distillation, the yeast can be separated and used to inoculate (pitch) the substrate coming in for the next fermentation. In the case of starch materials or a whole-grain mash, the recovery of the yeast from fermented mashes is not possible and a separate yeast mash must be processed: the by-product is used as animal feed and sold as distillers' dried grains, instead of as corn gluten.

Classical fermentation is achieved in three main steps. During the first 12–24 h, yeast cells multiply rapidly aerobically by consuming oxygen present in the mash. In the middle phase (12–48 h), predominant alcohol formation occurs with the postsaccharification of oligosaccharides, and the multiplication of yeasts falls off, accompanied by release of

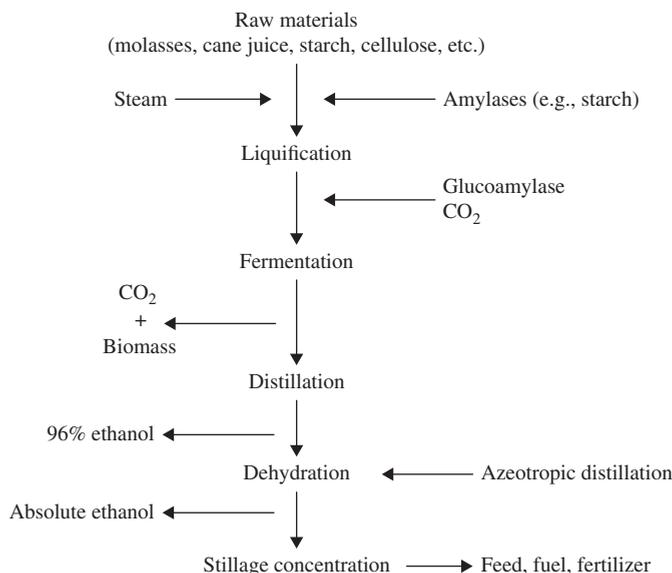


Figure 3.6 Scheme for a continuous flow fermentation process.

heat and rise of temperature to 40°C. Finally, there is an asymptotic decrease of alcohol formation, along with insignificant yeast growth at the final stage (48–72 h). Ethanol entrained by the carbon dioxide evolving from fermentation is recovered by a CO₂ washing process. The fermentation time can be shortened to 36–48 hours by using a higher pitching temperature and a larger amount of yeast mashes. Since aseptic conditions with complete sterilization of the very large mash volume have been considered impossible, batch fermentation normally is carried out without complete sterilization of media and equipment. Occasionally, some contamination by LAB occurs, reducing alcohol yield as much as 20%. Such contamination is more likely when stillage backset is used, since the contaminating organisms accumulate and become acclimatized to the fermentation conditions.

Recently, continuous processes have been developed in which the raw mash is fed in at the same rate as spent mash is removed, some yeast in the spent mash being fed back into the tank. This method is more rapid (8–18 h), depending on temperature and sugar content, because the yeast culture is continuously in an active state, and, also, more rigid process control can be maintained with respect to temperature and pH. There are three types of continuous process: the conventional continuous stirred-tank reactor (CSTR), the CSTR coupled with a cell recycler and the immobilized cell reactor. The last two processes show improvement over the conventional CSTR because the use of high cell densities results in a faster rate of fermentation. These processes make use of modified fermentors such as the tower, and dialysis, plug, hollow fiber, and pressure rotor devices. Agitation by stirring or gas sparging was found to be important for successful flow fermentation.

3.3.4 Economics

Distilling, rectifying, and blending of spirits; ethyl alcohol production reached a volume of 160.2 billion liters of alcohol in 2006, a large portion of which was used in beverage blending. The United States is the world's largest producer of ethanol, having produced over 13 billion gallons in 2012 alone. Together, the US and Brazil produce 87% of the world's ethanol. The vast majority of US ethanol is produced from corn, while Brazil primarily uses sugar. The United States accounted for 25% of world consumption. In the United States (1981), about 775 ML was used for industrial solvent and chemical applications, 269 ML as fuel and 38 ML for exportation. In the United States, the production of *bioethanol* or *gasohol* from the fermentation of corn grain is increasing rapidly and is creating an increase in the crop production affecting the corn price. The market sale of 113 ML of fuel alcohol in 1981 resulted in a decline in the prices of synthetic ethanol. Gasohol is successfully marketed as a premium fuel, superunleaded with ethanol, and is expected to increase the demand for the new fermentative ethanol capacity.

Economic considerations include the capital cost of the plant, the energy input, and the selling price of ethanol. The most significant cost item under operating expenses is the cost of raw materials, which accounts for as much as 80% of the total. Energy saving can be greatly reduced by employing integrated plant energy schemes. Mashing, cooking, saccharification, and fermentation have also been improved by continuous operation. Furthermore, improvements to the technology may be possible, but their contribution to the overall efficiency and economics of grain-based fermentation plants may be only minor. Although a great deal of research activity has been directed toward developing cellulose as a raw material for fermentation, it has yet to prove cost-competitive with the sugars and starches. Lactose from whey has also been used but with limited success, mainly because of the limited supply of yeast species that are capable of utilizing lactose and are tolerant to high concentrations of alcohol.

Summary

The substrate necessary for the production of ethanol is derived from various starting materials such as molasses, corn, wood, and sulfite waste liquor. The selection of the raw material determines the manufacturing process and the selling price of the final product. The yeast *S. cerevisiae* is used for fermentation and converts glucose, a hexose sugar, to ethanol by an anaerobic pathway. Fermentation has been carried out by two different types of process: batch and continuous. After fermentation, the ethanol is recovered and concentrated by distillation and rectification. In the near future, the world must face the reality that remaining nonrenewable energy sources will no longer suffice for our needs. It is wise, therefore, that we continue to prepare for that eventuality by developing alternate technologies for the utilization of renewable resources. The use of bioethanol for internal combustion engines appears to be very promising for the alcohol industry. Metabolic engineering will make major contributions to yeast and *Zymomonas* technology over those made by classical genetic modification. It is also important to develop strains that can secrete both α -amylase and glucoamylase for better conversion of starch directly to ethanol.

Several yeasts, in particular *S. cerevisiae*, have been widely used in genetics and cell biology, largely because *S. cerevisiae* is a simple eukaryotic cell, serving as a model for all eukaryotes, including humans, for the study of fundamental cellular processes such as the cell cycle, DNA replication, recombination, cell division, and metabolism. Yeasts are also easily manipulated and cultured in the laboratory, which has allowed for the development of powerful standard techniques, such as yeast two-hybrid, synthetic genetic array analysis, and tetrad analysis. Many proteins important in human biology were first discovered by studying their homologs in yeast; these proteins include cell cycle proteins, signaling proteins, and protein-processing enzymes. On 24 April 1996, *S. cerevisiae* was announced to be the first eukaryote to have its genome, consisting of 12 million base pairs, fully sequenced as part of the Genome Project after seven years and the involvement of more than 100 laboratories to accomplish. The second yeast species to have its genome sequenced was *S. pombe*, which was completed in 2002. It was the sixth eukaryotic genome sequenced and consists of 13.8 million base pairs. As of 2012, over 30 yeast species have had their genomes sequenced and published.

3.4 Bread and related products

3.4.1 Introduction

Bread making began as an art. The leavening action of sourdough by spontaneous fermentation was largely due to its natural mixed populations of LAB (heterofermentative lactobacilli) and some kind of yeast. The ancient Egyptians, some 6000 years ago, appear to have been the first to use leaven to make bread and to use ovens for baking bread. From Roman times to the middle of the nineteenth century, there was no production of yeast intended for use in bread making. Distiller's yeast had been prepared in pressed form for bakers as early in 1781, but the first Vienna process was developed for baker's yeast in 1846. The air process, introduced in 1877, was based on Pasteur's observation that yeast growth is stimulated by aeration. Modern production of baker's yeast began from 1915 to 1920 with the introduction of a fed-batch process (*Zulauf* process), which was carried out in semicontinuous operation with incremental feeding of wort. This method resulted in a high yield and improved storage stability. The continuous fermentation process has further developed,

but it has not displaced the fed-batch method, which is still continuously practiced. For all bread and baked products, leavening is achieved by microbial fermentation.

3.4.2 Ingredients and formulations

Breads cover a wide different variety of products, with variations in recipe, processing, shape, weight, and so on, but they contain the same four basic ingredients: wheat flour, salt, yeast or sourdough, and water. For the most part, however, conventional white pan bread (Table 3.7) and most specialty breads include optional ingredients to enhance overall product quality: namely, fat or shortening, sugars, milk powder, eggs, honey, syrups, fruits, spices and other aroma compounds such as cocoa, and fats. Other additives, including enzymes, swelling agents, emulsifiers, oxidizing agents, and reducing agents, have been developed to compensate for variations in the processing characteristics of flours. These ingredients contribute to loaf volume, crumb softness, grain uniformity, silkiness of texture, crust color, flavor, and aroma as well as to nutritive value. Specified amounts in the production of various bread products are legally defined by the US Food and Drug Administration. A representative formula for white pan bread (Table 3.7) includes flour (100%), water (55–65%), yeast (1–3%), salt (1.75–2.25%), sweetener solids (0–8%), shortening (0–8%), dairy blends (0–4%), yeast food (0–0.5%), protease (0–0.25%), emulsifier (0–0.5%), dough conditioner (0–0.5%), and preservative (0–0.2%). Flour is the major ingredient in bread making and influences the cost of baked products.

Wheat flour contains a unique protein called *gluten* which, when hydrated with water, form a viscoelastic protein structure under mixing and other processing conditions. During baking, the dough protein coagulates and the hydrated starch partially gelatinizes to form the structure of the bread. Bread-making flour must contain wheat protein of the required quantity and quality for the desired product. Water hydrates the flour proteins, which are partially absorbed by the starch and form a water phase in the dough, where soluble solids such as sugars, salt, and proteins are dissolved, and yeast cells are dispersed. The major role of yeast is leavening or aeration of the dough mass by anaerobic fermentation. The rate of

Table 3.7 Ingredient formulations for white pan bread: bulk fermentation, sponge, and dough

Ingredients	Bulk fermentation % (on flour weight)	Sponge (%)*	Dough (%)*
Flour	100	25	75
Yeast	1	0.7	2.0
Salt	2	0.5	1.5
Fat [†]	0.7	–	0.7
Soya flour [†]	0.7	–	0.7
Malt flour [†]	0.2	–	0.2
Water [‡]	57	14	44

*Percent of total flour.

[†]Optional “improving” ingredients.

[‡]Mix to a clear dough at 21 °C and leave to ferment for 12–16 h before adding to dough stage.

Table 3.8 Lactic acid bacteria and yeast occurring in sourdoughs

Lactobacilli		
Homofermentative	Heterofermentative	Yeasts
<i>L. acidophilus</i>	<i>L. brevis</i>	<i>Saccharomyces cerevisiae</i>
<i>L. casei</i>	<i>L. brevis</i> var. <i>lindneri</i>	<i>Saccharomyces exiguus</i>
<i>L. delbrueckii</i>	<i>L. buchneri</i>	<i>Pichia saitoi</i>
<i>L. farciminis</i>	<i>L. fermentum</i>	<i>Candida crusei</i>
<i>L. plantarum</i>	<i>L. fructovorans</i>	<i>Torulopsis holmii</i>
	<i>L. sanfrancisco</i>	

CO₂ production depends on the fermentation activity, concentration, and composition of dough ingredients, as well as on environmental factors (temperature, pH, etc.). The yeast also helps bring about essential changes in the gluten structure, which has been developed (or has matured correctly) to allow it to retain the gas produced. Moreover, yeast fermentation contributes greatly to the flavor of bread through the production of complex microbial metabolites. During oven heating, the *Maillard reaction* between reducing sugars and the dough proteins also creates flavor products in the crust. As mentioned earlier, baker's yeast is available in several forms: yeast cakes, bulk yeast, yeast cream, active dry baker's yeast, and instant active dry yeast. The LAB also produces acids, which contribute to the final flavor and enhance the storage properties. Sourdough starter cultures are commercially available; they contain from 2×10^7 to 9×10^{11} sourdough bacteria per gram and 1.7×10^5 to 8×10^6 yeasts per gram. These are strains specially adapted to dough as their medium and usually belong to the genus *Lactobacillus*. Some sourdough bacteria and yeasts are listed in Table 3.8.

In Germany and the United States, sour breads are produced from rye flour, which is difficult to bake without souring. Doughs containing rye swell during souring, becoming suitably elastic and able to retain gas. The enzymes from sourdough microorganisms strongly degrade pentosans during souring, which lowers the viscosity of rye dough. In modern sourdough bread making, the entire process has been simplified and automated. In San Francisco sourdough bread, heterofermentative lactobacilli, *Lactobacillus sanfrancisco*, and two yeasts, *Torulopsis holmii* and *Saccharomyces exiguus*, most commonly occur. *L. sanfrancisco* grows only on maltose and xylose: arabinose, glucose, galactose, saccharose, rhamnose, and raffinose are not fermented. *T. holmii* ferments glucose, sucrose, galactose, and raffinose but not galactose.

Salt flavors bread by enhancing other flavor products in the loaf and has a retarding effect on fermentation. Fats are used in bread making process primarily for the tenderness and shortness that are important in such products as biscuits, wafers, cookies, and many types of cakes. The shortening effect is due to the formation of a fat film between the starchy and flour gluten layers. The shortening effect is greater for liquid fats than for harder fats. Sugar promotes fermentation and browning.

3.4.3 Production processes

Bread requires a complicated series of operations to produce a particular bread type. After selection and formation of flour and other ingredients, mixing of the diverse ingredients into the dough is essential to obtain a homogeneous mass. Mixing initiates the long series

of complex changes and interactions of the diverse components by applying physical work and ultimately, dough results. The two major objectives of the dough-mixing process are the thorough and uniform dispersion of ingredients to form a homogeneous mixture and the induction of the physical development of the gluten structure in the dough. Significant physical changes during the initial mixing stages are hydration of the flour particles and the incorporation of oxygen. As the dough develops, many complex physical and biochemical changes occur that transform the dough into a complex viscoelastic polymer gel.

In North America, there are four basic methods for making wheat bread dough. Two of the traditional and most popular methods are the *sponge-and-dough process* and the *straight-dough process*. Other, newer methods are *continuous dough mixing* and *no-time dough*. Among mixing methods being practiced in modern bakeries, the most prevalent in North America is the sponge-and-dough process, which involves two mixing stages: one of the sponge and the other of the dough. The sponge mixing stage aims at homogeneous ingredient dispersion and flour dehydration within a relatively short period (4 h). It is followed by intensive mixing of the final dough in horizontal or continuous mixers. The straight-dough method is a single-step process, in which all the ingredients are mixed together and complete dough development must be achieved in a single batch.

Several variations and modifications of the sponge-and-dough and straight-dough methods have evolved over the years. The continuous mixing process, which involves the use of a pre-ferment in the dough mix, has failed to gain widespread consumer acceptance and is currently used on a limited scale for the production of soft hamburger and hot dog buns. The so-called no-time dough processes either involve ultra speed dough mixing or rely on chemical dough development using reducing and oxidizing chemical agents such as ascorbic acid. They are not subject to bulk fermentation and have made inroads into commercial development in the United States only as the Do-Maker and Amflow systems. The primary purpose of both methods is to circumvent the lengthy bulk fermentation stage by imparting to the dough the desired physical character. This principle has been applied to the Chorleywood bread process in the United Kingdom, but operating parameters are very different. The Do-Maker development system is suitable for standard white pan bread and hamburger buns.

The final step in bread making is *baking*, where the raw dough piece is transformed into a light, porous, digestible, and flavorful product under oven heat. Modern ovens are generally designed to convey the baking loaf on trays or a deck through a series of zones, permitting exposure for definite times at different temperatures (191–230 °C) and humidity conditions. Ordinary white pan bread requires a baking time of about 1 min/oz. of dough at 218–232 °C, with steam injection for the first 0.5–2 min of baking. Thus, a pound loaf of bread, scaled to 18 oz. of dough, will take about 18 min to bake. Since it is impossible to establish optimal baking conditions with a high degree of precision, these can be determined only by a practical study of actual oven performance in individual plants. The most apparent effects of oven heat on the dough are expansion of loaf volume, crust formation, the inactivation of yeast and enzymes, the coagulation of dough protein, and partial starch gelatination, as well as extensive dough stabilization. These transformations are accompanied by the formation of new flavor and aroma substances: caramelized sugars, pyrodeoxtrins, melanoidins, aldehydes, ketones, esters, acids, and alcohols. The rate and duration of heat, humidity level, and length of baking time all exert a vital influence on the final quality of the bread. Many of the chemical and physical changes that take place during baking are still not well understood, and research on this area has been significant in recent years.

Cooling is an important part of bread making, since proper cooling is necessary before slicing and packaging to prevent deformed loaves and undesirable moisture condensation inside the package. The general consensus on optimum bread cooling is that the interior

crumb temperature should be reduced to 35–40 °C in as short a time as possible without excessive moisture loss. Bread cooling is normally maintained to the legal limit of 38% moisture. Among the three cooling methods practiced commercially – convection, conditioned air, and vacuum – convection cooling, which is the simplest method, is by far the most prevalent. Though this system does not provide accurate control of moisture loss by the cooling loaf, some adjustments can be made to regulate the overall cooling.

In *conditioned air cooling*, the product is exposed to conditioned air that is maintained at dry-bulb and wet-bulb temperatures that will produce effective loaf cooling within 90 min. Recommended cooling conditions include air temperatures (22–25.5 °C), humidity (85%), and air velocity to bring a temperature rise (8–11 °C) in the air at the exhaust point. Under such conditions, a 1.25-lb loaf of bread will cool to an internal temperature of 32 °C in 90 min. In this system, both temperature and humidity of the cooling medium are held constant, and thus the rate of moisture loss from the cooling loaf is predetermined at the start of the cooling cycle.

The *vacuum cooling* method involves the application of vacuum to the bread, greatly accelerating the vaporization of free moisture from the product, and loss of the latent heat of vaporization. This series of events has a rapid cooling effect on the product. This method of cooling is particularly good for products that are very unstable and prone to collapse before they have cooled, but it finds very limited application at present.

Bread slicing principles have stayed fairly static, but the biggest change in packaging has been the move from wax and cellulose film wrapping to bagging of bread. Packaging films and laminates are available for all requirements, from the perforated packaging of crusty bread to sealed containers for breakfast goods.

3.4.4 New developments

Losses resulting from bread staling, which is starch retrogradation, are of great economic importance, and thus practical efforts to retard the process have centered mainly on the modifications of the method of bread production and on the use of antistaling agents and moisture-retaining substances in the dough formulation (emulsifiers and amylases). The growth of retail baking in supermarkets using the no-time dough processes has been considerable. The small-capacity, semiautomatic equipment allows clean, quick operation, has a low labor requirement, and offers consistent product quality. Complete dough additives have also been produced in sachet form containing all ingredients except flour, yeast, and water. The main products in in-store bakeries are bread, rolls, and brown and continental breads. Many in-store bakeries also make use of commercially available frozen unbaked products such as croissant and Danish pastry.

Bake-off units, which only bake the product, have been opened in supermarkets, convenience stores, airport terminals, shopping centers, and bus and railway stations. A bake-off unit normally uses two types of product: frozen unbaked dough and part-baked bread. The dough is processed to the final molding stage, with the minimum opportunity for yeast fermentation activity, blast frozen at –18 °C, and packed in polythene containers. The fast-freezing of the dough is designed to prevent damage to the yeast, which would result in poor gassing activity in the defrosted dough. For part-baked products, the baking conditions are adjusted to give the minimum of crust formation and color by baking initially at a low temperature and then at a high temperature to set the dough structure quickly; the product is removed from the oven before the crust has colored. The biotechnology of bread

making has also been simplified by improved wheat quality resulting from the manipulation of multi-protein gluten complex.

Summary

Advances in bread making technology over the last 30 years or so have been considerable, involving ingredients, mixing processes, dough development methods, automated processing equipment, and a wide range of products. Bread and related products contain four basic ingredients: wheat flour, yeast, salt, and water. Optional ingredients often are included to enhance overall quality of the product. Microbial leavening agents (yeast and LAB) are used for most breads, rolls, and some sweet goods; but many sweet goods and cakes are leavened chemically. The rate of CO₂ production during pan fermentation depends on the intrinsic properties of yeast, the composition and formulation of dough ingredients, and environmental factors. Instantized ingredients, antistaling agents, retail baking, and the bake-off units are some of new developments in bread making technology.

Bibliography

- Agrawal, M., Z. Mao, and R. R. Chen. 2011. Adaptation yields a highly efficient xylose-fermenting *Zymomonas mobilis* strain. *Biotechnol. Bioeng.* 108: 777–785.
- Bakker, J. and R. J. Clarke. 2011. *Wine: Flavour Chemistry*, 2nd ed. 440 pages. Wiley-Blackwell.
- Bartowsky, E. J. and A. R. Borneman. 2011. Genomic variations of *Oenococcus oeni* strains and the potential to impact on malolactic fermentation and aroma compounds in wine. *Appl. Microbiol. Biotechnol.* 92: 441–447.
- Boulton, C. 2013. *Encyclopaedia of Brewing*. 716 pages. Wiley-Blackwell.
- Boussiba, S., A. Vonshak, Z. Cohen, and A. Richmond. 2000. Procedure for large-scale production of astaxanthin. United States Patent 6022701.
- Cauvain, S. P. and BakeTran. Eds. 2012. *Breadmaking: Improving Quality*, 2nd ed. Woodhead Publishing Series in Food Science, Technology and Nutrition No. 229, pp. 832. Woodhead Publisher, UK.
- Feldmann, H. 2011. *Yeast: Molecular and Cell Biology*. p. 348, Wiley-Blackwell, UK.
- Gibson M. 2010. *The Sommelier Prep Course: An Introduction to the Wines, Beers, and Spirits of the World*, p. 361. John Wiley & Sons, Inc., Hoboken. ISBN 978-0-470-28318-9.
- Guan, Y., Y. Zeng, W. Bai, and Y. Sun. 2013. Utilization of *Candida utilis* cells for the production of yeast extract: effects of enzyme types, dosages and treatment time. *Adv. J. Food Sci Technol.* 5: 551–556.
- Guerin, M., M. E. Huntley, and M. Olaizola. 2003. *Haematococcus astaxanthin*: applications for human health and nutrition. *Trends Biotechnol.* 21: 210–216.
- He, J. and W. Zhang. 2011. Techno-economic evaluation of thermo-chemical biomass-to-ethanol. *Appl. Energy* 88: 1224–1232.
- Johnson, J. C., Ed. 1983. Other food additives: yeast and related products. In *Food Additives – Recent Developments*, pp. 377–402, Noyes Data Corp., Park Ridge, NJ.

- de Jong B., V. Siewers, and J. Nielsen. 2012. Systems biology of yeast: enabling technology for development of cell factories for production of advanced biofuels. *Curr. Opin. Biotechnol.* 23: 624–630.
- Kim, S. J., S. H. Baek, K. S. Lee, and J. S. Hahn. 2013. Cellulosic ethanol production using a yeast consortium displaying a minicellulosome and β -glucosidase. *Microb. Cell Fact.* 12: 14.
- Legras J. L., D. Merdinoglu, J. M. Cornuet, and F. Karst. 2007. Bread, beer and wine: *Saccharomyces cerevisiae* diversity reflects human history. *Mol. Ecol.* 16: 2091–2102.
- Lee, B. H. 1991. Bioconversion of starch wastes. In *Bioconversion of Waste Materials to Industrial Products*, A. M. Martin, Ed., pp. 265–291. Elsevier Applied Science, New York.
- Lee, K. S., M. E. Hong, S. C. Jung, S. J. Ha, B. J. Yu, H. M. Koo, S. M. Park, J. H. Seo, D. H. Kweon, J. C. Park, and Y. S. Jin. 2010. Improved galactose fermentation of *Saccharomyces cerevisiae* through inverse metabolic engineering. *Biotechnol. Bioeng.* 108: 621–631.
- Lin, Y. and S. Tanaka. 2006. Ethanol fermentation from biomass resources: current state and prospects. *Appl. Microbiol. Biotechnol.* 69: 627–642.
- López-Martínez, G., R. Pietrafesa, P. Romano, R. Cordero-Otero, and A. Capece. 2013. Genetic improvement of *Saccharomyces cerevisiae* wine strains for enhancing cell viability after desiccation stress. *Yeast* 30: 319–330, PMID 23576041.
- McBryde, C., J. M. Gardner, M. de Barros Lopes, and V. Jiranek. 2006. Generation of novel wine yeast strains by adaptive evolution. *Am. J. Enol. Vitic.* 57: 423–430.
- Mosier, N., C. Wyman, B. E. Dale, R. Elander, Y. Y. Lee, M. Holtzapple, and M. Ladisch. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour. Technol.* 96: 673–686.
- Nagodawithana, T. 1991. Yeasts, In *Encyclopedia of Food Science and Technology*, Y. H. Hui, Ed., pp. 2877–2904, John Wiley & Sons, Inc., New York.
- Nevoigt, E. 2008. Progress in metabolic engineering of *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* 72: 379–412.
- Pagliardini, J., G. Hubmann, S. Alfenore, E. Nevoigt, C. Bideaux, and S. E. Guillouet. 2013. The metabolic costs of improving ethanol yield by reducing glycerol formation capacity under anaerobic conditions in *Saccharomyces cerevisiae*. *Microb. Cell Fact.* 12: 29.
- Prielhofer, R., M. Maurer, J. Klein, J. Wenger, C. Kiziak, B. Gasser, and D. Mattanovich. 2013. Induction without methanol: novel regulated promoters enable high-level expression in *Pichia pastoris*. *Microb. Cell Fact.* 12: 5.
- Reed, G. and T. W. Nagodawithana. 1991. *Yeast Technology*, 2nd ed., Van Nostrand Reinhold, New York.
- Ribéreau-Gayon, P., D. Dubourdieu, B. Donéche, and A. Lonvaud-Funel. 2006. *Handbook of Enology: The Microbiology of Wine and Vinifications*, 2nd ed., John Wiley & Sons, Ltd, Chichester, UK.
- Rodicio, R. and J. J. Heinisch. 2013. Yeast on the milky way: genetics, physiology and biotechnology of *Kluyveromyces lactis*. *Yeast* 30: 165–177.
- Ruiz, P., P. M. Izquierdo, S. Seseña, E. García, and M. L. Palop. 2012. Malolactic fermentation and secondary metabolite production by *Oenococcus oeni* strains in low pH wines. *J. Food Sci.* 77: 579–585.
- Sainz, M. B. 2011. Commercial cellulosic ethanol: the role of plant-expressed enzymes. *Biofuels* 2011: 237–264.

-
- Scaife, M. A., A. M. Burja, and P. C. Wright. 2009. Characterization of cyanobacterial β -carotene ketolase and hydroxylase genes in *Escherichia coli*, and their application for astaxanthin biosynthesis. *Biotechnol. Bioeng.* 103: 944–955.
- Shaw, A. J., K. K. Podkaminer, S. G. Desai, J. S. Bardsley, S. R. Rogers, P. G. Thorne, D. A. Hogsett, and L. R. Lynd. 2008. Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield. *PNAS, USA.* 105: 13769–13774.
- Smit, A. Y., W. J. du Toit, M. Stander, and M. du Toit. 2013. Evaluating the influence of maceration practices on biogenic amine formation in wine. *LWT – Food Sci. Tech.* 53: 297–307.
- Vincenzini, M., S. Guerrini, S. Mangani, and L. Granchi. 2009. Amino acid metabolisms and production of biogenic amines and ethyl carbamate. In *Biology of Microorganisms on Grapes, in Must and Wine*, pp. 167–180. Springer, Heidelberg.

4

Bacteria-Based Processes and Products

4.1 Dairy products

4.1.1 Introduction

Fermented dairy products are enjoying increased popularity as convenient, nutritious, stable, natural, and healthy foods. Lactic acid bacteria (LAB) are intimately associated with food, feed, and health. For this reason, they have become established as a major target for modern biotechnological research and development. The characteristic aroma, flavor, and texture of fermented dairy foods are often due to the growth of LAB. The LAB form a diverse group of microorganisms, such as the genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, and *Pediococcus*, which are Gram-positive and catalase-, reductase-, oxidase-negative, as well as nonmotile and non-spore-forming. Current taxonomies of LAB and bifidobacteria are reviewed (Björkroth and Koort, 2011; Sonomoto and Yokota, 2011). Increasingly, *Bifidobacterium* is associated with this group and is added to the traditional yogurt cultures. *Bifidobacterium* was often classified to LAB, but it belongs to the high-G+C species, Gram-positive *Actinobacteria* (*Actinomyces*, *Streptomyces*, *Arthrobacter*, *Micrococcus*, *Bifidobacterium*). As an anaerobic bacterium, it ferments glucose via the fructose-6-phosphate phosphoketolase pathway and not via glycolysis. The typical fermentation end products are acetate and lactate. Low concentrations of O₂ and CO₂ can have a stimulatory effect on the growth of these *Bifidobacterium* strains. Based on the growth profiles under different O₂ concentrations, the *Bifidobacterium* species were classified into four classes: O₂-hypersensitive, O₂-sensitive, O₂-tolerant, and microaerophilic. The primary factor responsible for aerobic growth inhibition is proposed to be the production of hydrogen peroxide (H₂O₂) in the growth medium. A H₂O₂-forming NADH oxidase was purified from O₂-sensitive *Bifidobacterium bifidum* and was identified as a *b*-type dihydroorotate dehydrogenase. The kinetic parameters suggested that the enzyme could be involved in H₂O₂ production in highly aerated environments.

Although these LAB have been well studied and have enjoyed longstanding use in the food industry, there are still rapid developments in the field, and new uses and properties

Table 4.1 Lactic acid bacteria and predominant species utilized in the production of fermented dairy products

Products	Species used*
<i>Cultured butter</i>	
Traditional	<i>L. lactis lactis</i> , <i>L. lactis cremoris</i> , <i>L. lactis lactis</i> var. <i>diacetylactis</i> , <i>Leuconostoc cremoris</i>
Recombined	<i>Lb. helveticus</i>
<i>Yogurt†</i>	
Traditional	<i>St. thermophilus</i> , <i>Lb. bulgaricus</i>
Bioyogurt	<i>St. thermophilus</i> , <i>Lb. bulgaricus</i> , <i>Lb. acidophilus</i>
Biogarde	<i>St. thermophilus</i> , <i>Lb. acidophilus</i> , <i>Bifidobacterium bifidum</i>
Yakult	<i>Lb. casei</i>
Acidophilus milk	<i>L. lactis lactis</i> , <i>L. lactis cremoris</i> , <i>L. lactis lactis</i> var. <i>diacetylactis</i> , <i>Leuconostoc</i> subsp., <i>Lb. Acidophilus</i>
Kefir	<i>L. lactis lactis</i> , <i>L. lactis cremoris</i> , <i>Lb. casei</i> , <i>Lb. caucasicus</i> , <i>S. kefir</i>
Koumis	<i>Lb. acidophilus</i> , <i>Lb. bulgaricus</i> , yeasts
Leben	<i>Lactococcus</i> , <i>Lactobacillus</i>
<i>Soft cheese</i>	
Quark‡	<i>L. lactis lactis</i> , <i>L. lactis cremoris</i>
Cottage	<i>L. lactis lactis</i> , <i>L. lactis cremoris</i>
Camembert and Brie	<i>L. lactis lactis</i> , <i>L. lactis cremoris</i> , <i>P. camembertii</i>
Roquefort or blue	<i>Lactic culture</i> , <i>P. roqueforti</i> or <i>caseicolum</i>
<i>Semisoft cheese</i>	
Muenster	<i>L. lactis lactis</i> , <i>L. lactis cremoris</i>
Stilton	<i>L. lactis lactis</i> , <i>L. lactis cremoris</i> , <i>Leuconostoc</i> subsp.
<i>Hard cheese</i>	
Cheddar	<i>L. lactis lactis</i> , <i>L. lactis cremoris</i>
Gouda/Edam	<i>L. lactis lactis</i> , <i>L. lactis cremoris</i> , <i>L. lactis lactis</i> var. <i>diacetylactis</i> , <i>Leuconostoc</i> subsp.
Emmenthal	<i>St. thermophilus</i> , <i>Lb. bulgaricus</i> , <i>Lb. helveticus</i> , <i>Propionibacterium</i> spp.
Parmesan	<i>L. lactis lactis</i> , <i>L. lactis cremoris</i> , <i>St. thermophilus</i> , <i>Lb. bulgaricus</i>
Mozzarella	<i>L. lactis lactis</i> , <i>St. thermophilus</i> , <i>Lb. bulgaricus</i>

**L.*, *Lactococcus*; *Lb.*, *Lactobacillus*; *S.*, *Saccharomyces*; *P.*, *Penicillium*; *St.*, *Streptococcus*.

†Bioyogurt and Biogarde are trade name products containing *Lb. acidophilus* and *Bifidobacterium* developed for therapeutic effect, most notably in Scandinavia.

‡Quarg is a more recent product obtained by fermenting milk to a pH of 4.3–4.8 with mixed mild cheese starters and by removing whey and concentration of the solids to be eaten or to be used as an ingredient in cooking.

are being discovered, thus expanding the horizons for these bacteria. LAB are the principal organisms involved in the manufacture of cheese, yogurt, sour cream, and cultured butter. LAB and the predominant species utilized in the production of fermented dairy products are listed in Table 4.1. Many different products based on lactic cultures are known in different parts of the world, and more recently, lactic cultures per se are being marketed for their health and nutrition benefits. Certain health benefits have been reported, as well; claims include reduction of lactose intolerance, stimulation of nonspecific immune response, alleviation of diarrhea symptoms, cancer prevention, irritable bowel syndrome, and cholesterol reduction, but benefits are not clearly established, except for lactose intolerance. The detailed aspects can be found in a separate chapter on “Probiotics and Functional Foods.” LAB may contribute to the production of safer foods by inhibiting the growth of microbial pathogens and by removing chemical or toxic contaminants. LAB are

Table 4.2 Contributions of lactic acid bacteria as natural ingredients, health/nutrition additives, and specialty foods

Items	Applications
<i>Ingredients</i>	
Preservatives (nisin, bacteriocins, lactoperoxide–thiocyanate)	Preservation of various foods
Enzymes	
Proteinases, exopeptidases, esterases	Cheese ripening, enzyme-modified cheese flavors, new protein hydrolysates, functional and bioactive peptides
Lactases	Lactose-hydrolyzed whey syrups
Superoxide dismutases	Antioxidant for lipid
Polysaccharides (dextran)	Gums and thickeners, culture viscosity stabilizers
Flavors (diacetyl, acetoin)	Butter and yogurt flavors
<i>Health/nutrition</i>	
Probiotic cultures (<i>Lactobacillus acidophilus</i> , <i>Lactobacillus casei</i> , <i>Bifidobacterium bifidum</i>)	Lactose digestion, control of intestinal pathogens, stimulation of immune system, reduction of hypercholesterolemia, tumor inhibition, reduction of protein allergenicity, reduction of osteoporosis, increase in vitamin B complex (yogurt, Bifidus, and Kefir)
<i>Specialty foods</i>	
Reduction of toxic compounds/antibiotics	Removal of pesticides in wine/penicillin in milk
Foods for the elderly and babies	Immunostimulating, easily digestible, less constipating pasted foods, cereal-carrying cultures
Pet foods	Bioprotection ingredients against pathogens, upgrade by-products
Emergency foods	Dried foods containing live lactic acid bacteria for disaster scenes and unsanitary conditions
Ice cream	<i>Bifidobacteria</i> spp. and <i>Lactobacillus acidophilus</i> added

used in the development of new foods aimed at specific niches (e.g., foods for the elderly, for babies, for pets; breakfast foods; and bioprotection against pathogens: bioprotecting ingredients) in Table 4.2.

LAB are easily cultured on inexpensive feedstocks to produce secondary metabolites such as bacteriocins, nisin, enzymes, biomass, organic acids, and vitamins. *Bacteriocins* are antimicrobial proteinaceous compounds produced by LAB; more recent developments in their genetic and biochemical characterizations have been described. Nisin produced by some *Lactococcus lactis* strains and pediococin produced by *Pediococcus pentosaceus* are currently available in the market as food preservatives (see Chapter 5). They are effective bacteriocidal agents against Gram-positive bacteria, but other bacteriocins capable of inhibiting Gram-negative spoilage bacteria and food pathogens should be considered. The addition of lactic cultures has also been proposed to reduce undesirable gas production as well as to activate the *lactoperoxidase–thiocyanate* (SCN) system, which inhibits the development of psychrotrophic bacteria in milk. Immobilized cell technology has the potential of extending this approach to non-cheese-making milks as well as to produce cheese flavors, whey fermentation, and storage of cultures. Polysaccharide-producing LAB are used for the manufacture of fermented milk products with a ropy or mucoid texture. Also, many indigenous fermented foods and feeds, especially starch-containing materials, depend on amylolytic LAB. Several species of *Lactobacillus* such as *amylovorus* and *amylophilus* produce amylases and glucoamylases.

The economic importance of this group of microorganisms clearly demands the development of new technology to improve and stabilize the desirable characteristics of these bacteria. Recent advances in understanding the genetics of LAB are expected to yield specific strains designed not only to ripen cheese quickly but also to resist bacteriophage infection. Many desirable new traits and properties have been obtained by these techniques. Complicated nutritional requirements and enzyme regulations applying to LAB often make classical genetics tedious and strain improvement more difficult.

4.1.2 Basic knowledge of manufacture of dairy products

4.1.2.1 Cultured butter and buttermilk Cultured butter is made from milk fat to which a mesophilic starter culture has been added to enhance its flavor, *diacetyl*. Diacetyl is generated by metabolizing citrate and enhances storage qualities. Historically, *L. lactis* or mixed cultures that contained *L. lactis*, *Leuconostoc citrovorum*, and *Leuconostoc dextranicum* were used. The fat (cream) is separated from the skim milk by centrifugation of milk. The cream is pasteurized and inoculated with selected starter cultures. The ripened cream is then churned. The cream separates again into cream butter and its by-product sour buttermilk, which has limited use because of its high acidity.

An alternative process has been developed to produce cultured butter without the formation of sour buttermilk. In this process lactose-reduced whey inoculated with *Lactobacillus helveticus* and skim milk inoculated with a starter culture to produce aroma compounds and lactic acid are added to the pasteurized cream. The cream is further churned and worked. The resulting butter is known as sour aromatic butter. Sweet buttermilk is not as high in acidity as sour buttermilk.

4.1.2.2 Yogurt Yogurt is one of the older fermented milk products. It is produced by inoculating milk with *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Initial acid production is largely due to *St. thermophilus*, but the characteristic yogurt flavor is produced by *Lb. bulgaricus*. Both yogurt cultures may produce extracellular polymers, which contribute to the viscosity of yogurt. It is desirable that the starter cultures be present in about equal numbers in the cultures to ensure the characteristic flavor, consistency, and odor; otherwise *Lb. bulgaricus* becomes dominant.

Standardized milk, with a fat content of 0.5–3.0% and milk solids amounting to 14–16%, is homogenized and pasteurized by heating to 85 °C for 30 minutes (95 °C for 5–10 min or 120 °C for 5 s). After cooling to the incubation temperature, the batch is inoculated with starter cultures and incubated at 30 or 45 °C. Depending on the marketing strategies, inoculated milk is packed into either retail containers (set yogurt) or bulk containers (stirred yogurt). *Set yogurt* is allowed to ferment within its container, whereupon the containers are stored in a refrigerated room. *Stirred yogurt* is fermented in bulk, homogenized through a homopump, and then packed into containers.

Pasteurized yogurt was developed to overcome the problem of continuing acidification during storage by pasteurizing the yogurt after fermentation. Recently, it has been found that biologically active yogurts contain either *Lactobacillus acidophilus* or *B. bifidum* (or *longum*) besides *St. thermophilus*, such as Biogurt, Biogarde, and Bifigurt, have been developed. These probiotic starter cultures may colonize on the human intestinal tract. The most commonly used probiotic *Bifidobacterium* in yogurts or other dairy products are *Bifidobacterium animalis*, *B. bifidum* and *Bifidobacterium infantis*. A number of different types of harmless bacteria can be used to culture yogurt, and most yogurts are made using a mixture

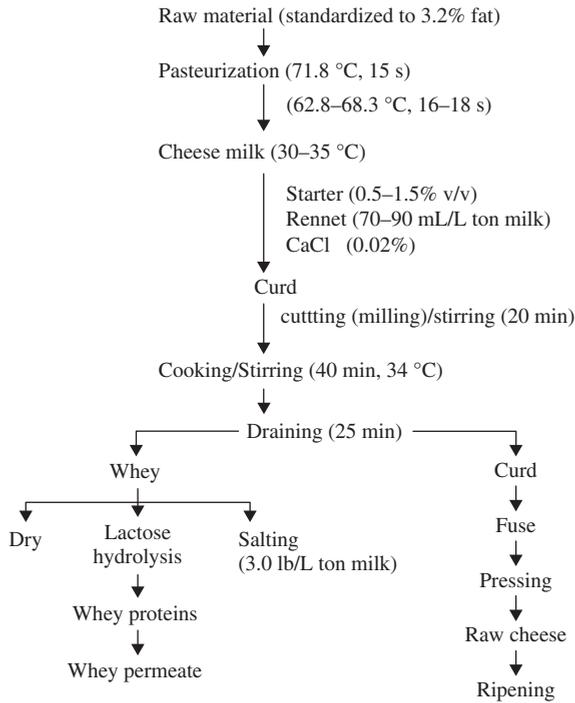


Figure 4.1 Basic steps of most cheese varieties (e.g., Cheddar).

of different bacteria. *Bifidobacterium* yogurt is common, though not as common as yogurts made with other types of live cultures. Between 1998 and 2003, fermented dairy products grew at six times the rate of total dairy products, and during this same period, probiotic drinking yogurt was the fastest growing segment of the dairy sector. Euromonitor data shows that between the years 2003 and 2008, global value sales of probiotic yogurt (both spoonable and drinks) grew from US\$ 6.8 billion to US\$ 13.7 billion. By 2013, its value is forecast to increase by double.

4.1.2.3 Cheese Cheese making is essentially a dehydration process in which milk casein, fat, and minerals are concentrated 6- to 12-fold, depending on the variety. Although the manufacturing protocols for individual varieties differ, the basic steps common to most varieties are acidification, coagulation, dehydration, and salting (Figure 4.1). Acid production is the major function of the starter bacteria. Lactic acid is responsible for the fresh acidic flavor of unripened cheese and is of importance in coagulation of milk. Coagulation of the casein is accomplished by the concerted action of rennet (limited proteolysis) and by acidification. Starters play other essential roles in the production of volatile flavor compounds (e.g., diacetyl, aldehydes), the synthesis and release of the intracellular proteolytic and lipolytic enzymes involved in the cheese ripening, and the suppression of pathogens and other spoilage microorganisms. The coagulated curd forms a gel that traps any fat present. Dehydration involves the post-coagulation treatments that break/cut the gel, resulting in expulsion of whey (whey contains most of the water, milk sugars, proteins).

For most ripened cheeses, the curds are cooked in whey until the temperature of the mixture reaches 37–41 °C, depending on the variety. Salting serves many functions in controlling microbial growth and activity, reducing the water activity (A_w), controlling the rate of proteolysis of both α_{s1} - and β -casein, and regulating the formation of physical changes in cheese protein. Salt prevents further growth of the starter bacteria and causes the uncoupling of lactose fermentation. Lactic acid starter cultures are added to vat milk to give about 10^6 – 10^7 colony-forming units (cfu)/mL.

The amount and type of starter may vary significantly depending on the type of cheese and characteristics desired. In most cheese types, an overnight pH change is in the range 4.95–5.3; in addition, there is generally a shift in the population of starter organisms to lactobacilli and in some cases pediococci. In young cheese, there are inhibitory and stimulatory factors for lactobacilli, but as the cheese ripens most of the inhibitory effect is known to disappear. These stimulatory factors for lactobacilli seem to originate primarily from α_s -casein products, since β -casein is not extensively degraded.

Microbiological and biochemical changes during cheese ripening Cheese ripening refers to the storage of cheese under controlled conditions of time, temperature, and humidity during which the desired body, flavor, and texture develop. The cheese-ripening and flavor profiles are complex and none of them, by itself, is characterized sufficiently to permit duplication of a complete flavor through a mixture of pure compounds. About 86 odor-active compounds are currently identified from Cheddar cheese. Fresh cheese is often tough and rubbery, but during ripening it acquires a smooth texture or soft consistency by way of primary biochemical changes involving glycolysis, proteolysis, and lipolysis. These changes are overlapped by a number of secondary catabolic changes, including deamination, amination, transamination, decarboxylation, desulfurylation, β -oxidation, and esterification.

The agents involved in the ripening of cheese are likely to be the enzymes derived from rennin, as well as indigenous milk enzymes (e.g., plasmin), and starter, nonstarter, and secondary inocula such as propionic acid (PPA) bacteria, *Brevibacterium linens*, and molds (*Penicillium roqueforti*, *Penicillium candidum*). Lactic starter culture reaches maximum growth within few weeks. Nongrowing cells are slowly autolyzed by their hydrolytic enzymes, and the products (sugar, nucleic acid, etc.) are utilized by *nonstarter lactic acid bacteria* (NSLAB). The widely accepted *component balance theory* states that the flavor of Cheddar is the outcome of a synergistic odor effect of the right blend of some of these compounds in balanced proportions. Sulfur compounds, methyl ketones, aldehydes, esters, alcohols, lactones, and free fatty acids (FFAs) that are generated from numerous sequential and simultaneous biochemical reactions involving milk lipids and proteins form the principal components of cheese aroma. Many flavor compounds are chemical interactions of low pH and low oxidation–reduction potential.

The three primary events that occur during cheese ripening (i.e., glycolysis, proteolysis, lipolysis) are mainly responsible for the basic textural changes of cheese curd and for the basic cheese flavors. However, numerous secondary changes occur simultaneously that are mainly responsible for the final aspects of cheese flavor as well as for the modification of cheese texture. The primary biochemical changes in cheese ripening are now well characterized but the secondary events are known only in general terms. However, cheese flavor development is a very complicated and dynamic biochemical process, thus the mechanism is still not known. The type and component of milk, processing conditions and microorganism and enzyme present in the cheese matrix all contribute cheese flavor development (Figure 4.2).

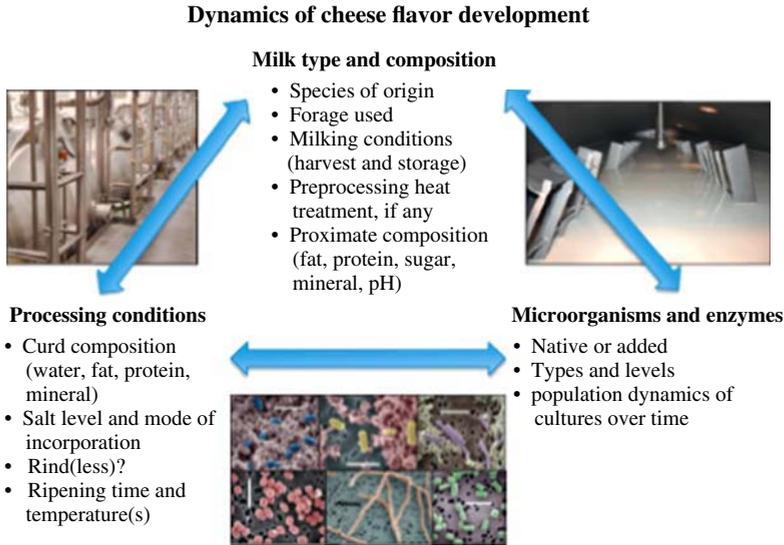


Figure 4.2 Dynamic nature of cheese flavor development. *Source:* Steele et al., 2013. Reproduced with permission of Elsevier.

Transformation of lactic acid Normally, saturated lactate is converted to acetate and other carbonyl compounds by oxidative activities (NADH oxidase) of NSLAB such as lactobacilli and pediococci. *Propionibacterium* species (e.g., *Propionibacterium shermanii*, *Propionibacterium freudenreichii*) convert lactate to propionate and acetate. The concentrations of lactate in Camembert, Swiss, and Cheddar are about 1.0%, 1.4%, and 1.5%, respectively. The fate of lactate during cheese ripening has not been studied well. Cheddar cheeses contain D-lactate that appears to be formed from residual lactose by lactobacilli or racemization of L-lactate. The metabolism of lactate is significant in surface-mold-ripened cheeses such as Camembert and Brie. The concentration of lactate as L-lactate in these varieties at 1 day is about 1.0% and is produced exclusively by the mesophilic starter. Secondary organisms such as *Geotrichum candidum* and *Penicillium caseicolum* quickly colonize the surface of these cheeses. *G. candidum* and *P. caseicolum* eventually metabolize lactate to CO₂ and H₂O, causing an increase in pH.

Transformation of amino acids The fate of amino acids in ripening cheese is important for the development of cheese flavor. Many free amino acids are catabolized by decarboxylation, deamination, transamination, desulfurylation, and so on, and these can undergo a variety of changes (Figure 4.3). The principal products obtained from the catabolism of amino acids are (i) amines resulting from decarboxylation, (ii) ammonia, acids, keto acids, carbonyls, and alcohols resulting from deamination, (iii) other amino acids produced by transaminations, and (iv) hydrogen sulfide, dimethyl sulfide, methanethiol, thio esters, and other sulfur compounds produced by desulfurylation and demethiolation. The secondary flora generally play an important role in the catabolism of amino acids and contribute to the characteristic flavor of a given cheese variety. Volatile sulfur compounds, especially methanethiol, appear to play a pivotal role in the typical aroma of Cheddar cheese. The precise role of

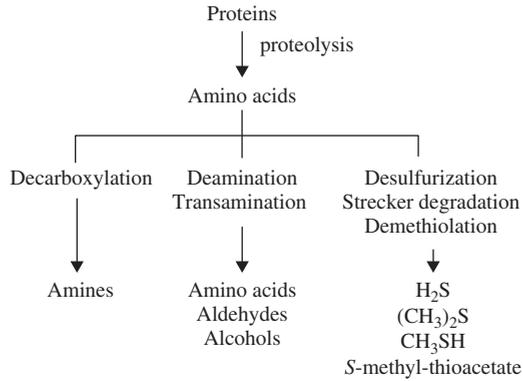


Figure 4.3 Secondary biochemical changes during cheese ripening.

methanethiol is not known, but the reactions and interactions between methanethiol and other compounds result in the formation of flavor compounds.

Transformation of fatty acids Cheddar cheese prepared from skim milk does not develop full typical flavor, indicating that fat is required for the development of characteristic cheese flavor. When milk is pasteurized (72 °C/15 s), much of the milk lipase is destroyed, but the starter and lactobacilli esterase and lipases act on mono- and diglyceride fractions to liberate FFAs. The mono- and diglycerides can also be hydrolyzed by carboxyesterases or by mono- and diglyceride lipases. FFAs play a major role in flavor of many cheese varieties, especially for strongly piquant Italian cheeses (Romano, Parmesan, Provolone). FFAs are transformed to methyl ketones, secondary alcohol, lactones, and so on (Figure 4.4). In general, the synthesis of esters and the formation of methyl ketones are the most important transformations of FFAs in cheese ripening. A variety of enzymes such as carboxyesterases or arylesterases, which are present in yeasts, LAB, and other microorganisms, can synthesize esters in a slightly aqueous–alcoholic medium. This synthetic ability is less well documented for cheese microflora than for those of beverages. However, the numerous esters

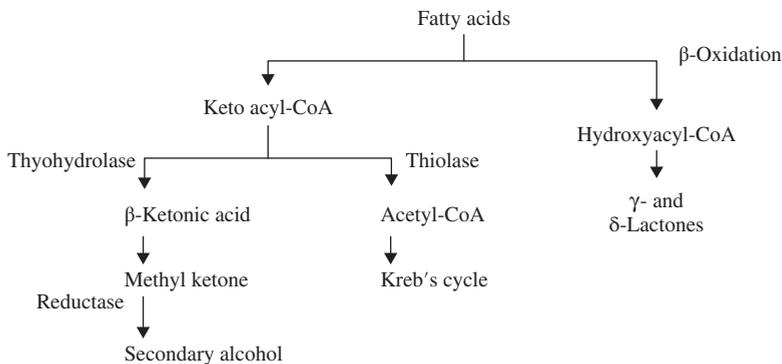


Figure 4.4 Transformation of fatty acids.

formed during cheese ripening and their participation in cheese defects clearly demonstrate the existence of this activity. Oxidation of FFAs common to certain molds of *Aspergillus* and *Penicillium* leads to the formation of further flavor compounds such as methyl ketones through the actions of thiohydrolase and α -ketoacyl decarboxylase. *P. roqueforti* has strong lipolytic activity and produces fatty acids, methyl ketones, the most important of which is 2-nonanone, and other flavor compounds.

4.1.3 Metabolic systems in lactic acid bacteria

Renewed interest in fermentations by LAB is due to their technological, nutritional, and therapeutic aspects and to their status as natural ingredients. These simple, low-cost preservatives offer the advantages of predigestion of protein, presence of polysaccharides, elimination of antinutritional factors, and availability of minerals.

Growth and activity of LAB, moreover, are often stimulated by the creation of selective/elective conditions (low E_n , low A_w , low pH, low sugar, low temperature, high salt) that retard the growth and activity of many endogenous spoilage organisms and produce stable foods. The complexity of relations among microflora, manufacturing environments and techniques, and milk sources and quality, reflects the varieties that exist in the products. More than 20 million tons of fermented dairy products are annually produced worldwide with the help of LAB as well as other bacteria genera (e.g., *Propionibacterium*), yeasts (*Saccharomyces*), and molds (*Penicillium*) (Table 4.1). The LAB contain no cytochromes, and thus energy production depends solely on substrate-level phosphorylation, mainly from carbohydrates.

4.1.3.1 Carbohydrate and citrate metabolism Lactose (galactose- α or β -1,4-glucose) in milk ($\approx 4.8\%$) is a major carbon and energy source and is converted into lactic acid by LAB. Two different mechanisms for transport of sugars (glucose, lactose, sucrose) are known in LAB (Figure 4.5). In most homofermentative mesophilic lactococci (especially *L. lactis*) and lactobacilli (*Lactobacillus casei*), free glucose and galactose are transported by two separate systems: (i) a *phosphoenolpyruvate-lactose phosphotransferase* (PEP-PTS^{lac}), which is mediated by Enzyme II^{lac} (LacE) and Enzyme III^{lac} (LacF), and (ii) a *lactose permease*, which obtains its energy from the hydrolysis of ATP. The resulting glucose is then catabolized by the glycolytic Embden–Meyerhof (EM) pathway. Intracellular lactose-6-phosphate is subsequently cleaved by phospho- β -galactosidase (LacG) into glucose and galactose. In most other lactic bacteria, such as *Lb. acidophilus*, *St. thermophilus*, and *Leuconostoc*, lactose is taken up by the lactose permease system and lactose is hydrolyzed by β -galactosidase into glucose and galactose. Galactose 6-phosphate is catabolized via the *D-tagatose-6-phosphate pathway*, and free galactose is then utilized by the enzyme of the *Leloir pathway*.

The relatively low concentration of citrate in milk (≈ 8 mM) belies the importance of its metabolism in cheeses, but it plays a role in the production of aromatic compounds such as diacetyl and in the formation of characteristic eyes in some curds, especially Dutch cheese. *L. lactis lactis* var. *diacetylactis* and certain *Leuconostoc* species (e.g., *lactis*, *cremoris*) are responsible for flavor/aroma production. Diacetyl is the important compound adding flavor to butter, buttermilk, cottage cheese, quarg, and yogurt. Eye formation in Gouda cheese is contributed by CO₂ produced by LAB. Citrate is transported by citrate permease and cleaved by citrate lyase into oxaloacetate and then to diacetyl through complex enzyme systems (Figure 4.6). The citrate lyase in *Leuconostoc lactis* is inducible, while in *L. lactis lactis lactis* var. *diacetylactis* this enzyme is constitutive. Insufficient flavor and/or

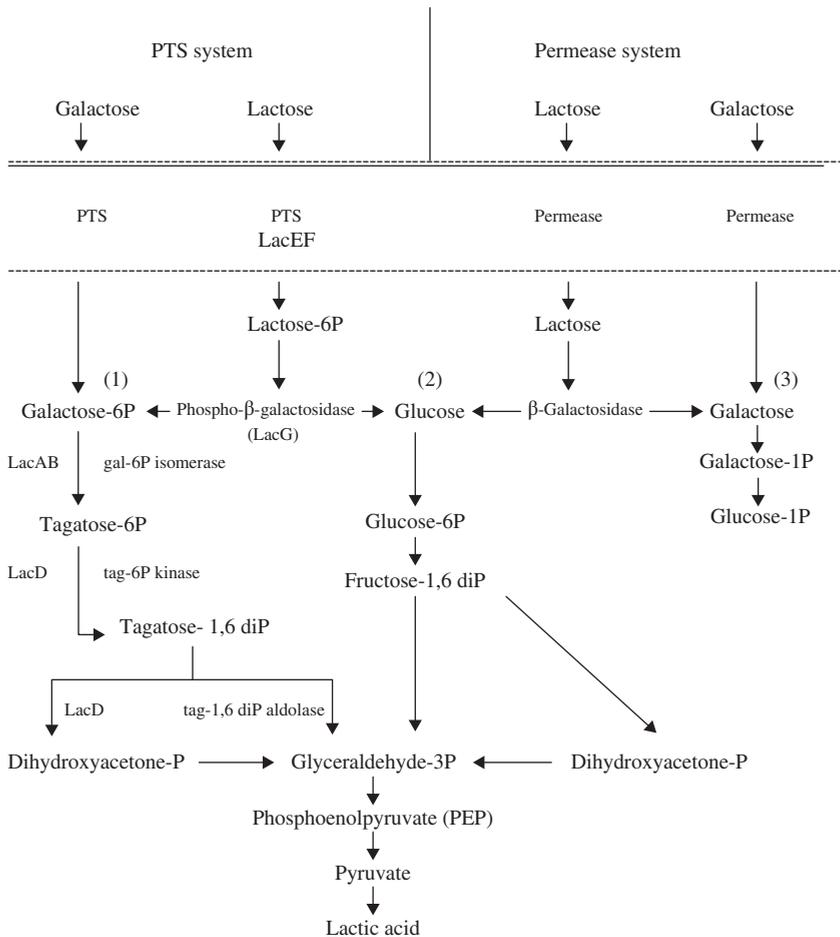


Figure 4.5 Catabolic pathways of lactose and galactose by lactococci: 1, the tagatose-6-phosphate pathway; 2, the Embden–Meyerhof pathway (glycolysis); and 3, the Leloir pathway.

CO₂ production in cultured dairy products are usually due to low concentration of citrate in milk or to the loss of citrate-fermenting ability associated with loss of the citrate plasmid.

4.1.3.2 Protein metabolism The proteolytic system is crucial for the growth of LAB in protein-rich substrates because these microorganisms are nutritionally very fastidious. Although their proteolytic capacity is weak compared with other bacteria (e.g., *Bacillus*, *Pseudomonas*) and varies greatly among strains and species, LAB possess a complex proteolytic system composed of enzymes of different types and locations. Thus, mixed cultures consisting of proteolytic and nonproteolytic strains are used to stimulate diverse flavor compounds via decarboxylation, de- and transamination, and desulfurylation (e.g., *Propionibacterium* in Swiss-type cheeses, *Penicillium* spp. in Camembert).

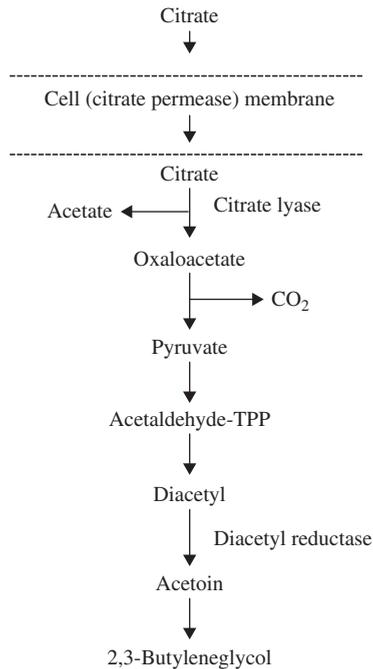


Figure 4.6 Citrate metabolism in lactic acid bacteria.

The hydrolysis and uptake of proteins involves three identifiable steps (Figure 4.7): (i) proteolysis of native proteins to peptides by extracellular serine proteinases located on the cell envelope, (ii) further hydrolysis by peptidases localized in or on the cell wall or cytosol, eventually to individual amino acids, and (iii) transportation into the cell by peptide transport system(s) (e.g., ATP hydrolysis by a proton-translocating ATPase or an end product efflux). Peptidases up to 13 *exo*- and *endo* peptidases such pepC, N, O, X, T, Q, V, DA exist in *L. lactis* localized in or on the cell wall or cytosol (Lopez-Kleine and Monnet, 2011). Peptides and amino acids are important in cheese ripening and cheese flavoring. Free amino acids are the precursors of numerous aromatic volatile compounds, and the water-soluble, nonvolatile fraction that contains the peptides and free amino acids has a marked flavor.

Biochemical and immunological investigations suggested the existence of several cell wall proteinases. On the basis of pH and temperature optima, three types of lactococcal proteinase were identified: PI and PIII, which are active at acid pHs and 30 and 40 °C, respectively, and PII, which is active at neutral pH as well as at 30 °C. Further classification of lactococcal proteinases based on the distinctive hydrolysis patterns of α_{s1} -, β - and κ -casein showed two enzyme specificities: PI, present in *L. lactis cremoris* HP and Wg2, which degrades β -casein with only weak hydrolysis of α -casein, and PIII, present in *L. lactis cremoris* AM1 and SK11, which degrades α_{s1} -casein in addition to β - and κ -caseins. Polyclonal and monoclonal antibodies against the proteinases of *L. lactis cremoris* showed that among four proteins, A, A', B, and C, component A is present in all strains.

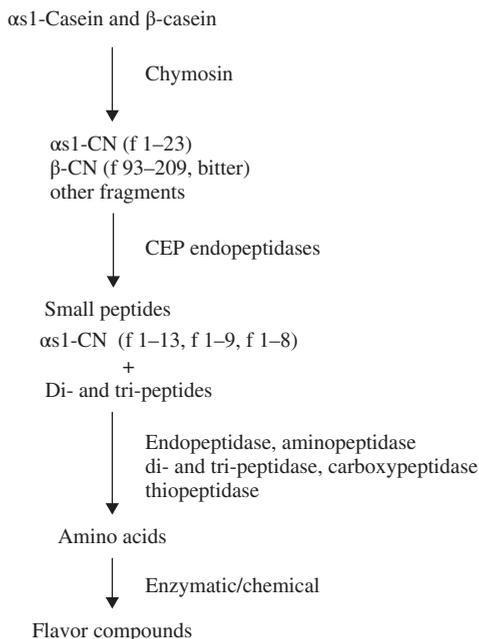


Figure 4.7 General proteolytic system of lactic acid bacteria in cheese.

Despite the limitations of the experimental techniques used in cell fractionation, there is strong evidence for the existence of different peptidases isolated from intracellular, cell membrane, and cell-wall-bound peptidases. The principal peptidases in LAB are exopeptidases such as amino-, di-, and tripeptidases, which cleave one or two amino acids from the free N-terminus of the peptide chain. Other exopeptidases such as arylpeptidyl amidase, aminopeptidase P, proline-specific peptidases (proline iminopeptidase, *x*-prolyldipeptidyl aminopeptidase, prolinase, prolidase), endopeptidase, and carboxypeptidase have all been found in LAB.

4.1.3.3 Lipid metabolism LAB are only weakly lipolytic and esterolytic toward mono- and diglycerides compared with milk triglycerides and their enzymes, but they contribute to the background flavor of many dairy products. Intracellular and extracellular lipases and esterases from LAB are known, but localization studies have not been well analyzed. Other secondary microflora contribute more significantly to the hydrolysis of milk fat and the production of FFAs.

4.1.4 Genetic modification of lactic acid bacteria

4.1.4.1 Plasmid biology of lactic acid bacteria Many metabolic properties essential for growth of LAB in milk are unstable, and that instability is related to the unusually large numbers of *plasmid DNA* present (most *cryptic*) in lactic cultures. Spectacular advancement of the genetics of LAB has been achieved, especially for lactose and protein metabolism. Other functions associated with plasmids include citrate utilization, polysaccharide or nisin production, and phage resistance (Table 4.3).

Table 4.3 Plasmid encoded functions of lactic acid bacteria

Activity	<i>Lactococcus/ Streptococcus</i>	<i>Lactobacillus</i>	<i>Leuconostoc</i>
Lactose utilization	+	+	+
Protein utilization	+	+	-
Citrate utilization	+	-	+
Phage resistance	+	-	-
Bacteriocin production	+	-	-
Antibiotic production	-	-	-
Antibiotic resistance	+	+	-
Exopolysaccharide production	+	-	+
Cell aggregation	+	-	-
UV resistance	+	-	-

Lactococcal lac plasmids The genes encoding the PEP-PTS^{lac}, the tagatose-6-phosphate pathway, and phospho- β -galactosidase enzymes in lactococci are located on conjugal plasmids. The stabilization of lactose-PTS and proteinase (*prt*) genes (56.5 kb plasmid) into the chromosome was established by transduction. The *lacG* gene encoding the *p*- β -galactosidase was cloned and the nucleotide sequences of the *lacG* gene from *L. lactis* and *Lb. casei* were determined. Various promoters from LAB were isolated either by shotgun cloning upstream of promoterless chloramphenicol acetyltransferase (CAT) genes or by characterization of the expression signals adjacent to cloned genes. The lactococcal -35 and -10 consensus sequences TTGA and TATAAT are similar to those of *Escherichia coli*, but are usually separated by 17 nucleotides. An increasing number of inducible systems have been identified, but no molecular mechanisms underlying this regulation have been studied.

Lactococcal proteinase plasmids Loss of proteolytic function was often correlated with loss of lactose metabolism. In *L. lactis lactis* NCDO712, a 56.5 kb plasmid harbored the lactose-PTS and proteinase genes. Plasmid (13.5–100 kb) was implicated in proteinase production, and shortening and deletion of the proteinase genes were achieved. Three proteinase genes from *Leuconostoc cremoris* stains (Wg2, SK11, NCDO76S) have been cloned, expressed into a proteinase-negative strain of *L. lactis* using pWV01 vector, and sequenced with an overall homology of 98%. Natural transfer systems using conjugation, transduction, and transformation (protoplast, electroporation) provided evidence for plasmid linkage of proteinase determinants. The proteinases are attached to the cells via a membrane anchor present at the extreme C-terminal end of the molecule. Compared with the genetic knowledge that has been obtained about the plasmid-encoded proteinase system, little is known of the chromosomally linked peptidases from LAB.

4.1.4.2 Genetic transfer systems The main genetic transfer systems that can be used in LAB systems are *in vivo* conjugation, transduction, transformation and cell fusion, and *in vitro* recombinant DNA techniques (Table 4.4). Transposable elements contain either the functions that are necessary for transposition (inverted repeats flanking the transposase gene) or additional information such as that encoded by genes imparting drug resistance. Transposable elements in lactose fermentation, nisin production, and malolactic fermentation from LAB are known. Various heterologous genes in *Lactococcus*,

Table 4.4 Genetic transfer systems in lactic acid bacteria

Process	Major use	Limitation
<i>In vivo</i>		
Conjugation		
Donor/recipient cell-to-cell contact	Transfer of transmissible plasmids	Cell surface recognition required
Conjugal plasmid transfer at high frequencies in lactococci and leuconostocs	Genetic transfer of chromosomal DNA with homologous DNA Mobilization of nontransmissible plasmids	The host DNA must be homologous for recombination to occur
Transduction		
Bacteriophage-mediated transfer of DNA	Useful technique for making fine structural changes in genetic material	Only small fragments of DNA transfer
Transformation		
Uptake of naked DNA by host	General DNA transfer procedure	Plasmid DNA maintained, but chromosomal DNA must be homologous for recombination to occur
Three systems (protoplast, whole cell, electroporation)	Electroporation technique is current choice for plasmid DNA transfer into lactic acid bacteria	
<i>In vitro</i>		
Recombinant DNA		
Insertion of foreign DNA into a plasmid or phage cloning vector; transformation into the host; screening of the target gene	Transfer of single gene or groups of linked genes from any source without barrier to a desirable host strain	No theoretical limitation, but must overcome stability and expression of introduced gene; must meet safety requirements

St. thermophilus, and *Lactobacillus* species are expressed and some are secreted in *L. lactis* (Table 4.5).

4.1.5 Applications of genetic engineering

During the last decade, several important applications of genetic engineering in the dairy starter culture industry have been identified to improve and stabilize desirable characteristics and to produce new products (Table 4.6). Some examples include flavor and texture enhancement as well as in accelerated ripening of cheese, stabilization of plasmid-encoded genes by chromosomal integration insertion, development of bacteriophage-resistant cultures, production of bacteriocins and other natural antimicrobial compounds, production of biogums (which improve body and texture in fermented milk products), achievement of enhanced tolerance to freeze-drying of lactic cultures, achievement of enhanced probiotic effect, and development of a safe host for the production of food-grade enzymes and heterologous proteins. Other significant activities are also

Table 4.5 Cloning and expression of heterologous genes in lactic acid bacteria or *Lactococcus lactis*

Genes (donor)	Host (cytoplasmic or secreted)
Bovine prochymosin	<i>L. lactis</i> (secreted)
Neutral protease (<i>B. subtilis</i>)	<i>L. lactis</i>
Egg white lysozyme	<i>L. lactis</i>
β -Lactamase (<i>E. coli</i>)	<i>L. lactis</i> (secreted)
α -Amylase (<i>B. stearothermophilus</i>)	<i>Lb. plantarum</i>
α -Amylase (<i>B. licheniformis</i>)	<i>L. lactis</i> (secreted)
Phage lytic enzyme (<i>Listeria monocytogenes</i>)	<i>L. lactis</i>
β -Galactosidase (<i>E. coli</i>)	<i>L. lactis</i>
β -Galactosidase (<i>Clostridium acetobutylicum</i>)	<i>L. lactis</i>
β -Glucuronidase (<i>E. coli</i>)	<i>L. lactis</i>
Thaumatococcus (plant)	<i>S. thermophilus</i>
Brazzein (sweet protein)	<i>L. lactis</i>
Galactokinase (<i>E. coli</i>)	<i>S. thermophilus</i>
Catechol 2,3-oxygenase	<i>S. thermophilus</i>
Cholesterol oxidase (<i>Lactococcus</i>)	<i>S. thermophilus</i>
Cholesterol oxidase (<i>Streptomyces</i>)	<i>Lb. casei</i>
Aminopeptidase (<i>Lb. lactis</i>)	<i>Lb. casei</i>
Aminopeptidase N (<i>Lb. helveticus</i>)	<i>L. lactis</i> (secreted)
Levanase (<i>B. subtilis</i>)	<i>Lb. plantarum</i>
Luciferase (<i>Vibrio harveyi</i> , <i>Vibrio fischeri</i>)	<i>L. lactis</i> , <i>Lb. casei</i>
Green fluorescent protein (<i>Aequoria victoria</i>)	<i>L. lactis</i>
Endoglucanase (<i>Clostridium thermocellum</i>)	<i>Lb. plantarum</i>
Lipase (<i>Staphylococcus hyicus</i>)	<i>L. lactis</i> (secreted), <i>Lb. curvatus</i>
Dextran sucrose (<i>Leuconostoc mesenteroides</i>)	<i>L. lactis</i> (secreted)
Pediocin PA-1 (<i>Pediococcus acidilactici</i>)	<i>L. lactis</i> (secreted)

Source: Le Loir et al., 2005. Microbial Cell Factories, 4, 2–15; Author's compiled data.

ongoing in additional areas for specialty markets, including the development (i) of low fat fermented dairy products by constructing specialized culture systems that satisfy low fat cheese making constraints, (ii) cold-sensitive yogurt starter cultures, and (iii) Mozzarella cheese does not brown as much as the traditional variety. *Antifreeze proteins* found in fish, cold-tolerant insects, and plants were genetically overproduced in microorganisms for additives to frozen foods, or incorporated with the genome of the raw foods to retard ice crystal growth, or to prevent damage to agricultural crops (Peters et al., 1989; Feeny and Yeh, 1993). Commercially, there appear to be countless applications for antifreeze proteins, among which AquaBounty's genetically modified (GM) salmon (AquAdvantage Salmon) has been approved in 2013 for commercial use. In this work, a recombinant DNA construct (opAFP-GHc2), composed of a promoter from an ocean pout antifreeze protein (opAFP) gene and a protein-coding sequence from a Chinook salmon growth hormone (GHc2) gene were micro-injected into the fertilized eggs of wild Atlantic salmon (<http://www.digitaljournal.com/article/349660#ixzz2Xwax222w>). The research and prospects of having the GM salmon in the market has led to a heated debate which has lasted for several years. The safety issue will be dealt with in a separate chapter.

Nisin, a peptide secreted by some of *L. lactis* strains is by far the most successful example of a bacteriocin for use in food preservation. Its biosynthetic genes are encoded by a conjugative transposon, and the DNA sequence of these genes has been fully

Table 4.6 Possible targets of genetic engineering in modifying starter cultures*Improvement of existing catalytic functions and traits*

Development of lactic cultures having specific activities (proteolytic, acidifying, maturing, etc.)
 Elimination of culture rotation
 Stabilization of plasmids by insertion into chromosomes
 Development of phage-resistant cultures
 Enhanced slime formation (improved body and texture in fermented milk products)
 Enhanced tolerance to freeze-drying

Introduction and expression of heterologous genes

Proteases to replace rennin
 Safe hosts for production of food additives (e.g., enzymes, colors, preservatives, stabilizers, flavors)
 Phage resistance genes
 Antibiotic (nisin) genes
 Drug-resistance genes
 Cholesterol-degrading enzyme
 Added nutritional value
 Enhanced probiotic effect
 Antifreeze protein gene

New applications

Natural secondary metabolite production
 New fermented foods and feeds
 Vaccines and pharmaceuticals

determined. This information will lead to the development of strains that overproduce nisin for commercial purposes. Several protein-engineered nisin variants have also been produced (Rouse et al., 2012; Zendo, 2013).

Another success story is the development of *bacteriophage-resistant starters* (Szczepankowska et al., 2013). Conjugation has been used successfully to introduce a plasmid that encoded *restriction/modification* (R/M) and abortive phage defense mechanisms into a commercial strain that has since been used for Cheddar cheese manufacture. Other recent advances designed to help combat phage infection entail the use of *antisense RNA* technology, and so on. Molecules that bind with specific mRNAs can selectively inactivate genes. In tomatoes, antisense expression vectors can be used to inhibit an enzyme that breaks down plant cell walls, thus creating tomatoes that can ripen more slowly than untreated ones. Tomatoes with their antisense RNA50 modified are available in the market (Flavr Savr). Intensive genetics and microbiological studies are continuing to develop the flavors and enzyme systems involved in cheese ripening by overexpression of different peptidase genes in the lactococci or lactobacilli. Progress has also been made in the development of autolytic machinery and in the protein engineering of an autolytic phenotype using a cloned phage lysin gene.

4.1.5.1 Applications of lactic bacterial enzymes Through classical selection procedures, and more efficient fermentation and downstream processes, significant progress has been made with LAB and their enzymes, particularly for accelerated cheese ripening, enzyme-modified cheese (EMC) flavoring, and the production of protein hydrolysates. Cheese ripening is a slow microbial process that incurs high costs of refrigeration and warehousing, and interest charges involved in cheese storage represent a significant portion of the total production costs. Any development leading to accelerated cheese ripening, therefore, has direct economic importance and the potential to save millions of dollars. Another consideration of accelerated ripening is the opportunity to enhance

cheese flavor intensity in full and low fat cheeses, as opposed to the more common practice of using *direct vat inoculation (DVI)* or *direct vat set (DVS) starters* to combat phage attacks that decrease cheese flavor. Furthermore, the occurrence of *Salmonella* and *Listeria* food poisoning has doubled in recent years, bringing about the mandatory pasteurization of milk used for cheese making. Pasteurization, in turn destroys most of the desirable native lactic acid bacterial flora in milk, thereby retarding the cheese ripening process.

Acceleration of cheese ripening Present acceleration methods include addition of proteolytic and lipolytic enzymes, and use of modified starters and combinations, elevated ripening temperatures, and slurry methods. Use of higher ripening temperatures (10–12 °C instead of the normal 5 °C) appears to be the simplest approach and has the lowest cost, but its success depends on adequate quality control. Otherwise the cost savings are minimal because the downgraded cheese will be sold at a lower price. Enzymes or modified starters, added either to the cheese milk or to curd at the salting stage, are the most commonly used ripeners. Although most of the methods currently used have not gained universal acceptance, new strategies for the application of accelerated ripening systems are emerging with the advance of food biotechnology and increasing consumer demand for low fat cheese. These requirements include the need for typical cheese flavor in the production of processed cheeses, the need to improve the rheological properties of cheeses that are low or reduced in fat, the return of flavor ingredients to cheese produced with modern direct vat cultures, which often are lacking in flavor, and the development of available proteolytic and lipolytic enzyme products.

Several commercial enzymes, such as Flavor-Age (C. Hansens), Nature-Age (Miles), Accelerase (Imperial), and Neutrase (Novo, Nordisk A/S), are currently available for the purpose of accelerated cheese ripening. Application and incorporation of commercial enzymes have not been as successful as predicted, however, because of quality defects (bitterness, rancidity, poor texture, nontypical flavors, etc.), poor understanding of the cheese ripening mechanism, and suboptimal conditions (4–6 °C: selected by the industry to retard the growth of spoilage microorganisms). Also, there are many variables that affect cheese ripening.

Lipolytic enzymes Milk fat is broken down to FFAs and partial glycerides by esterases and lipases endogenous to the milk or of microbial origin. The FFAs contribute to desirable flavor, but too high an FFA concentration may induce a rancid off-flavor in the cheese. Acceleration of FFA formation induced by the addition of lipase or esterase gives a desirable flavor to Provolone, Romano, and Parmesan varieties, and feta cheeses, which are often produced from goat's milk. Pregastric lipases from calf and kid can be used for this purpose. Calf lipases, which liberate relatively more C₁₄ and C₁₆ fatty acids, give a more balanced mild flavor, whereas kid lipase produces a more piquant flavor. Only a few reports are available on the isolation and characterization of lipolytic and esterolytic activities in the cell-free extracts of LAB. Recently, there has been some interest in the lipase and esterase activities of various *Lactobacillus* species.

Proteolytic enzymes Proteolytic enzymes are required to achieve the acceleration of physical body breakdown as well as a faster flavor development of the cheese. Commercially available, "a package of proteolytic enzymes" is a mixture of protease and peptidase produced either from starter cultures, nonstarter cheese microflora, or from non dairy microorganisms. Accelerase (Imperial Products) produced from *L. lactis* is an enzyme system to reduce maturation time of full-fat cheese and to improve flavor and texture of low fat

cheeses. Aminopeptidase enzymes in Accelerase are leucine aminopeptidase (EC 3.4.11.1), dipeptidyl peptide hydrolase (EC 3.4.14), arginine aminopeptidase (EC 3.4.11.6), tripeptidase (EC 3.4.11.4), dipeptidase (EC 3.4.13.9.11), and phenylalanine aminopeptidase (EC 4.11). Savorase, an enzyme system used to produce natural cheese, meat, and savory flavors, is used in the process of forced ripening of cheese. Another enzyme system, Debitrase, is used to produce nonbitter protein hydrolysates from dairy, vegetable, meat, and fish proteins. Limitations of external addition of enzymes include loss of enzyme in whey when added to milk, reduction of cheese yield, and flavor defects. The addition of enzymes to curd is efficient only in the case of Cheddar, but it is often difficult to ensure a uniform distribution of the salt–enzyme mixture without hot spots, where excessive proteolysis and lipolysis occur.

Enzyme encapsulation within liposomes or milk fat capsules and attenuated bacterial cells (heat- or freeze-shocking) have been suggested as other alternatives. *Liposomes*, composed of phospholipids and other lipids sustaining a biomolecular configuration, are available in a wide range of vesicles varying in size, net charge, and sensitivity to pH and/or temperature. Heat treatment or freeze-shocking of bacterial cells under optimum conditions can induce cell lysis, which leads to loss of acidifying activities but permits retention of the enzyme systems. GM starter strains, which may be lactose-negative or proteinase-negative, were also added to accelerate cheese ripening. Recombinant DNA techniques also led to clone and express neutral proteinase from *Bacillus subtilis* in *L. lactis* and many aminopeptidases or proline-specific peptidases from LAB into *E. coli* or lactococci and lactobacilli. More recently, after a recombinant aminopeptidase of *Lactobacillus rhamnosus* was overexpressed up to 1000 fold in *E. coli*, the autolyzed and purified aminopeptidase (pepN) was microencapsulated using alginate and chitosan for Cheddar cheese ripening in which about 4 months of the acceleration was achieved with a good sensory and excellent flavor profile (Azarnia et al., 2010a,b).

New applications of enzymes

Low and reduced fat cheese To meet the increasing consumer demand for low-fat cheese, the commercial producers are interested in developing the methods of manufacturing low and reduced fat Cheddar cheese without compromising flavor and mouthfeel. Most of the current low-fat Cheddar cheese types give poor mouthfeel and mature slowly. A low or reduced fat cheese made with a combination of DVS starters and Accelerase has been reported to produce flavor and mouthfeel similar to that of a full-fat cheese but with fewer calories. DVS cultures are highly concentrated (10^{11} cfu/g) cell suspensions of defined strains in milk along with cryoprotectors (e.g., glycerol or lactose) frozen in liquid nitrogen.

High-flavor cheese High-flavor cheese, which is force-ripened for 6–10 weeks at relatively high temperatures (12–15 °C), is used in the production of processed cheese slices, sauces, and powders. This system allows cheese makers to get a very intense flavor quickly, although the product texture is crumbly and presents handling difficulties for further processing. The Accelerase and the Savorase enzyme systems may be used for very fast ripening of high-flavor cheese.

Full-fat cheese Accelerated ripening in full fat Cheddar is also applicable in countries such as Canada, which have traditionally made cheese from unpasteurized milk, aged for long periods at low temperatures (4–5 °C). As the pressure builds to switch to pasteurized milk, the use of relevant enzyme systems may boost the flavor of pasteurized milk cheese without requiring producers to raise the ripening temperature. Otherwise, off-flavors and/or

gas blowing by heterofermenting microorganisms such as *Clostridium* or *Lactobacillus* species (*Lactobacillus brevis* or *Lactobacillus fermentum*) might break out at high ripening temperatures.

Enzyme-modified cheese flavors The enzymes of LAB, when added to fresh cheese slurry and incubated in a controlled environment, produce within a few days an intense Cheddar cheese flavor without bitterness and off-flavors. This slurry, so-called EMC, can be used directly as flavoring for snack foods and crackers (Haileselassie et al., 1999; Kilcawley et al., 2005). Pizza sauce formulations and other enzyme-modified butter fats, creams, and cultured creams, as well as other novel flavors, are available for use in formulated foods. A wide range of new protein-based products is also possible through lactic enzymes to make flavored protein hydrolysates which can be debittered and given enhanced functional properties, as well as new bioactive and functional peptides.

4.1.5.2 Bacteriophages Because of the rapid multiplication of virulent bacteriophages, they may have an immense impact on dairy fermentation processes. Lactic starter cultures, especially mesophilic lactococci, have been found to contain lysogenic (temperate) bacteriophages, which are capable of integrating into the host genome without causing lysis. Although lytic bacteriophages are the main cause of problems in industrial fermentations, lysogenic bacteriophages also form a potential risk to a starter culture. Lysogenic starter cultures will replicate the prophage, the incorporated phage genome, along with their own chromosomes. Then the prophage switches to a lytic cycle, lysing the host spontaneously or as a response to a certain stimulus. The bacteriophage produced by this strain will undergo lysogeny with the same host bacterium.

During a working day in a modern cheese plant, each vat is used several times without decontamination between fillings, since steaming and cooling would take up a great deal of time and greatly reduce output. The following procedures should be considered to prevent bacteriophage development during cheese making.

1. The number of bacteriophages in the delivered milk should be less than 100 per milliliter, and the whey should be transported in the same container that is used to deliver the milk.
2. The cheese milk and whey cream used for standardization should be pasteurized (15–30 min at 62.8 °C or 1 min at 90 °C) to kill any bacteriophage present.
3. The starter should be injected into the cheese milk through separate pipework.
4. After each cheese milk fermentation process, the vat should be thoroughly rinsed with drinking-quality water.

To prevent bacteriophage development during starter production, bacteriophage contamination must not be allowed to occur, and measures to inhibit multiplication in the starter media should be taken into consideration. Possible sources of phage contamination in starter cultures are the milk medium/ingredients used in cultivating the starter, the vessel in which the starter is grown, the equipment used to process the culture, the inoculum, and the environment. External contamination could be minimized by applying positive pressure in the culture vessel head space and by filtering the air introduced into culture vessels using a *high efficiency particulate air* (HEPA) filter. *Phage inhibitory media* (PIM) used extensively in the United States contain phosphate, citrate, or other ion chelators to sequester calcium ions, which bacteriophage require for effective adsorption to host cell receptors.

Modern starter culture systems To reduce the likelihood of contamination by bacteriophage, which produce bitter off-flavors and reduce the ripening time required, two different modernized starter culture systems are widely used in Europe and New Zealand.

Starter rotating system Rotating pairs of single strains consisting up to eight bacteriophage-unrelated strains (designated A–H) were adopted in New Zealand. The aim of this system is to prevent bacteriophage from accumulating in a factory and causing contamination. One of the pair is a temperature-sensitive strain and the other is a temperature-insensitive one. The temperature-sensitive strain stops growing at the cooking temperature of 38 °C in the making of Cheddar cheese, but the other strain continues developing acid during cheese making as well as in maturation. This longer growth period allows greater opportunity for the development of bacteriophage infection.

The practice-derived (P) starter system The P-starters, mixed-strain starters propagated daily on farms and in factories, usually have a high bacteriophage resistance, which results in the natural selection of starter bacteria. The term “P-starters” is reserved for mixed-strain starters that have been obtained directly from practice and used after a minimum of transfers. P-starters usually contain a great number of phage particles (10^7 mL⁻¹), which contribute to maintaining insensitivity by preventing the growth of bacteriophage-sensitive mutants. Upon infection of a P-starter by bacteriophage, the sensitive bacteria are destroyed and become replaced by insensitive strains within 22 h at 20 °C.

Defense mechanisms against bacteriophages Mesophilic lactococci, which are widely used in the dairy industry, have been found to possess defense mechanisms against the attack of bacteriophages. Cell-wall resistance is usually associated with an alteration of the wall receptor sites and results in the failure of the bacteriophage to become attached to the cell wall. Intracellular protection may be realized through various mechanisms. One is restriction/modification, which operates by means of a restriction enzyme degrading the foreign bacteriophage DNA. Another system is abortive infection, or the failure of the bacteriophage DNA to be converted into a functional bacteriophage particle. Lysogenic immunity, by which the host cell carrying an integrated prophage is protected against superinfection by related temperate bacteriophages, may be regarded as a defense mechanism.

Several plasmid-linked R/M systems have been characterized during recent years, and other plasmid-associated phage resistance mechanisms such as inhibition of phage adsorption and abortive infection, have been detected.

4.1.5.3 Rennet Traditional cheese rennet produced from calf stomachs has been regarded as the unique enzyme for cheese making. Acid proteinases such as chymosin comprise the active component of rennet. Rennet coagulation involves two distinct stages: a proteolytic stage in which the casein micelle is destabilized by hydrolysis of κ -casein, primarily on the Phe₁₀₅-Met₁₀₆ peptide bond, to yield para- κ -casein micelles, and a calcium-mediated stage in which paracasein micelles undergo limited aggregation.

Rennet substitutes Calf rennet substitutes of animal origin (e.g., bovine pepsin, pig pepsin, chicken pepsin) and fungal acid proteases derived from *Mucor pusillus* Lindt (Enporase, Meito, Noury, Novadel), *Mucor miehei* (Fromase, Hannilase, Marzyme, Miki, Rennilase), or *Endothia parasitica* (Supraren, Surecud) have been used for cheese making. These products, however, have certain limitations. Pig pepsin is very sensitive to pH changes in the some usual ranges during renneting and is unstable above pH 6.0.

Chicken pepsin has strong proteolytic activity, which might give bitterness and weakens the cheese body. *Mucor* coagulants show a higher thermostability than other coagulants, a property that causes problems during whey processing and gives lower quality Parmesan and Emmenthal cheeses. Also, the proteolytic activity of casein in the cheese differs and is somewhat stronger than that of calf rennet.

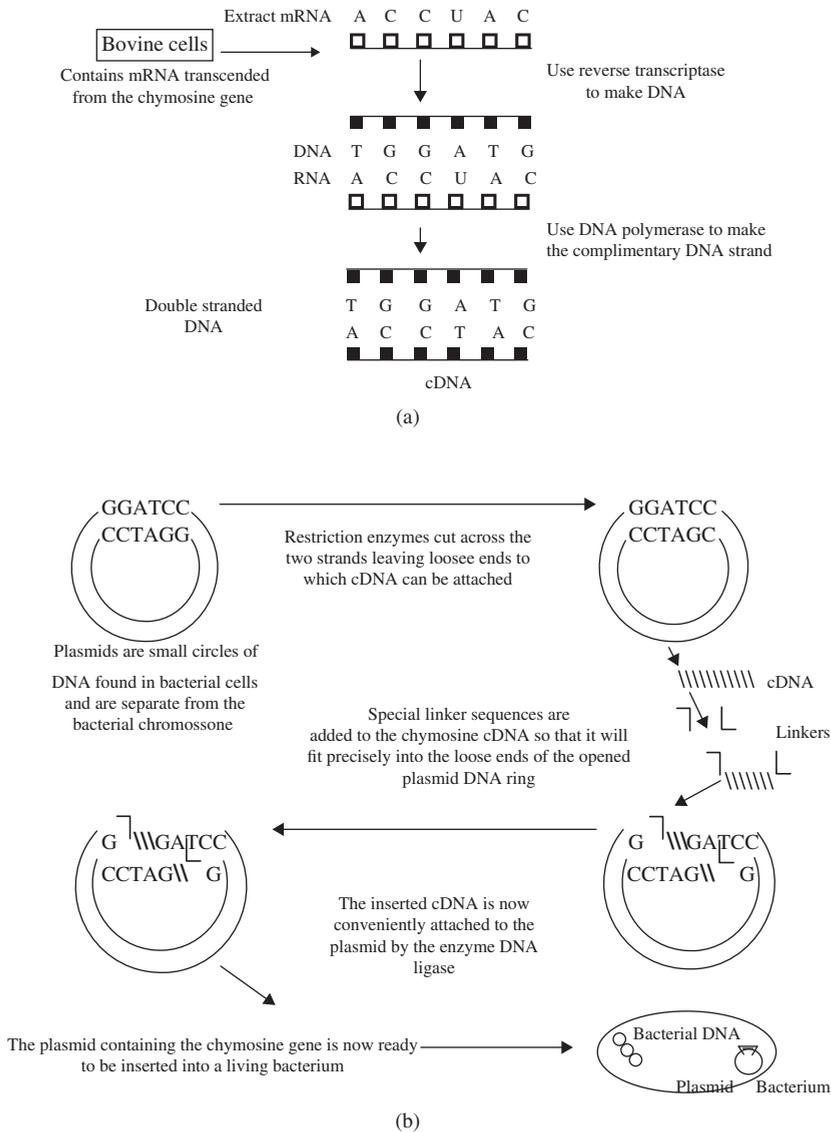


Figure 4.8 The overall process of chymosin production using recombinant *K. lactis*. (a) preparation of cDNA and (b) insertion of cDNA into a vector.

Recombinant rennet Three recombinant chymosin products have recently been introduced on the market: the Chymogens, Maxiren, and CHY-MAX[®]. Chymogen, from the Hansen laboratory in collaboration with Genecor, USA is produced via the controlled fermentation of *Aspergillus niger* var. *awamori*. Chymogen S was created to meet a demand for a product of the same enzyme composition as calf rennet, which contains Chymogen (90%) and bovine pepsin (10%). Other recombinant chymosins, such as Maxiren (Gist-Brocades, the Netherlands) from the yeast *Kluyveromyces lactis* (Figure 4.8) and CHY-MAX[®] (Pfizer, US) from *E. coli*, that purchased by Chr. Hansen have also been produced for cheese making. However, CHY-MAX[®] by *E. coli* is no longer available and is produced by fermentation of the fungus *A. niger*.

By 2008, approximately 80–90% of commercially made cheeses in the United States and Britain were made using recombinant rennet or Fermentation-Produced Chymosin (FPC) (www.gmo-compas.org; 2011). The most widely used is produced either by the fungus *A. niger* (trademark CHY-MAX[®], Chr Hansen) or produced by *K. lactis* (trademark MAXIREN[®], DSM). In all cases, the cheese quality of recombinant rennet was just as good as that of cheese made with the natural calf rennet. The proteolytic patterns during ripening were similar. Besides maintaining cheese quality, the recombinant product gave better process control (since it is less susceptible to variations in milk quality and pH).

Summary

Conversion of milk sugar (lactose) to lactic acid by LAB is second in importance only to alcohol fermentation in the food industry. Fermented dairy foods are enjoying increased popularity as convenient, nutritious, stable, natural, and healthy products. The characteristic aroma, flavor, and texture of fermented dairy foods are often due to the growth of LAB. Today, LAB include the genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, and *Pediococcus*, with the first two types being most frequently used in the manufacture of dairy products such as cheese, yogurt, sour cream, and cultured butter.

The major metabolic pathways for the catabolism of lactose, casein, and citrate by lactococci have been well investigated, but details of the secondary biochemical changes during cheese ripening are still not known. Significant progress has been made with LAB and their enzymes, particularly for accelerated cheese ripening.

The economic importance of this group of microorganisms clearly demands the development of genetic engineering technology to improve and stabilize the desirable characteristics of these starter cultures. Many desirable new traits and properties have already been identified and obtained by gene transfer systems. However, new technologies present safety concerns. Despite the safety issue, practically useful products of GM bacteriophage-resistant starter cultures and recombinant rennet are available on the market.

4.2 Meat and fish products

4.2.1 Introduction

In meat processing, microorganisms had long been regarded as the enemy because they cause spoilage and can present a threat to public health if they are pathogenic.

Fermentation is one way to prolong the shelf life of meat and fish products and has been known since ancient times. Sausages probably are the oldest form of processed food. The ancient Egyptians actually recorded the preservation of meat by salting and sun drying. The ancient Babylonians, Greeks, and Romans had also used sausage as a food source during times of war. It was not until about 1921 that it was recognized that microorganisms contribute to the production of sausages. In the 1940s and 1950s, the use of pure microbial starter cultures began, and some patents were awarded. In the 1960s, commercial meat starter cultures became available to processors, and in 1968, frozen culture concentrates became commercially available for the meat industry. Use of these culture resources was not widespread until the early 1980s, however, mainly as a result of the resistance of producers, who clung to the traditional ways and feared a loss in the quality and consumer acceptance of their final product. Today the importance of the use of starter cultures is recognized in most of North America and in some European countries.

Fermented fish products constitute a major portion of the diet and increase protein intake for a large number of the world's population. The products have an amino acid composition different from that normally found in cereals and therefore complement this staple (cereals), ensuring good utilization of dietary nitrogen. In northern Europe fermented fish products are used mainly as condiments, while in areas such as Southeast Asia such products form the principal part of the diet. Evolution and development of fermented fish products in some tropical countries are due to a number of factors, including the following: fishing is encouraged in countries that have a long coastline; the provision of fresh fish to potential inland consumers may be difficult; and seasonal variations in the availability of fish are typical. Salt, which has been used as a preservative since ancient times, provides a relatively easy and economical method of maintaining the fish supply. Traditional methods have evolved, and well-defined markets are present today that are peculiar to particular regions. Some of the basic methods of preservation involve dehydration, sun drying, and pickling in brine.

4.2.2 Fermented meat products

Fermented dry sausages are defined as chopped or ground meat products that, as a result of bacterial action, reach a pH of 5.3 or less and are then dried to remove 25–50% of the moisture, resulting in a moisture-to-protein ratio no greater than 2.3:1.0. *Semidry fermented sausages*, which are the same that the moisture level is decreased by 15%, are packaged soon after the completion of the fermentation–heating process. They are generally smoked during fermentation, and the moisture-to-protein ratio must be no greater than 3.7:1.0. Two reviews of fermented meats and probiotic aspects have recently been published (Fadda et al., 2010; Rouhi et al., 2013).

4.2.2.1 Classification of fermented sausages In the United States, fermented sausages are classified as dry or semidry on the basis of final moisture content of the product and the descriptive terminology used in industry (Table 4.7). The European system comes from Germany (Table 4.8) and is based on the temperature treatment the product receives. Other classification systems often used in the United States are based on the ethnic origin of the sausage (Germanic, Italian, or Lebanese), the moisture-to-protein ratio, and the composition (moisture, fat, protein, salt, sugar, pH, total acidity, and yield).

Table 4.7 Classification of various fermented sausages

Class	Moisture content (%)	Characteristics	Examples
Dry sausage	25–45%	Cured, air-dried; sometimes smoked; mold-ripened	Pepperoni Genoa salami
Semidry sausage	40–50%	Cured, air-dried; usually smoked	Lebanon bologna Summer sausage Cervelat
Fresh smoked sausage	–	Fresh meat, cured or uncured; smoked, not cooked; must be cooked prior to consumption	Country style Mettwurst Kielbasa
Cooked sausage	–	Cured or uncured; cooked and smoked	Frankfurters Bologna

Source: Adapted from Bacus, J. 1984. Utilization of Microorganisms in Meat Processing. Research Studies Press, Letchworth, UK.

Table 4.8 Classification of fermented sausages in Europe (non-heat treated)

Group	Product	Examples
A1	Raw sausage	Rohwurst
A1,* nonfermented	Fresh sausage, beef and pork	Bratwurst, Thuringer,* Nurnberger*
A1, fermented	Beef and pork	Frischwurst, Mettwurst, Braunschweiger, [†] Thuringer, [†] Cervelat, [†] Teewurst [†]
A11, fermented	Dry sausage, salami types	Dauerwurst, Salami, Cervelat, [†] Plockwurst [†]

*Not smoked.

[†]Smoked.

Source: Adapted from Bacus, J. 1984. Utilization of Microorganisms in Meat Processing. Research Studies Press, Letchworth, UK.

4.2.2.2 Production process

Ingredients (%) of dry and semidry fermented sausages are:

Lean meat (pork, beef)	55–70
Fat	25–40
Curing salts	~3
Fermentable carbohydrate	0.4–2
Spices, flavorings	~0.5
Other: starter culture, ascorbic acid, nitrite	~0.5

The manufacture of dry and fermented sausages proceeds as follows. The meat is formulated by breaking (i.e., grinding, chopping, and mixing) with the fat, to give the desired fat content. This is done at cold temperatures to avoid smearing the fat. The spices, flavorings, curing salts, carbohydrate, nitrite, and starter culture are mixed in, and the mixture is stuffed into the proper sausage casings (cellulose, collagen, or natural) at a temperature of -2.2 to 1.1 °C. In the traditional process, there was an additional step before stuffing called *pan curing*, in which nitrate-reducing bacteria induced the conversion of nitrate to nitrite. Since nitrite is now added directly, this step is no longer required, and the characteristic pink color

of cured meats (nitroso-hemochrome) is formed without pan curing. After stuffing, the sausages are hung in a maturing room. It is in this room that the fermentation takes place.

The environmental conditions can vary widely during fermentation depending on the type of sausage being made. It is not the intent here to describe these conditions for the literally hundreds of different varieties of sausages. Traditionally, the temperature can range from 15.6 to 23.9 °C and the relative humidity from 80% to 90%. The temperature is raised over the course of fermentation: for dry sausages, up to 37.8 °C; for semi-dry sausages, up to 43 °C. The nature of the fermentation will depend on which microorganisms are present. The dominant microorganisms in most sausages are *Lactobacillus* spp., which are generally homofermentative in that they produce only lactic acid as a product that gives the characteristic tangy flavor. Heterofermentative microorganisms can produce an assortment of end products including lactic acid, ethanol, carbon dioxide, and acetic acid, which can add to the uniqueness of a sausage; or they can be unwanted, depending on the type of product. Following fermentation, sausages are fully cooked, partially cooked, and/or placed in a drying room. Fermentation may continue during this process depending on the growth characteristics of the particular bacteria, temperature, pH, carbohydrate level, and degree of heat penetration.

4.2.2.3 Starter cultures To ensure products of consistent flavor, texture, and shelf stability, as well as to improve product safety, most processors have developed pure microbial cultures to control the fermentation of their sausage product. It is evident that with a starter culture, the pH drops much more rapidly, hence the whole manufacturing process is accelerated, leading to economical gains for the processor. The majority of starter cultures are natural isolates of the desirable microorganisms found in the sausage normally.

Starter cultures can come in fresh, frozen, or freeze-dried forms, and they can be single or mixed cultures of selected strains of microorganisms with definite characteristics that are beneficial in the manufacture of certain sausages. They are grown under closely controlled conditions in a liquid medium, concentrated to smaller volume, and then placed in frozen storage or other suitable medium (lyophilization) to preserve their viability and activity. The normal inoculum level ($\approx 10^6$ organisms per gram of product) theoretically inhibits any growth of undesirable microorganisms. A wide variety of species have been used as starter cultures (Table 4.9) and investigated for their potential for use as starter cultures (Table 4.10). Many of the bacteria used are LAB, but are also nonlactic acid bacteria, which are used in combination with LAB, that contribute unique properties. Even *Staphylococcus* spp. and *Micrococcus varians* (*Kocuria varians*) exhibited nitrite and nitrate reductase activities, which are important for the reddening of the sausages by forming nitrosomyoglobin as well as catalase activity that counteracts the formation of hydrogen peroxide, thus preventing color defects and rancidity (Hammes et al., 2003).

The following starter culture characteristics are ideal for sausage production: salt tolerance, fast growth, nitrite tolerance, optimum temperature for growth is ~ 32 °C, and the culture should be homofermentative, nonproteolytic, nonlipolytic, nonpathogenic, non-toxic, and with no off-flavor production. An inactivation temperature of ~ 60 °C is most desirable. Potential of fermented sausage-associated LAB to degrade biogenic amines has also been studied during storage (Kongkiattikajorn, 2013).

Starter improvement Culture research by genetic engineering techniques or by natural selection offers the potential for the selective elimination of such undesirable fermentation end products as excessive CO₂, while retaining the desirable flavor compounds. *Staphylococcus carnosus*, a strain routinely used for dry sausage production in Europe,

Table 4.9 Bacteria used as starter cultures in meat, poultry, and fish products

Products	Bacteria
<i>Semidry fermented meat sausages</i>	
Lebanon bologna	Mixture of <i>Pediococcus cerevisiae</i> / <i>Lactobacillus plantarum</i>
Summer sausage	<i>P. cerevisiae</i> or mixture/ <i>Lb. plantarum</i>
Cerevelat	<i>P. cerevisiae</i> or mixture/ <i>Lb. plantarum</i>
Thuringer	<i>P. cerevisiae</i>
Teewurst	<i>Lactobacillus</i> species
Pork roll	<i>P. cerevisiae</i>
<i>Dry fermented meat sausages</i>	
Pepperoni	<i>P. cerevisiae</i> / <i>Lb. plantarum</i>
Dry sausage	<i>P. cerevisiae</i>
European dry sausage	<i>Micrococcus</i> species or <i>Micrococcus</i> / <i>Lactobacillus</i> species
Salami	<i>Micrococcus</i> / <i>Lactobacillus</i> species or <i>Lb. plantarum</i>
Hard salami, Genoa	<i>Micrococcus</i> species <i>Micrococcus</i> species/ <i>P. cerevisiae</i> ; <i>Micrococcus</i> species/ <i>Lb. plantarum</i>
<i>Fermented poultry sausages</i>	
Semidry turkey sausage	<i>P. cerevisiae</i>
Dry turkey sausage	<i>P. cerevisiae</i> or <i>P. cerevisiae</i> / <i>Lb. plantarum</i>

Source: Hammes et al., 2003. Reproduced with permission of Taylor & Francis Group.

Table 4.10 Some bacteria investigated for their potential use as starter cultures**Lactobacillaceae**

<i>Lactobacillus</i>	<i>Lb. plantarum</i> , <i>Lb. acidophilus</i> , <i>Lb. casei</i> , <i>Lb. fermenti</i> , <i>Lb. brevis</i> , <i>Lb. buchneri</i>
<i>Lactococcus</i>	<i>L. lactis</i> , <i>L. diacetylactis</i> , <i>L. acidilacti</i>
<i>Pediococcus</i>	<i>P. cerevisiae</i> , <i>P. acidilacti</i> , <i>P. pentosaceus</i>

Micrococcaceae

<i>Micrococcus</i>	<i>M. lactis</i> , <i>M. aurantiacus</i> , <i>M. candidus</i> , <i>M. varians</i> (<i>Koccurio varians</i>), <i>M. epidermidis</i> , <i>M. conglomeratus</i> , <i>M. aquatilis</i>
--------------------	--

Actinobacteria *Streptomyces griseus*, *Bifidobacterium* spp.

Source: Hammes et al., 2003. Reproduced with permission of Taylor & Francis Group.

is being investigated as a host strain for recombinant DNA in the genetic organization of certain staphylococci. More recently, strains of *Pediococcus cerevisiae*, *Pediococcus acidilacti*, *Lactobacillus plantarum*, *Lb. casei*, and *M. varians* were also examined with the intent of preparing mixed cultures of compatible strains for meat fermentation. The addition of 0.18% nitrite was slightly inhibitory, while addition of 7% of a spice mix proved stimulatory to the mixed cultures. Antibiosis was observed between lactobacilli and pediococci during fermentation. Micrococci did not produce substances inhibitory toward other cultures and was insensitive to bacteriocins produced by lactobacilli or pediococci. The most rapidly fermenting strains in the mixed cultures did not give the most active culture, and mixed cultures for meat fermentation must be chosen for compatibility by means of disk assays to evaluate antibiosis between potential strains.

In the most recent research on dry fermented sausages in Spain, unexpected difficulties with starter cultures imported from northern Europe have been experienced, mainly as a result of the inhibitory action of contaminating microorganisms from

different environmental conditions. Finally, two microorganisms, *Lactobacillus sake* and *Lactobacillus curvatus*, which are insensitive to the inhibitory action of contaminated microorganisms as starter cultures, have been isolated.

4.2.3 New developments

Studies of the biotechnology of fermented meat technology have lagged behind research on other fermented foods because meat is more difficult to examine than fermented fluids such as milk products and alcoholic beverages. And, practically, it is almost impossible to experiment with sterilized or pasteurized raw materials, since the effects of random microbial contaminants in raw meat cannot be eliminated. Raw meat normally contains several million contaminants per gram, and the influence of these substances on fermentation cannot be overlooked.

There are basically two paths along which development has progressed. Commercial cultures of *P. cerevisiae* and *Micrococcus aurianticus* were simultaneously introduced to the American and European meat industries, respectively. The *Pediococcus* strains were added for their lactic acid producing capability, while the *Micrococci* were used as a control mechanism to reduce nitrates to nitrites, to effect the curing reaction. *Pediococci* do not reduce nitrate, and a mixed cure of nitrate and nitrite has replaced the traditional nitrate cure in many American processes. The Europeans depend more on chance inoculation to carry out the fermentation, and in general the fermentation times are slower, with lower temperatures being used, and thus a higher final pH. American processors use a higher temperature with a faster fermentation rate and a lower final pH. In the United States, therefore, research has focused on rapid acid-producing strains of pediococci and lactobacilli.

Successful meat fermentation can now occur in as short a time as 6–8 h, given the proper formulation, starter culture, and process controls, compared with the traditional 3–5 days. Since one effect of this rapid fermentation is the inhibition of flavor-producing microorganisms, various control mechanisms in the formulation and process are being explored to optimize the effects of both these microorganisms and the lactic acid producing bacteria. This can be achieved to a certain extent by altering the inoculation ratio and/or varying the processing schedule to initially favor one of the lower temperature cultures (which are generally the flavor-producing ones) before elevating the process temperature to accelerate acid production. In Europe, research has focused on the equipment and environmental controls and a wide variety of starter cultures to effect unique flavor and color development. Also in Europe, the possibility of using starter cultures for meat products of other types (hams, dry-cured products) is being investigated.

The variety of fermented meat products available around the world is nearly equal to that of cheese. With meat products, raw fermented sausages could constitute an appropriate vehicle for such microorganisms into the human gastrointestinal tract. It may be possible to select probiotic strains to produce the probiotic fermented meats with all the sensory attributes and health benefits.

4.2.4 Fermented fish products

In general, when fish and dry salt are packed in layers and left for a long time, cellular liquid will be extracted to form a pickle. If this is left in contact with the fish and the proteolytic enzymes of the fish are active enough, a fish sauce will develop. If the liquid is drawn off at intervals, or the period of contact is kept short, it is possible to produce a fish paste. If the

period is much shorter and the amount of salt used is limited, the result will be salted fish that have undergone softening.

Three types of fermentation can occur: (i) traditional fermentation carried out by the enzymes and entrails of fresh fish in the presence of high concentrations of salt, (ii) traditional fermentation carried out by the combined action of fresh fish enzymes and entrails and microbial enzymes in the presence of salt (the microbial enzymes are added as a starter, in that they are usually present on some form of cereal-like cooked rice or maize), and (iii) nontraditional processes in which products are obtained by accelerating the rate of fermentation with enzymes or by chemical hydrolysis.

4.2.4.1 Fish sauces Fermented fish sauces have been consumed since ancient times, and the earliest reported is *garum*, which was highly prized in the Roman era. This product was made from the blood and viscera of mackerel, and fermentation was due to the proteolytic enzymes from the viscera. *Garum* is thought to be different from the sauces produced today in that it was decanted to give a clear liquid, and the residue was made into a thicker sauce called *alec*. In Southeast Asia, the use of fermentation as a method of preserving fish has been of importance since earliest times. Table 4.11 lists some of the different fish sauces found in the world, along with the basic process and fish species used.

Table 4.11 Fish sauces

Name	Fish species	Ratio of fish to salt; fermentation time	Country
Shottsur	<i>Astroscopeus</i> spp. (sandfish)	5:1 salt + malted rice and koji (3:1)	Japan
Uwo-shoyu	<i>Clupea</i> spp. (sardine)	6 months	
Ika-shoyu	<i>Omnastrephis</i> spp. (squid)		
Saewu-jot	Shrimp	4:1 salt, 6 months	Korea
	<i>Sardinella</i> spp., <i>Jelio</i> spp., <i>Carangidae</i> spp., <i>Engraulis</i> spp., <i>Teuthis</i> spp.	4:1 salt, 3–12 months	Hong Kong
Nam-pia	<i>Stolephorus</i> spp., <i>Ristrelliger</i> spp., <i>Cirrhinus</i> spp.	5:1–3:1 salt, 5–12 months	Thailand
Ketjap-Ikan	<i>Stolephorus</i> spp., <i>Clupea</i> spp., <i>Leiognathus</i> spp.	3:1–4:1 salt, 3–12 months	Indonesia
Budu	<i>Stolephorus</i> spp.	5:1–3:1 salt + palm sugar and tamarind, 3–12 months	Malaysia
Patis/Bagoong	<i>Stolephorus</i> spp., <i>Clupea</i> spp., <i>Decapterus</i> spp., <i>Leiognathus</i> spp.	3:1–4:1 salt, 3–12 months	Philippines
Nuoc-man/Mam	<i>Stolephorus</i> spp., <i>Ristrelliger</i> spp., <i>Engraulis</i> spp., <i>Decapterus</i> spp., <i>Dorosoma</i> spp., <i>Clupea</i> spp.	3:1–3:2 salt, 3–12 months	Cambodia/ Vietnam
Colombo-cure	<i>Ristrelliger</i> spp., <i>Cybiium</i> spp., <i>Clupea</i> spp.	Gutted fish with gills removed and tamarind added, 6:1 salt, 12 months	India and Pakistan
Garos	<i>Scomber colias</i>	Liver only, 9:1 salt, 8 days	Greece
Pissala	<i>Aphyia pellucida</i> , <i>Gobius</i> spp., <i>Engraulis</i> spp., <i>Atherina</i> spp., <i>Meletta</i> spp.	4:1 salt, 2–8 weeks	France
Anchovy	<i>Engraulidae encrasicholus</i>	Beheaded and gutted fish, 2:1 salt, 6–7 months	Many

The uneviscerated fish is normally mixed with salt in the ratio of 2:1 or 5:1 (fish:salt), and is then fermented in tanks buried in the ground for at least 6 months. The supernatant liquid is drained off, filtered, and ripened for about 3 months under the sun to obtain better aroma and color. The finished product is a liquid protein, which contains a good source of amino nitrogen, Ca, P, Fe, other organic nutrients, and vitamin B complex.

With traditionally produced sauces, the most important factors in consumer acceptability are flavor and aroma. Aseptically produced sauces do not produce the aroma typically associated with the respective sauces, and thus it was proposed that microorganisms were involved. The proteolytic enzymes responsible for the protein degradation that occurs may have endogenous or microbial origins. Some earlier studies have provided conflicting results and further study is needed in this area.

Among the large numbers of bacteria and yeasts present in fish fermentation, microflora such as *Bacillus*, *Micrococcus*, *Lactobacillus*, and *Pediococcus* genera are most common. The high concentration of salt limits microbial growth, particularly of pathogens, and therefore reduces spoilage. The surviving microflora must thus be at least halotolerant or halophilic bacteria. These halophilic bacteria appear to be involved in the development of aroma, since the aseptically produced fish sauces did not give the typical aroma associated with them (Beddows, 1985).

The production cycle for fish sauce is long, and thus many attempts have been made to speed up the production time by adding exogenous enzymes such as bromelain, ficin, papain, trypsin, and microbial proteases. The organoleptic properties of fish sauces with added enzymes are not satisfactory, however. Others have accelerated production of fish sauces by adding some halophilic *Bacillus* C₁ or LAB (Steinkraus, 2005).

Recently, research has focused on the chemical components of fish sauces produced in various parts of Japan (Steinkraus, 2005). The chemical components of fish sauces compare to those of fermented plant materials. There is a similarity in that both contain salty and amino acid substances as well as the umami amino acids. Other works (Lüche and Earnshaw, 1991) explored the potential use of starter cultures for the production of chilled fish products. *Leuconostoc* spp. and *Lb. plantarum* showed a wide inhibitory range among 61 isolates of LAB, which were phenotypically identified and characterized based on gas production, carbohydrate fermentation, production of off-odors, peroxide production, antagonistic activity, and growth at 2, 5, and 10 °C.

Biogenic amines have been found to be produced during processing of foods which include fishery products and other fermented foods. Biogenic amines including histamine are produced as a result of microbial decarboxylation of dietary amino acids in animal, plants, and microorganism, and are reported to be toxic to humans. Common toxic symptoms of biogenic amines in humans are nausea, respiratory distress, hot flushes, sweating, heart palpitation, headache, a bright red rash, oral burning, and hypertension as well as hypotension (Mah and Hwang, 2009). There are few reports on the inhibition of histamine formation in fish sauce; rice bran nuka (Kuda and Miyawaki, 2010) and starter culture (Zaman et al., 2011). Koji and histidine (Lee et al., 2013) have been applied to accelerate the fermentation of fish sauce for the purpose of shortening its manufacturing period.

4.2.4.2 Fish pastes Much more widely produced and eaten than fish sauces, fish pastes are usually consumed in small quantities, as condiments with rice dishes. The process of fermentation is much shorter than for sauces, and in addition, larger fish species as well as fish that are too low in enzyme activity can be used for fish sauce production. Table 4.12 shows some of the fish pastes that have their origins in Asia.

Very few data are available on the role, if any, of microorganisms in the production of fish pastes. Whether they assist in the breakdown of tissue or in the development of flavor and aroma is a subject of speculation.

Table 4.12 Fish pastes and their ingredients

Name	Ingredients	Country
Kapi	Marine shrimp, salt	Thailand
Pla-mam	Freshwater fish, salt, roasted rice, pineapple	
Pla-chao	Freshwater fish, salt, glutinous rice, KNO ₃	
Kung-chao	Marine or freshwater shrimp, salt, color, roasted rice, sesame	
Blachan	Shrimp, salt	Malaysia
Bagoong	Fish or shrimp, salt, color	Philippines
Trassi	Fish, salt; sun dried	Indonesia
Nga-Ngapi	Fish, salt	Burma
Shiokara	Squid or skipjack; salt; mustard rice	Japan
Sidal	Small fish, salt; dried, crude fish oil	Pakistan and India

4.2.4.3 Salted fish A number of salted fish products are prepared in a way that facilitates microbial and biochemical action. The degree of proteolysis is much less than for fish paste production. In Japan, a product is made from sea bream and sandfish, which are gutted and cured with 20–30% salt. The fish are mixed with a fermenting cereal such as rice, and LAB thrive to lower the pH over a month or two. The fish is then desalted and the liquid is drained off. Boiled rice and koji are mixed in and a secondary fermentation occurs, giving the characteristic flavor of the product, as the yeasts in the koji become very active. In this case, lactic acid aids in preservation, and the bulk of the flavor comes from the koji and secondary fermentation.

The production of anchovies in certain countries usually involves some fermentation. The fish are gutted and beheaded and placed in a vat with salt. The contents are weighed down to extract the pickle as it forms, and the fish are matured for 6–7 months at ~16 °C.

Summary

Fermented meat and fish products form only a small portion of the wide variety of the fermented foods available today, and research in these sectors tends to be relatively limited, mainly because of the nature of the substrate. As our understanding of the complex processes that occur during fermentation increase and as we are able to optimize production through techniques such as genetic engineering, it should become possible to create an economical and sustainable food supply for generations to come.

4.3 Vegetable products

4.3.1 Introduction

The fermentation of vegetables, a practice that originated in the orient, has been used as a means of preserving food for more than 2000 years. In the third century BC., large-scale production of fermented vegetables (cabbages, radishes, turnips, cucumbers, etc.) was reported during the construction of the Great Wall of China. The most important fermented vegetables found on today's market are pickles, sauerkraut, and olives. Carrots, cauliflower, celery, okra, onions, and sweet and hot peppers are also sold as fermented vegetable products.

Soybeans have been an important source of protein, fat, and flavor for oriental people for thousands of years. Many foods are prepared from fermented soybean, including soy sauce, miso, tempeh, and natto. Vegetable products are prepared not only by bacterial fermentation but also predominantly by fungi in particular species of *Aspergillus*. In making soy sauce and miso, a koji is prepared in a preliminary fermentation that is used in a second stage to ferment a combination of cooked soybeans and cereal. The cereal is usually wheat when making soy sauce and rice when making miso. Iru, a product derived from locust beans fermented by *B. subtilis* and *B. licheniformis*, is the most important food condiment in Nigeria.

4.3.2 Fermented vegetable products

4.3.2.1 Sauerkraut *Sauerkraut*, a major fermented vegetable food in Europe and the United States, is produced from the natural lactic acid fermentation of cabbage that has been shredded and salted. The procedure for producing sauerkraut is as follows:

1. The cabbage is allowed to wilt for a day or two at room temperature.
2. The cabbage is washed carefully, and all damaged areas are removed.
3. The cabbage is finely sliced, and salt is added to a final concentration of 2.25–2.50%, being evenly distributed to avoid high salt pockets. The addition of salt purges the cabbage, and subsequently liquid containing the carbohydrate (2.9–6.4% of the total weight of the cabbage) is squeezed out. How much lactic acid will be produced during the fermentation is determined eventually by the amount of sugar present in the liquid.
4. The shredded, salted cabbage is allowed to soften for a while to prevent breakage of the shreds during packaging in the fermentation vats.
5. The shreds are packed densely but gently, and weights are applied to the surface of the cabbage until enough liquid has been extruded to cover the surface entirely.
6. Conditions are made anaerobic to prevent spoilage by yeasts and molds by placing plastic sheets over the surface of the brine and filling it with water or brine.
7. Fermentation takes place at 18.3 °C or lower, since this temperature favors the growth of heterofermentative LAB over the other bacteria and therefore gives the sauerkraut a superior taste.

At the beginning of the fermentation, some oxygen will remain in the shredded cabbage. Plant cells, aerobic bacteria, yeasts, and molds will consume this remaining oxygen and die off, as the supply diminishes. The facultative anaerobes will then increase in number. First, coliform species (e.g., *Enterobacter cloacae*) and *Flavobacterium* species produce gas and volatile fatty acids, and start producing flavors. As the acidity increases, these organisms are normally replaced by *Leuconostoc mesenteroides*, which will become the predominant organism. This heterofermentative organism will produce lactic acid as well as acetic acid, ethanol, and mannitol esters, which impart a bitter flavor, and carbon dioxide. *Lc. mesenteroides* is also responsible for the good flavor, diacetyl, attributed to sauerkraut. As the acidity continues to increase and reaches 0.7–1.0%, *Lc. mesenteroides* dies off and is replaced by *Lb. plantarum*, a homofermentative organism that produces lactic acid as its principal end product. *Lb. plantarum* is also responsible for the degradation of the undesirable mannitol esters produced earlier by the *Leuconostoc* species. Under favorable conditions, *Lb. plantarum* produces lactic acid until an acid concentration of 1.5–2.0% is reached. At that point, the fermentation should be completed within 1–2 months, when the total acidity has reached 1.7–2.3%. The product should be stored at low temperatures

or pasteurized to prevent the growth of spoilage organisms and subsequent deterioration of the product.

A recent study on the role of antimicrobial proteins in the ecology of traditional fermented foods shows that nisin, which is produced by some strains of *L. lactis lactis*, might be involved in controlling the rise of *Lb. plantarum*. *Lc. mesenteroides*, being resistant to nisin, will therefore have a competitive advantage during the first part of the fermentation. Spoilage may result from improper fermentation temperature, incorrect salt concentration, and/or improper anaerobic conditions. Too high a fermentation temperature might result in the inhibition of *Lc. mesenteroides* and the growth of *P. cerevisiae*, which produces unfavorable results. Too low a fermentation temperature would allow *Enterobacter* and *Flavobacterium* species to remain the predominant organisms. Too long a fermentation would result in the elimination of *Lb. plantarum*, and the ascendancy of *Lb. brevis* will further increase the acidity of the sauerkraut and produce undesirable flavors and aroma. Too low a salt concentration would result in complete spoilage of the vegetable product by any anaerobes present in the cabbage. Too high a salt concentration would enable *P. cerevisiae* to grow freely, since it is highly tolerant to salt, whereas some of the undesirable organisms might break down the cell structure. Dark brown or black sauerkraut may result from oxidation of various compounds to chromogenic compounds by undesirable bacterial enzymes. Pink sauerkraut might result from the growth of red asporogenous yeasts, which grow well in the presence of air and high concentrations of salt. Slimy sauerkraut can also result from the presence of encapsulated varieties of *Lb. plantarum*; this product can be rinsed to make it edible, although it is not acceptable for sale to the public. A good final product should be a light-colored, crispy food having 1.72–2.3% lactic acid, a pH of 3.4–3.6, and a pleasant aroma and taste produced by diacetyl.

4.3.2.2 Cucumber pickles There are two different processes for producing pickled cucumbers. Salt or salt stock pickles are used for sour, sweet–sour, and mixed pickles, and pickle relishes. Dill pickles are produced differently from salt stock pickles.

Salt stock pickles Immature cucumbers, in good conditions, are washed and placed in barrels where they are then brined. The wash water should preferably contain chlorine dioxide, which has been found to be 10 times more efficient than chlorine in killing microorganisms in the wash water. Cucumbers should not remain in this solution for extended periods, however, since oversoaking can result in poor fermentation later. Salt stock cucumbers can be produced in one of two ways. The *low salt method* uses a brine containing 30 salometer (or 8%) NaCl. The cucumbers are added to this brine along with 9 kg of salt per 100 kg of cucumbers. Subsequently, the salt concentration is increased by 2 salometer each week till it reaches 50 salometer. The salt concentration is then increased by 1 salometer each week till it reaches 60 salometer. The salt concentration is crucial, since a concentration of less than 6% would allow the growth of spore-forming bacteria. The *high salt method* is similar except that the suspending brine contains 10.5% salt (40 salometer). Salt is added weekly to raise the concentration by 3 salometer until 50 salometer is reached (13.2% NaCl). In both methods, the cucumbers are held down, under the surface of the brine, during the entire fermentation. Open tanks are covered by plastic sheets. The fermentation is completed within 6–9 weeks. Potassium sorbate (0.03%) may be added to acidify the brine. This, along with constant air purging, results in salt stocks with the lowest incidence of all types of bloater damage.

Dill pickles There are two types of dill pickle: the overnight dill and the genuine dill pickle. Both involved the addition to the fermentation vat of the herb dill. Both have a much lower salt concentration than the salt stock pickles, and both have added acetic acid. Overnight dill pickles are prepared by adding 45 gallons of a 20 salometer (5.3% salt) brine solution to 10 lb of cured dill weeds, 1 lb of mixed spices, a gallon of 100 grain acetic acid, and cucumbers. The fermentation takes place at 3.3 °C until the lactic acid percentage has reached 0.3–0.6%. The temperature must remain low because the salt and acid concentrations are relatively low. Genuine dill pickles are prepared by adding to a 32 salometer (7.5–8.5% salt) brine solution, the same concentration of spices, but only a quart of acetic acid per 45-gallon barrel. The fermentation should take place around 16 °C but it can go up to 29 °C. The fermentation should be stopped when the lactic acid concentration has reached 1.0–1.5%. The fermentation takes around 5–6 weeks at room temperature.

Now we turn to the flora involved in the fermentation of the salt pickles. Initially, organisms such as *Pseudomonas* spp., *Flavobacterium* spp., *Alcaligenes (Acromobacter)* spp., and *Bacillus* spp. will grow and multiply. These are considered to be spoilage organisms. If the salt concentration is low, other organisms, such as coliforms, *Lc. mesenteroides*, *Streptococcus faecalis*, and *P. cerevisiae*, will also grow and produce acid in the first few days of the fermentation. Therefore, unlike the fermentation of sauerkraut, *Leuconostoc* spp. never predominate in the initial stages of the fermentation. If the salt concentration is not too high, *Lb. brevis* will begin to grow and contribute to the acidity of the fermentation fluids. In the low salt brine, a mixture of low acid tolerant species of *Leuconostoc* and the high acid tolerant species of *Lactobacillus* and *Pediococcus* will predominate during the intermediate stages of fermentation. In the final stages, *P. cerevisiae*, *Lb. brevis*, and *Lb. plantarum* predominate. As the salt concentration exceeds 8%, *P. cerevisiae* lose its activity and the two lactobacilli complete the fermentation. The final product should have 0.9% lactic acid and a pH of 3.3. The flora involved in the fermentation of genuine dill pickles initially comprises the soil bacteria, which are soon inhibited by the acid produced by *Lc. mesenteroides*, *S. faecalis*, and *P. cerevisiae*. Subsequently, these organisms are replaced by *Lb. plantarum*, which will mainly produce lactic acid. *Lb. brevis* may contribute to the final lactic acid concentration of 1.0–1.5%.

The major problem with pickles is because of fermentative yeasts. These organisms may cause gas production inside the cucumbers resulting in *bloaters*. The major yeast species responsible for this are *Brettanomyces*, *Hansenula*, *Saccharomyces*, and *Torulopsis*; *Lb. brevis* also has been observed. Another type of spoilage caused by bacteria is blackening of pickles. Organisms such as *Bacillus nigrificans*, which are found on cucumbers, are able to produce hydrogen sulfide if the process water contains high concentrations of iron or copper ions. Pickles may be preserved by refrigeration or pasteurization (internal temperature of 74 °C for 15 min).

4.3.2.3 Olives Like cucumbers, olives are fruits that are classed as vegetable substances and are fermented in a similar fashion to other vegetable products. The olives are harvested when fully developed but still green. Fermented, ripened olives also exist, but they are less popular and are processed differently. There are three major types of green fermented olives. The *Spanish* types are brined after having received a lye treatment. The green olives are submerged in a 1.25–2.0% lye solution at 15.6–21.1 °C until the solution has penetrated half to three-quarters of the way toward the pit. They are then leached to remove the lye by changing the water several times. This treatment removes some of the bitterness of the

olives that is due to the glucoside oleuropein. *Greek*-type olives are also subjected to a lye treatment, which is followed by an aeration step, to produce the highly colored to jet-black olives. These olives are placed in a high salt brine of 7–10% at the beginning and gradually increased to a final 15% salt concentration. *Siciliano*-type olives are not subjected to the lye treatment but simply brined in a final low salt concentration of 7–8%. Fermentation, which is relatively fast because the sugar has not been removed by lye treatment, is stopped when the acidity reaches 0.4–0.6%.

The microbial population responsible for the fermentation of olives differs from that of sauerkraut and pickles mainly because the higher salt concentration of the brine prevents many salt-sensitive strains from growing and gives superior advantages to salt-tolerant strains. For instance, *Lc. mesenteroides*, which is not very resistant to high salt concentration, will not dominate at any stage of the fermentation. On the other hand, *Lb. plantarum*, which is much more tolerant to salt, will be the major fermenter. Yeasts are also very salt-resistant, and if the concentration of the brine reaches 40 salometer, they become the dominant fermenting organisms and produce mainly acetic acid. If the fermentation is allowed to continue, spoilage may occur.

Olives are apparently more sensitive to spoilage than sauerkraut and pickles. The addition of a starter culture (pure culture fermentation) of *Lb. plantarum* has been suggested as a way to reduce the chances of spoilage by undesirable organisms. The maintenance of anaerobic conditions is especially important when producing fermented olives, since yeast proliferation is a major problem in this industry. Yeast, along with mold growth, causes the production of off-flavors. Gas pockets may also result from the proliferation of yeasts and other organisms. The major organisms involved in that type of spoilage are species from the genera *Saccharomyces*, *Hansenula*, *Bacillus*, *Aeromonas*, and *Clostridium*.

4.3.2.4 Other bacteria-based vegetable products Other fermented vegetable products, such as cauliflower, celery, carrots, and onions mixed with pickled cucumbers, are found on the market. Depending on the region, you might also find fermented beets, okra, and mustard leaves. These vegetables are fermented in brine or by dry-salting, as with sauerkraut. Usually leafy vegetables are dry-salted, to ensure that they remain crispy. Sliced beets, cauliflower, tomatoes, and okra tend to become slimy during fermentation because they have a relatively high sucrose content, which is transformed into dextran during the process. A vegetable-like celery will not exhibit this feature because its sugar content is much lower. The natural fermentation of these vegetables is very similar to that of sauerkraut. *Lc. mesenteroides* initiates the fermentation, which will be continued by other LAB, mainly *Lb. plantarum*. The successful fermentation of green peas with strains of *Pediococcus* or *Lactobacillus* added to brine as starter cultures and the fermentation of onions with strains of pure culture *Lactobacillus* are known. The fermentation process also greatly reduces the levels of antinutritional and toxic components such as phytates, trypsin inhibitor, cyanogenic glycosides, nitrates, and nitrites found in some raw plants and vegetables (Steinkraus, 2005; Reddy and Pierson, 1994).

Fermented vegetable juices are commercially available in many European countries. These juices are prepared by adding 2–3% LAB starter culture per total volume of vegetable juice. Many lactic fermented cabbages, carrots, beets, radishes and other vegetables other than sauerkraut are also vacuum packaged for commercial sales to overcome refermentation and prolong shelf life (www.caldwellbiofermentation.com).

4.3.3 Fermented soy products

4.3.3.1 Soy sauce Soybean products have been staple food in the Asian diet for centuries. Although intake of soy remains low in most Western populations, the use of soy isoflavone supplements has become commonplace, and soy ingredients have become very popular. While unfermented soy contains many undesirable compounds such as phytates, protease inhibitors, hemagglutinin, saponin, soyatoxin, oxalates, goitrogens, and esterogens and soy proteins are allergenic, soy is also loaded with the antioxidant isoflavones, genistein, and daidzein. Soy isoflavones behave like mild estrogens and may reduce hot flashes in women, but can lead to breast cancer (Wu et al., 2013). Soy isoflavones have a direct correlation with increased thyroid disease. Isoflavones are a type of phytoestrogen, which is a plant compound resembling human estrogen, that have been found to have adverse effects on various human tissues. Soy phytoestrogens are known to disrupt endocrine function, may cause infertility, and may lead to breast cancer in women. Genistein and daidzein, the soy phytoestrogens most widely studied, have been shown to possess anticancer properties, but more studies are needed to better understand and elucidate all pathways mobilized by genistein and daidzein (Adjakly et al., 2013). During the fermentation process, the enzymes produced by the bacteria and yeast break down and become easier to digest. This also makes the protein content of unfermented soy protein easier to digest.

Soy sauce is a dark brown liquid, with a salty taste and a sharp flavor, which is made by fermenting soybeans, wheat, and salt. It is an all-purpose seasoning agent used in the preparation of foods as well as for a table condiment in oriental and many other countries. The fermentation of soy sauce is essentially the hydrolysis of proteins, carbohydrates, and other constituents of soybeans and wheat to peptides, amino acids, sugars, alcohols, acids, and other low-molecular compounds, catalyzed by the enzymes of mold, yeast, and bacteria. In addition to the fermentation technique, two other processes are followed. One is a chemical method, in which acid hydrolyzes the proteins and the carbohydrates, and the other is a combination of the two. Figure 4.9 shows the production steps in brewing soy sauce.

During the initial stage of mash fermentation (*moromi*), proteins and carbohydrates are hydrolyzed by *Aspergillus oryzae* or *Aspergillussoyae*. Osmophilic bacteria gradually lower the pH from about 6.7 to about 5.0. *Pediococcus soyae* is the predominant flora in the mash. In the course of low temperature fermentation, *Saccharomyces rouxii*, a dominant osmophilic yeast in the alcoholic stage of fermentation, produces about 2.5% alcohol. Yeasts and bacteria continue fermentation in the mash. A good soy sauce has a salt concentration of about 18%. Its pH is between 4.7 and 4.8; below that, the product is considered to be too acid, suggesting acid produced by undesirable bacteria. A ratio of greater than 50% of amino acid nitrogen to total soluble nitrogen is evidence of quality.

Soy sauce is made either by fermentation or by acid hydrolysis. Some commercial sauces have both fermented and chemical sauces. Some brands of soy sauce are made from acid-hydrolyzed soy protein instead of brewed with a traditional culture. This takes about three days. Although they have a different flavor, aroma, and texture when compared to brewed soy sauces, they have a longer shelf-life and are usually made for this reason. The clear plastic packets of dark sauce common with Chinese-style takeout food typically use a hydrolyzed vegetable protein formula. Some higher-quality hydrolyzed vegetable protein products with no added salt, sugar or colorings are sold as low-sodium soy sauce

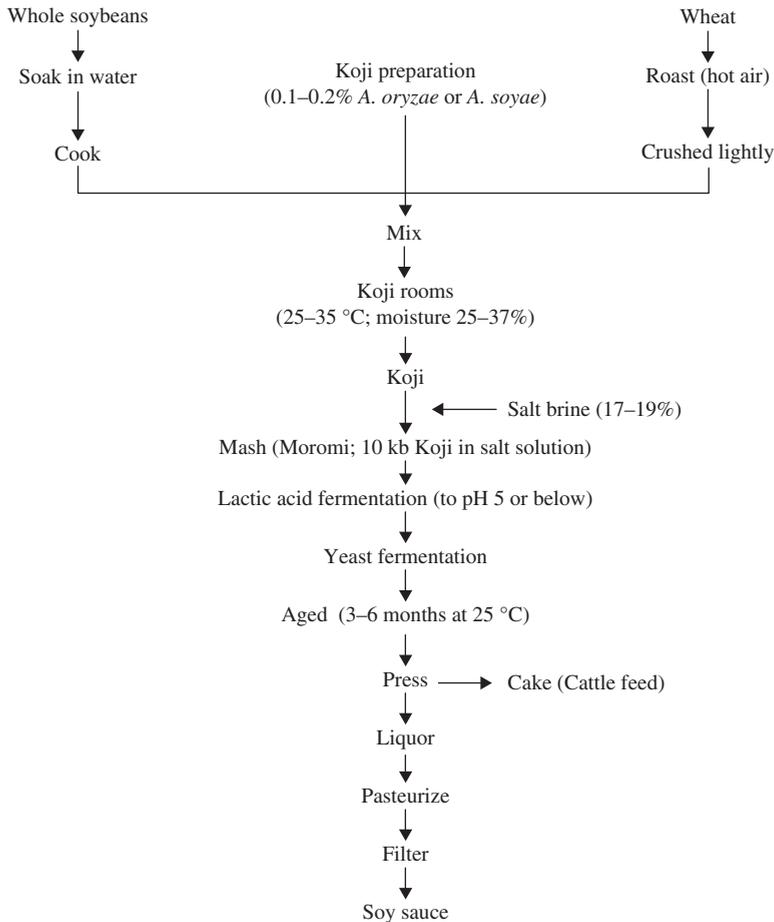


Figure 4.9 Flowchart for soy sauce manufacture.

alternatives called “liquid aminos” in health food stores, similar to the way salt substitutes are used, but these products are not necessarily low in sodium.

For the health aspects, soy sauce may contain ethyl carbamate (EC), a Group 2A carcinogen, 3-MCPD (3-monochloropropane-1,3-diol) and 1,3-DCP (1,3-dichloropropane-2-ol), that cause a food scare in Vietnam (2007) (<http://afp.google.com/article/ALeqM5iiMrrBvLTLGX3P0uXmSqeT1Zjayw>). Health Canada has established 1.0 part per million (ppm) as a guideline for importers of these sauces, in order to reduce Canadians’ long-term exposure to these chemicals. This is considered to be a very safe level. As most varieties of soy sauce contain wheat, to which some people have a medical intolerance, but gluten is not detectable in the finished product.

4.3.3.2 Miso *Miso*, a food prepared by the fermentation of soybeans and salt with or without a cereal, is produced in a number of countries in Asia. The original meaning of *miso* is “immature shoyu” that is a paste resembling peanut butter in consistency and smooth in

the texture. The ingredients used to produce miso may include any mix of soybeans, barley, rice, buckwheat, millet, rye, wheat, hemp seed, and cycad, among others. Lately, producers in other countries have also begun selling miso made from chickpeas, corn, azuki beans, amaranth, and quinoa. Fermentation time ranges from as little as five days to several years. The wide variety of Japanese miso is difficult to classify, but is commonly done by grain type, color, taste, and background.

In miso manufacture, methods differ from variety to variety, but the basic process is that charted in Figure 4.10. Briefly, it involves the cleaning and cooking of soybeans; preparation of rice koji; mixing of soybeans, salt, koji, and inoculum; fermentation under anaerobic conditions; and blending and packaging of the product for market. Miso manufacture is essentially two successive fermentations. First koji is prepared under aerobic conditions from strains of *A. oryzae* and *A. soyae*. Then follows an anaerobic fermentation involving yeasts and bacteria. During the second fermentation the enzymes convert the rice into dextrin, maltose, and glucose, which serve as fermentable sugars for the yeasts and bacteria. The soybean protein is converted to peptides and amino acids. One of the chief amino acids produced is glutamic acid, which gives miso its delicious flavor. Soybean oil is converted in part to fatty acids.

Some suggested that miso can help treat radiation sickness, citing cases in Japan and Ukraine where people have been fed miso after the Chernobyl nuclear disaster and the atomic bombings of Hiroshima and Nagasaki (<http://web.archive.org/web/20060524132713/http://www.clearspring.co.uk/pages/site/products/miso>). Some experts suggest that miso is a source of *Lb. acidophilus*. Lecithin, a kind of phospholipid caused by fermentation, which

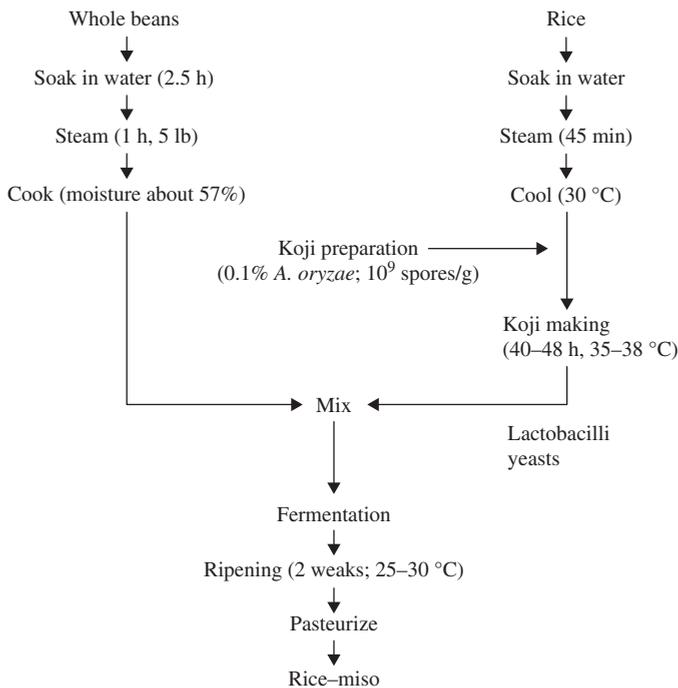


Figure 4.10 Flowchart for rice-miso manufacture.

is effective in the prevention of high blood pressure, but miso is also relatively high in salt which can contribute to increased blood pressure in the small percentage of the population with sodium-sensitive pre-hypertension or hypertension. There is no definitive evidence that high sodium intake leads to negative clinical conditions such as hypertension in healthy persons. Clinical evidence indicates wide-population heterogeneity in response to sodium.

4.3.3.3 Natto In the natural fermentation of soybeans, molds usually dominate, but one of the new products in which bacteria predominate during fermentation is *natto*. *Bacillus natto*, also identified as *B. subtilis*, is claimed to be responsible for the characteristic odor and persistent musty flavor of this organism. Natto is covered with a viscous, flavorful, and slimy glutamate polymer. There is no change in fat and fiber contents of soybeans during a 24-h fermentation period, but the carbohydrate almost totally disappears. A great increase in water-soluble and ammoniacal nitrogen was noted during fermentation as well as during storage. The amino acid composition remained the same. Boiling markedly decreased the thiamine content of natto approximately to the same level of soybeans. Riboflavin in natto greatly exceeds that in soybeans. Vitamin B₁₂ in natto was found to be higher than in soybeans.

Soybean cooked syrup (SCS), a by-product of natto, is rich in nutrients such as functional oligosaccharides, fibers, and proteins. This product, however, has been treated as wastewater and discharged by natto production companies in Japan. A study (Matsuda et al., 1992) suggested that the SCS could be used to produce a cheeselike food by fermenting the syrup with LAB. Cultures of *Lb. casei rhamnosus* were shown to be especially valuable starter bacteria for this fermentation, since this strain has an ability to decompose diazin and genistin, the SCS components that produce unpleasant tastes.

Today's mass-produced natto is sold in small *polystyrene* containers containing 40–50 g. Natto contains no cholesterol, almost no sodium, but is a significant source of iron, calcium, magnesium, protein, potassium, vitamin B6, B2, E, K2 and more. When *B. subtilis* natto breaks up soy protein, the bacteria creates chains of polyglutamic acid, Gamma polyglutamic acid. The unique part of this polypeptide chain is that the peptide bond is between Nitrogen and R-group's carboxyl acid.

For the health benefits, both pyrazine, a flavor compound, and *nattokinase* (serine protease) seem to reduce the likelihood of *blood clotting*. Studies have shown that oral administration of nattokinase in enteric capsules leads to a mild enhancement of fibrinolytic activity in rats and dogs. It is, therefore, plausible to hypothesize that nattokinase might reduce blood clots in humans – although *clinical trials* have not been conducted. The nattokinase in natto has the ability to degrade amyloid fibrils, suggesting that it might be a preventative or a treatment for amyloid-type diseases such as *Alzheimer's* (<http://www.wisconsinagconnection.com/story-national.php?Id=409&yr=2009>). Natto contains large amounts of vitamin K₂, which is involved in the formation of calcium-binding groups in proteins, assisting the formation of bone and preventing osteoporosis and also contains vitamin pyrroloquinoline (PQQ), which is important for the skin. Nattō also contains daidzein, genistein, isoflavone, phytoestrogen, and the chemical element selenium, but their effect on cancer prevention is uncertain.

4.3.3.4 Tempeh One of the most important fermented soybean foods, originating in Indonesia, is *tempeh* or *tempe*, a cakelike product made by fermenting soybeans with *Rhizopus*. When fried in oil, it has a pleasant flavor, aroma, and texture. Unlike most of the other fermented soybean foods, which usually are used as flavor agents or relishes, tempe serves as a main dish in Indonesia, where it constitutes a source of low cost

protein. In Indonesia, 750,000 tons of soybeans per year are consumed as tempeh. In the United States, Europe, and Japan, some food companies produce tempeh on a large scale of up to 2–6 tons per week (SoyInfo center, <http://soyinfocenter.com/index.php>). Among the nonsalted fermented soybeans, only tempeh has been accepted worldwide.

Strains of tempeh molds produce various amounts of amylase, pectinase, lipase, and proteases. Among the strains suitable for tempeh fermentation, *Rhizopus arrhizus* NRRL 1526 appears to produce the highest amount of pectinase. All strains of *R. oligosporus* have little or no pectinase activity. Lipase is also produced by molds in tempeh fermentation. Fatty acids are liberated by hydrolysis of soybean lipids, but there is no further utilization of these fatty acids. Either the mold does not possess the enzyme systems needed to metabolize these fatty acids or the fatty acids cannot permeate the cytoplasmic membrane of *Rhizopus*. Proteases are much more important enzymes and have an optimal pH at 3.0 (type predominating in submerged cultivation) or 5.5 (type predominating in tempeh fermentation). The enzymes are stable at pH 3–6 and have high milk-clotting activity.

The soy carbohydrates in tempeh become more digestible as a result of the fermentation process and resulting oligosaccharides associated with gas and indigestion are greatly reduced by the *Rhizopus* culture. The starter culture often contains beneficial bacteria that produce vitamins such as B₁₂ though it is uncertain whether this B₁₂ is always present and bioavailable. The fermentation process also reduces the phytic acid in soy, which in turn allows the body to absorb the minerals that soy provides.

In tempeh extract, strong thrombolytic activity (about 450 IU/g dry weight) was observed (Peng et al., 2005). The content of γ -aminobutyric acid, which improves blood flow to the brain and which inhibits high blood pressure, was also increased after tempeh fermentation (Aoki et al., 2003). Tempeh contains not only fiber, saponins, and isoflavones of soybean origin, but also superoxide dismutase which eliminate active oxygen. There is report on phytoestrogen, equol derived from soybean isoflavone which showed a suppressed effect on prostate cancer (Horii, 2008).

4.3.3.5 Sufu *Sufu* is a soft cheese-type product made from cubes of *soybean curd* (*tofu*) by the action of microorganisms. Three steps are normally involved in making *sufu*: preparing *tofu*, molding, and brining. Soybeans are first washed, soaked overnight, and then ground with water. The finely ground mixture is strained through a coarse cloth to separate the soybean milk from the insoluble residue. After the soybean milk has been heated to boiling, calcium or magnesium sulfate is added to coagulate the proteins. The coagulated milk is then transferred into a cloth-lined wooden box and weighted on top to remove whey. A soft, but firm cakelike curd (*tofu*) forms. *Tofu*, which has a high content of water ($\approx 90\%$), hence can be consumed directly and is eaten extensively throughout the far east. But the water content of *tofu* for making *sufu* is lower (8.3%) than that of *tofu* consumed directly. Otherwise, the product is likely to be spoiled by bacterial growth.

In preparation for molding, *tofu* is cut into small cubes ($2 \times 2 \times 4 \text{ cm}^3$). The cubes are immersed in an acid saline solution of 6% sodium chloride plus 2.5% citric acid for 1 h, and then subjected to hot air sterilization at 100 °C for 15 min. This treatment prevents the growth of contaminating bacteria but does not affect the growth of fungi needed in making *sufu*. After cooling, the cubes are inoculated over their surface with pure culture of an appropriate fungus grown on filter paper impregnated with a culture solution. The inoculated cubes are incubated at 20 °C or lower for 3–7 days, depending in the culture. The freshly molded cubes, known as *peptize*, have a luxurious growth of white mycelium and no disagreeable odor. The *peptize* is 74% water; 10.9% insoluble protein, 1.3% soluble protein, 4.3% liquid, and 9.5% trace components.

The last step in making sufu is brining and aging. The peptizes are placed in brining solutions of various types depending on the flavor desired. The most common brine contains 12% sodium chloride and rice wine having about 10% ethyl alcohol. The immersed cubes are allowed to age for about 40–60 days. The product is then bottled with brine, sterilized, and marketed as sufi. *Actinomucor elegans*, *Mucor hiemalis*, *Mucor silvaticus*, and *M subtilissimus* produce sufu having a good quality, but *A. elegans* appears to be the best mold for sufu fermentation and is the one adopted commercially.

4.3.4 New developments

Recently, fermented soybean foods have attracted attention as physiologically functional foods containing isoflavones, fibrinolytic enzymes, antioxidants, free-radical scavenging activities, angiotensin I-converting enzyme inhibitors, as well as reducing effect of menopausal symptoms, and so on. Biotechnological and genetic engineering techniques are useful to improve postharvest quality and also help to increase the nutritional value of vegetables. Genetic engineering techniques have been applied to improve *Lb. plantarum* and *P. pentosaceus*, which have been used for the fermentation of vegetables. Conjugal plasmid transfer has been reported between streptococci and pediococci, and plasmids have been observed in *Lb. plantarum*. The development of starters is largely confined to pickles at the present time, however, since the complex interrelated reactions occurring during sauerkraut fermentation are difficult to reproduce. Many tissue and cell culture techniques are now available for vegetable breeding. Carrot appeared to be an excellent model plant for the development of synthetic seeds. The future challenge will be to devise means of using genetic engineering technology to improve fermentation without losing desirable traits. Further optimization of starter cultures either by conventional modification or by recombinant DNA manipulations may result in increased levels of safety of fermented foods. As EC, known as urethane, is a genotoxic carcinogen in animals and a possible human carcinogen and this toxicant occurs naturally in alcoholic beverages and most fermented foods, efforts are underway in many countries to reduce this compound during fermentation.

Summary

There are two types of fermentation: natural fermentation, which is performed by the organisms naturally found in vegetables, and fermentation controlled by the amount of salt that is added and the acid produced. The organisms found in the latter type of fermentation will compete among one another, and depending on their tolerance to either salt or acid, the sensitive bacteria will be outcompeted. The pure culture fermentation consists of inoculating brine with a starter culture. This is produced in certain cases to ensure a proper fermentation and to reduce the chances of spoilage. Brining and fermentation were primary methods for preserving vegetables before the advent of canning and freezing. They are still primary methods of vegetable preservation in use all over the world. Many countries are interested in food uses of soybean for its high content of good nutritional quality protein. Fermentation also reduces phytate and other toxic components in plant foods. In developing countries, the current goal of fermented soybean food research is the preparation of native food by modern technology to give uniform products that are cheap, nutritious, free of pathogens, and completely acceptable to the local population. Physiology studies of LAB isolates from different foods combined “omics” approaches would allow a better understanding of how cell behavior is tuned by biotic and abiotic factors.

4.4 Vinegar and other organic acids

4.4.1 Introduction

An organic acid is an organic compound with acidic properties. Organic acids have been utilized for a long time by the food industry as food additives and preservatives to prevent deterioration and extend the shelf life of perishable food ingredients. The production of different organic acids (acetic acid, lactic acid, succinic acid, etc.) through fermentation of biomass sugars can be a primary or secondary coproduct in a bio-refining system. They have been widely used in foods, beverages, pharmaceuticals, cosmetics, detergents, plastics, resins, and other biochemical or chemical products (Rodríguez Couto and Sanromán, 2006). Vinegar (acetic acid) and other organic acids are used as acidulants and flavor compounds in processed liquid foods, and as chemical feedstocks. Organic acids may be produced by chemical synthesis, by fermentation, or by extraction from natural products. Fermentative production is restricted to citric acid and lactic acid. However, some acids, such as lactic acid and acetic acid, are also produced simultaneously by microbiological and chemical methods. Malic acid and fumaric acid are produced by fermentation but have not been used commercially owing to lack of demand.

Other acids used widely in the food industry are itaconic acid produced by *Aspergillus terreus*, and gluconic acid, which is used as gluconolactone, produced by *A. niger*. Gluconolactone is used as a slow-acting acidulant in baking powders, and in meat processing, and so on, but gluconic acid is more readily produced by the electrochemical or enzymatic transformation of glucose. Tartaric acid produced by precipitation during wine production or by fermentation (using oxidation of maleic acid by immobilized cells of *Alcaligenes levotartaricus*), is used in the food industry as an acid carrier in baking powder, sweeteners, ice cream, lemonades, various fruit products, and baked goods.

Except for citric acid, which is produced entirely by fermentation, there is always great competition between fermentation and chemical processes. Citric acid is by far the most important organic acid, with a worldwide production exceeding 1,700,000 metric tons annually in 2007. More than 50% of this volume was produced in China. More than 50% was used as an acidulant in beverages and approximately 20% was used in other food applications. Another 20% was used for detergent applications, and 10% was used in the production of nonfood related products such as cosmetics, pharmaceuticals, and in the chemical industry. The worldwide production of alcohol vinegar (10% acetic acid) is assumed to be about 6.5 million tons of pure acetic acid annually in 2013, among which 1 million tons from Europe and 2.5 million tons from USA. Lactic acid ranks among the high-volume chemicals produced microbially, with an annual world production volume in the range of 259,000 (Martinez et al., 2013). World lactic acid production has expanded 10-fold in the last decade due, in large part, to increased demand for green bioplastic products derived from lactic acid, including ethyl lactate and polylactic acid (PLA). Lactic acid has a worldwide production of about 259,000 metric tons and is produced by both fermentation (40%) and by chemical synthesis (60%).

4.4.2 Acetic acid

Acetic acid is the main organic acid from which vinegar is produced. Traditional production of vinegar was achieved from beer or wine mash by the *Orleans process*, a slow surface process developed in France, in the region of Orleans. A quick process with a flowing liquid, known as the *German process*, was developed and became the modern *trickling generator*.

The *Frings reactor*, which is similar to the trickling generator, uses beechwood shavings bearing adhering colonies of *Acetobacter* spp. or *Gluconobacter*. The commercial strains are *Acetobacter aceti*, *Acetobacter pasteurianus*, and *Acetobacter peroxidans*. *Gluconobacter oxydans* and several subspecies are also used commercially. Ethanol-based raw materials, such as wine, whey, malt, or cider, which do not require other nutrients, are trickled into the reactor from the top, and the bacteria oxidize the ethanol to acetic acid. When potato or grain spirits or technical alcohol is used, nutrients such as grain hydrolysates, ammonium phosphate, magnesium sulfate, calcium citrate, and calcium pantothenate must be added to obtain optimal growth of *Acetobacter*. The raw material is fed into the reactor at the same rate at which the effluent leaves it. The residence time of the liquid depends on the rate at which ethanol is reduced to acetic acid. Mass transfer is achieved through direct contact with air that is circulating within. This technology is very similar to that based on modern immobilized cell principles, because fermentative bacteria are fixed by natural means to a carrier surface inside the reactor. The advantages of this method are low costs, higher yields, less space occupied by the tank, and low evaporation losses. Today, high-yielding strains may produce up to 13–14% acetic acid.

Further technological advance has resulted in the *submerged culture* process, which resembles the modern stirred-tank reactor. As in all oxidative reactions, however, oxygen availability is often the limiting factor. Acetic acid bacteria will consume on average 7.75 L of O₂ per gram of cell per hour, and less than 5% O₂ in the gas phase leads to the stoppage of the fermentation. Although continuous processes have been described with good yields, they are very seldom used in practice. Some of the advantages of submerged cultivation over the generator are the higher efficiency of alcohol conversion, the greater holding capacity of the reactor, and low capital cost (due to fully automated operation). Although immobilization techniques have been developed to ensure a high cell density, it is difficult to achieve consistently high viability and a high oxygen transfer rate. Since the product obtained in the submerged process is turbid because of the suspension of bacteria, the product must be filtered by plate filters and decolorized by potassium ferrocyanide. Recently recombinant DNA techniques have been used to clone the aldehyde dehydrogenase (ALDH) gene into *A. aceti* to produce transformants that have increased tolerance to acetic acid as well as greater productivity. Comparison of the acetic acid productivity of two transformants of *A. aceti* (Table 4.13) indicates that the modified *A. aceti* significantly improved the productivity and specific growth rate. More recently, Sakurai et al. (2013) and others demonstrate that wild type *A. aceti* NRRC 14818 and *Gluconacetobacter diazotrophicus* Pal5 possess isocitrate lyase and malate synthase which

Table 4.13 Comparison of acetic acid productivity between two transformants of *Acetobacter aceti*

Property	<i>A. aceti</i> (pMV24)*	<i>A. aceti</i> (pAL25)†
Acetic acid productivity (g/L/h) at 20 g/L acetate	1.8	4.0
Specific growth rate at 30 g/L acetate	0.072	0.142
Maximum acetic acid concentration, g/L	68.4	96.6

*pMV 24 is a plasmid vector developed for *Acetobacter*.

†pAL 25 is the recombinant plasmid constructed by inserting a gene encoding the 75 kDa subunit of ADH complex of *A. polyoxogenes* into pMV 24.

Source: Fukara et al. 1992. Reproduced with permission of Wiley.

constitute the glyoxylate pathway, but several acetic acid bacteria utilizing for vinegar production lack these genes. Thus, lack of the glyoxylate pathway in *A. aceti* could be advantageous for industrial vinegar production.

Recently apple cider vinegar (ACV) was ranked as the best of all the home remedies to relieve the symptoms of occasional heartburn or acid reflux and also the benefits of ACV include weight loss (Tomoo et al., 2009); better skin tone, detoxification, and so on. However, excessive intake of vinegar can cause hypokalemia, hyperreninemia, and osteoporosis. ACV contains chromium which can alter insulin levels. ACV is highly acidic, that can cause esophageal injury (Hill et al., 2005).

Acetic acid is produced industrially both synthetically and by bacterial fermentation. About 75% of acetic acid made for use in the chemical industry is made by the carbonylation of methanol. The biological route accounts for only about 10% of world production, but it remains important for the production of vinegar, as many food purity laws stipulate that vinegar used in foods must be of biological origin. As of 2003–2005, total worldwide production of virgin acetic acid was estimated at 5 Mt/a (million tons per year), approximately half of which was then produced in the United States. European production stood at approximately 1 and 0.7 Mt/a were produced in Japan (http://en.wikipedia.org/wiki/Acetic_acid) Another 1.5 Mt were recycled each year, bringing the total world market to 6.5 Mt/a. Since then the global production has increased to 10.7 Mt/a (in 2010). The two biggest producers of virgin acetic acid are Celanese and BP Chemicals.

4.4.3 Citric acid

Citric acid has a pleasant acid taste, is very soluble in water, and finds many applications in the food, pharmaceutical, and cosmetics industries. The dominant use of citric acid is as a flavoring and preservative in food and beverages, especially soft drinks. Citrate salts of various metals are used to deliver those minerals in a biologically available form in many dietary supplements. The buffering properties of citrates are used to control pH in household cleaners and pharmaceuticals. In the United States the purity requirements for citric acid as a food additive are defined by the Food Chemicals Codex.

Citric acid occurs naturally in almost all living things and is the predominant acid in citrus fruits and in many vegetables. Originally this acid was extracted from citrus. Since 1893 scientists have known that citric acid is produced by *Penicillium glaucum*, and the first successful fermentation in surface culture was started in 1923. Fermentation processes using deep-vat fermenters began in the 1930s. Although many strains excrete traces of citric acid as primary metabolites in the tricarboxylic acid (TCA) cycle, only mutants of *A. niger* growing on carbohydrates (sucrose or molasses) or *Candida lipolytica* growing on paraffin substrates are used in the commercial production of citric acid. The production of undesirable side products such as oxalic acid and gluconic acid can be effectively suppressed by these mutants. The presence of metal ions in the raw materials caused drastically reduced yields and required removal by precipitation using hexacyanoferrate or by ion exchange resin (Sodeck et al., 1981). Thus, *A. niger* accumulates high concentrations of citric acid when substrate concentration is high and phosphate or metal limited. The role of metal ions in this respect is not fully understood. Other yeasts also produce high yields of citric acid using different carbon sources such as glucose, molasses, hydrocarbons, acetate, and alcohols, but they produce a mixture of citric acid and isocitric acid. Most of the citric acid used in foods is derived from carbohydrate fermentation by *A. niger*. Small quantities ($\approx 1\%$) of natural citric acid are still produced by extraction of citrus or pineapple.

Basically two types of process are used for the commercial production of citric acid: (i) surface (koji) processes employing solid substrates (wheat bran) or liquid nutrients

(sucrose, NH_4NO_3 , CaH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, ZnSO_4) and (ii) submerged processes using stirred fermentors or air-lift fermentors. The koji process is a traditional solid state process, similar in operation to the surface process. Submerged fermentation is more difficult technically than the surface process but may be batchwise, fed-batch, or continuous in operation. At the end of the fermentation, the mycelial mass is removed by filtration and washed. Oxalate is then precipitated as calcium oxalate at a pH less than 3.0. Citrate, precipitated from the broth as tricalcium citrate tetrahydrate, is filtered off and further purified by activated carbon and ion exchange resins. Optimization method on citric acid production by a novel *A. niger* isolate can be useful for the details (Berovic and Legisa, 2007; Lotfy et al., 2007).

4.4.4 Lactic acid

Lactic acid was the first organic acid manufactured by microbial fermentation, starting in 1880 and using *lactobacilli* of three species: *delbrueckii*, *leishmanii*, and *bulgaricus*. This process was conducted at 45–50 °C, using starch hydrolysates prepared with enzyme or acid. Today more than 50% of the lactic acid made is used in foods as an acidulant and preservative, while another 20% is used in the production of stearyl-2-lactylates and in the pharmaceutical industry.

Currently, the major manufacturers of lactic acid include Archer Daniels Midland Company (USA), NatureWorks LLC (USA), Purac (The Netherlands), Galactic S.A. (Belgium) and several Chinese companies such as the CCA (Changzhou) Biochemical Co. Ltd., Henan Jindan Lactic Acid Co. Ltd., and Musashino Chemical Co. Ltd (Japan company). Homofermentative LAB are preferred because of their ability to produce lactic acid alone, with no simultaneous production of carbon dioxide and other by-products. Thus, the substrate carbon is more productively utilized. A mole of glucose is reduced to two moles of lactic acid via the EM pathway. *Lactobacillus pentosus* has been used to ferment sulfite waste liquor (SWL), while *Lb. bulgaricus* produces lactic acid from whey permeate or ammonium lactate by continuous fermentation of deproteinized whey. The lactic acid fermentation of whey has been studied in batchwise, continuous, and immobilized cell processes using different bacterial strains (Mehaia and Cheryan, 1986). *Lactobacillus delbrueckii* produces 89 g/L/day in continuous culture, while immobilized *Lb. delbrueckii* cells in calcium alginate have been used in continuous culture, with a half-life of 100 day, yielding 90% L-(+)-lactic acid. In these processes, lactic acid is produced as the calcium salt, and thus the filtrate is treated with sulfuric acid to recover the product. As far as the animal nutrition is concerned, controlled lactic fermentation increases the shelf life, palatability and nutritive value of silage.

Lactic acid is used as a food preservative, curing agent, and flavoring agent. It is an ingredient in processed foods and is used as a decontaminant during meat processing. This acid is also used in beer brewing to lower the wort pH in order to reduce some undesirable substances such as tannins without giving off-flavors such as citric acid and increase the body of the beer. In winemaking, a bacterial process, natural or controlled, is often used to convert the naturally present malic acid to lactic acid, to reduce the sharpness and for other flavor-related reasons. This malolactic fermentation is undertaken by the family of LAB. Ammonium lactate is an excellent nonprotein nitrogen source, which is preferred in cattle to urea and ammonium citrate because it results in milk with higher nutritive value and does not require any expensive purification. According to forecasts, its production should increase significantly over the coming years mainly due to the PLA manufacturing, and it is

expected to reach 259,000 metric tons in 2012 (Mujtaba et al., 2012). The global market for lactic acid is forecast to reach approximately 329,000 metric tons by the year 2015 (Martinez et al., 2013).

4.4.5 Malic acid

Malic acid (DL-racemic mixture) is a naturally occurring acidulant, present in most fruits and vegetables. Malic acid is a low-acid food additive, which enhances the flavor, adds acidity to control the growth of bacteria, and adjusts the pH value of the alkaline product. Malic acid is added to fruity drinks, lemon-flavored ice tea mix, candy, ice cream, and canned fruit. Malic acid is used in processed cheese, chocolate milk and pudding, commercially prepared additives, processed meat and breakfast cereals.

The traditional method for producing L-malic acid was by extraction from apple juice (0.4–0.7% malic acid). Malic acid finds its application as an alternative to citric acid in fruit products and lemonades. Today malic acid is produced by two additional methods: (i) chemical synthesis, via hydration of maleic or fumaric acid at high temperature and high pressure, and (ii) enzymatic synthesis, whereby fumaric acid is transformed to L-malic acid. Although L-malic acid is commercially produced by fumarase from fumaric acid, it costs more than the chemically synthesized form. About 85% of the total world production of malic acid is carried out by chemical synthesis. Because of its favorable functionality in foods and competitive price, as well as new applications being developed in foods (e.g., encapsulation, mixes), this acid has the highest annual growth rate ($\approx 6\text{--}8\%$) among all organic acids.

Malic acid fermentation by *Aspergillus* species (e.g., *Aspergillus flavus*, *Aspergillus parasiticus*, *A. oryzae*, *A. niger*, *Aspergillus wentii*) was studied in a minimal medium containing glucose, salts, and calcium carbonate (Battat et al., 1990). Among these strains, malic acid fermentation by *A. flavus* was studied in detail. During fermentation, *A. flavus* produces unusual crystals composed of calcium malate, with minor amounts of calcium succinate and calcium fumarate. It was speculated that *A. flavus* excretes acids to the broth from the hyphae and these acids react with CaCO_3 present in the medium to form an insoluble calcium salt of acid residues that crystallize on the hyphae. *A. flavus* is not a food-grade organism, however, and it is important that future research on malic acid production be carried out with a food-grade organism, such as *A. niger*, in which the transformation and genetic systems are better developed to improve acid accumulation. The most common commercial process in Japan is by a continuous reaction of immobilized whole cells of *Brevibacterium ammoniagenes* or *Brevibacterium flavum*, where fumarase is present at a high activity. However, the L-malic acid formed is used for special purposes, thus not competing with DL-malic acid.

Recently, poly(β -L-malic acid) (PMA) similar to PLA as a natural biopolyester (bioplastic) has received attention for pharmaceutical applications and other potential uses. PMA production by 56 strains of the fungus *Aureobasidium pullulans* representing genetically diverse phylogenetic clades were studied (Manitchotpisit et al., 2012). Out of these research initiatives, several strains produced at least 4 g PMA/L, and several strains in clades 9, 11, and 13 made 9–11 g PMA/L up to 72% purity with no more than 12% contamination by pullulan. The molecular weight of PMA from *A. pullulans* ranged from 5.1 to 7.9 kDa. Results indicate that certain genetic groups of *A. pullulans* are promising for the production of PMA.

4.4.6 Fumaric acid

Fumaric acid is also a dicarboxylic acid, and is the least expensive among the food-grade acids in terms of cost and the quantities used in foods. Fumaric acid is used similarly in the food industry as an acidulant, accounting for about 20% of the total acidulant in the United States, and is especially suitable for using in dry beverage mixes, where its low hygroscopicity serves to extend the shelf life of other ingredients. This acid accelerates the fixation of color in cured meat and poultry products and can replace tartaric acid in the beverage and baking industries. This acid is used not only as an acidulant but also as candy coatings, emulsifying agents, and fat and dough conditioners. It is used for the production of L-malic acid by fumarase, and L-aspartic acid by aspartase, a component of aspartame sweetener.

The major drawback of fumaric acid is its low solubility in water, and thus its application is limited mainly to dry mixes. However, a quick-dissolving product, called *cold-water-soluble* (CWS) *fumaric acid* was introduced by the Monsanto Company to overcome this problem. This product contains 0.35 (w/w) dioctyl sodium sulfosuccinate. Another product, *quick dissolving* (QD) *fumaric acid*, which contains maltodextrin carrier (4.5–6%; w/w) was introduced by Miles. In the early 1940s, fumaric acid was produced by fermentation on a commercial scale using such fungal species as *Rhizopus oryzae*. However, more attractive chemical synthesis from maleic acid has since been developed. Molar yields of fumaric acid exceed 100%, compared with malic acid (15 mol%) and succinic acid (5 mol%). The biosynthesis of fumaric acid through both the oxidative branch and part of the reductive branch of the TCA cycle may explain the high molar yield of this acid by *R. oryzae*. Three enzymes – pyruvate carboxylase, fumarase, and NAD⁺–malate dehydrogenase – are localized in the cytosol and are likely to be responsible for the accumulation of malic acid. The characterization of these enzymes and the acquisition of details of the molecular biology of *R. oryzae* are essential for the elucidation of fumarate accumulation, and for their high expression in this fungus.

Fumaric acid is an interesting compound because of its numerous applications, not only in the food industry but also in the polymers industry. Its abilities to be converted into pharmaceutical products and act as starting material for polymerization and esterification reactions have led to the US Department of Energy to designate fumaric acid among the top 12 biomass building-block chemicals with potential to significantly enhance the economy (Xu et al., 2012). As the petroleum derivative has significantly increased in price, fermentation routes for fumaric acid production have been receiving more attention. *Rhizopus* sp. were identified as the best fumarate-producing strains among different microorganisms tested. Deng et al. (2012) studied simultaneous saccharification and fermentation (SSF) of cheap, raw bioresource-degermed corn powder (100 g/L total sugar) by mutant strains DG-3 and gained the maximum fumaric acid concentration of 32.18 g/L and productivity of 0.44 g/L h, respectively. Recently, an immobilized *R. arrhizus* RH-07-13 did not increase the yield of fumaric acid (wild type: 32.03 vs immobilized: 31.23 g/L) but fermentation time drastically reduced to 83.3% from 24 h compared to 144 h, which is the fermentation time when wild type strain was used (Gu et al., 2013).

Summary

The demand for organic acids is steadily increasing because of larger production volumes of acid foods. Citric acid and malic acid are the general-purpose acidulants, with fumaric acid being used in most foods where acidity is desired. All other acidulants fall into the category of specialty acids, which are limited in their functionality and range of application. The new

uses of L-malic acid in infusions and fumaric acid in animal feed have resulted in increased demand. There is a trend toward the use of organic acids in foods, and also toward producing them as naturally and economically as possible by means of biotechnological processes. Organic acids such as polymers of lactic acid (PLA), malic acid (PMA), fumaric acid (PFA), and PPA became important source of biodegradable polymers, which are already used in many fields. To become competitive, the biological production of organic acids must be further improved, especially with regard to regulatory mechanisms and genetic aspects, to permit a better understanding of the fundamental control mechanisms. The many functions and broad range of applications of food acidulants make selection of the most suitable acid for a given product a matter of serious concern.

4.5 Bacterial biomass

4.5.1 Introduction

To keep up with world population, projected at about 10 billion by the twenty-first century, food production will have to increase fivefold. Such an increase can be brought about by increasing either the area planted or the yield per unit area. Most of the agricultural land today is being rapidly diverted for other uses, limiting further the area that can be used for increasing food production. Genetic improvement of crop varieties is an alternative with its own limitations.

The term *single-cell protein* (SCP), coined to describe unconventional protein sources like those obtained from monocellular bacteria, algae, and yeasts, has been replaced by the term *microbial biomass protein* (MBP), to allow inclusion of higher fungi as unconventional sources of protein. *Biomass* refers to the organic cell substance of a living organism. Production of microbial biomass to satisfy world food demand, especially demand from the developing world, appears to be the most attractive alternative to date for at least six reasons:

1. Microbes require less time to grow than crops.
2. Production can be carried out in fermentors without requiring too much land or technical control.
3. Microbial biomass production processes are independent of the vagaries of nature.
4. Microbial biomass processes can be genetically manipulated with ease to improve production processes and product quality.
5. These processes have high protein contents (35–60%).
6. The nutritional value of MBPs is as good as that of other conventional foods rich in protein.

The use of microorganisms for human consumption dates back at least 6000 years to the time when *Saccharomyces* spp. were used in the production of bread, beer, and wine. The Aztecs consumed blue–green algae, which served as a major source of protein in their diet. The Egyptians and Greeks consumed fermented milk and cheeses produced by species of *Streptococcus* and *Lactobacillus*.

Molds have been used in the production of oriental fermented foods. Although baker's yeast has been produced on a large scale since the early 1900s, the first move toward growing microbes for food was made when the yeast *Candida utilis* grown on SWL, a by-product of the pulp and paper industry, was produced for use as a human and animal food supplement in Germany during World War II. In the late 1950s, commercial production of MBP on hydrocarbons was attempted. The British Petroleum Company was the first to

enter this field, followed later by others. In the 1970s, Shell developed a process for SCP production using methane as a substrate. Since then there have been many new technological developments in this area of research. In this chapter, “biomass” includes the cells of other microorganisms besides bacteria for the convenient treatment of the subject.

4.5.2 Microorganisms for the production of biomass

Numerous species of bacteria, and some species of actinomycetes, fungi, and algae, have been proposed as attractive candidates for the production of biomass protein. These organisms are capable of utilizing a wide range of carbon and energy sources for their propagation. Table 4.14 lists some organisms and substrates used in these processes. Desirable characteristics include genetic stability, high substrate conversion, high specific growth rate and productivity, easy separation, good quality and composition, and absence of pathogenicity.

Bacteria are able to use a wide variety of substrates ranging from simple sugars and complex carbohydrates to petrochemical products. The ability to utilize methane as a carbon source is a property almost exclusively present in bacteria. These bacteria, called the *methanotrophs*, may be found in anaerobic environments such as those observed in waste digestion processes. *Methylomonas methanooxidans*, *Methylophilus methylotrophus*, and *Methylococcus capsulatus* are examples of bacteria being used for the production of biomass protein from methane. These organisms, as well as actinomycetes (e.g., *Streptomyces* spp.), yeasts (e.g., *Candida* spp., *Hansenula* spp., *Torulopsis* spp.), and fungi (e.g., *Trichoderma* spp.), are also able to grow on methanol. Compared with the methylotrophic bacteria, however, the yeast strains assimilate methanol with a poor efficiency of conversion, higher oxygen demand, greater reaction heat, lower fermentation temperature, and lower protein content. Bacteria in general have faster growth rates

Table 4.14 Source organisms and substrate used for the production of bacterial biomass

Organism	Carbon or energy source
<i>Bacteria</i>	
<i>Cellulomonas</i> spp.	Bagasse
<i>Alcaligenes</i> spp.	–
<i>Methylophilus methylotrophus</i>	Methanol
<i>Methylococcus capsulatus</i>	Methane
<i>Yeast</i>	
<i>Candida utilis</i>	Ethanol, sulfite waste liquor
<i>Candida lipolytica</i>	<i>n</i> -Alkanes
<i>Kluyveromyces fragilis</i>	Cheese whey
<i>Saccharomyces cerevisiae</i>	Molasses
<i>Mold and higher fungi</i>	
<i>Cephalosporium eichorniae</i>	Cassava starch
<i>Paecilomyces varioti</i>	Sulfite waste liquor
<i>Penicillium cyclopium</i>	Cheese whey (lactose)
<i>Chaetomium cellulolyticum</i>	Agriculture and forestry waste
<i>Algae</i>	
<i>Scenedesmus acutus</i>	CO ₂ , sunlight
<i>Spirulina maxima</i>	CO ₂ , HCO ₃ ⁻ , CO ₃ ²⁻ sunlight

(generation times \approx 20–120 min) than yeasts, molds, and fungi (2–16 h or more). The cultivation of higher fungi for use as part of the normal human diet is not uncommon. The *sporophores* (fruiting bodies) of the basidiomycetic species *Agaricus bisporus* are common ingredients in many of our food items. The cultivation of higher fungi for use as a source of protein offers many advantages. However, the protein content is in general unfavorable and nutritional quality is poor compared to yeasts or bacteria. The productivity of fungal biomass production processes is also lower, in the range of 3–4 kg/m³. Many fungi are known to produce a range of undesirable metabolites (e.g., oxalic acid, mycotoxins).

Most bacterial SCP processes use pure cultures, but mixed cultures of *Cellulomonas* spp. and *Alcaligenes faecalis* have been shown to be more effective for the use of cellulose. *A. faecalis* in this mixed culture system is able to utilize the soluble sugars produced by the *Cellulomonas* spp., which have strong cellulase activity but do not possess β -glucosidase activity strong enough to be able to bring about complete utilization of cellulose. Another example of mixed cultures is the production of bacterial biomass from methane using the species of *Pseudomonas*, *Hyphomicrobium*, *Acinetobacter*, and *Flavobacterium*. When grown together, they had a higher growth rate, higher yield coefficient, and less foaming than pure cultures. Mixed cultures of yeast and fungi have also been used. For instance *Saccharomyces cerevisiae* and *Trichoderma viride* grown on cassava were found to produce better protein content than just the yeast alone. In the production of biomass from methane using a methane assimilating strain, *M. capsulatus*, addition of *Pseudomonas*, *Nocardia*, and *Moraxella* had a stabilizing effect, which was attributed to the ability of the auxiliary cultures to assimilate toxic metabolites of the main strain.

4.5.3 Raw materials for the production of biomass

The raw materials required for the growth and metabolism of microorganisms include a carbon or energy source, a nitrogen source, and supplementary nutrients. The carbon or energy sources that have been considered to be suitable for the production of MBP fall into two broad categories: substrates from renewable resources and substrates from nonrenewable resources (Table 4.15).

4.5.3.1 Substrates from renewable resources Substrates obtained from the renewable resources in the agricultural, forestry, and food-processing sectors are both cheaply available and abundant. Lignocellulosic materials, widely available in agricultural or forestry product residues, are the most abundant resources (estimated at 100 billion tons per year). These substrates are composed of simple sugars and complex carbohydrates. The simple sugars are the hexoses (e.g., glucose, galactose, fructose, mannose), the pentoses (e.g., xylose), and the dis-accharides (e.g., lactose, sucrose). The complex carbohydrates are starch, hemicellulose, and cellulose. The products constituting these compounds include potatoes, cassava, whey, SWL, and molasses. The chief disadvantage of these products is the high cost of collecting and transporting sufficient quantities to operate a processing facility large enough to be viable economically; such costs can be prohibitive in less-developed countries. Also, many of the substrates are available only seasonally, which means that in many regions SCP production facilities could operate during only a portion of the year.

Yeasts such as *Endomyces vernalis* and *S. cerevisiae* and fungi such as *C. utilis* are able to utilize pentoses and may therefore be employed in the production of biomass protein from SWL. The *Pekilo* process, the largest fungal biomass-based process, was developed in Finland at the Finnish Pulp and Paper Institute. It produces 15–16.5 tons dry weight of *Paecilomyces varioti* mycelium on SWL in 24 hours with a protein content of 55%.

Table 4.15 Substrates for microbial protein production

Material	Availability	Pretreatment	Yield (g/g substrate)	Use
Saccharides				
Molasses	Seasonal	Simple	0.25–0.33	Animal feed
Whey	Year-round	None	0.03	Fractionation
Sulfite waste	Year-round	Simple	0.008	Animal feed
Potato waste	Seasonal	None	0.5	Animal feed
Fruit	Seasonal	None/simple	0.03	–
Polysaccharides				
Starch	Seasonal	Hydrolysis	0.5–0.6	Food
Cellulose	Year-round	Hydrolysis	0.03	Fuel/animal feed
Hydrocarbons				
Methane	Year-round	None	0.3–1.4	Fuel/chemical feedstock
<i>n</i> -Paraffins	Year-round	Separation	1.0	Fuel/chemical feedstock
Alcohols				
Methanol	Year-round	None	0.25–0.5	Fuel
Ethanol	Year-round	None	0.6–0.7	Fuel
Propanol	Year-round	None	0.4	–
Other				
Acetate/Malate	–	Dependent on source	0.35	–

Source: Adapted from Davis 1974; Lee 1991.

Similarly whey, a by-product of the cheese industry, may serve as a suitable substrate for the lactose-utilizing yeast *Kluyveromyces fragilis*. Both whey and SWL have very high biological oxygen demand (BOD), causing serious problems to the industrial sectors producing them because of the high cost of disposal. Disposal is expensive because the high BOD cannot be tolerated by the environment. The conversion of these products to biomass protein not only helps in their disposal but also produces valuable protein.

The complex carbohydrates may serve as substrates for organisms that are capable of producing the enzymes necessary for the breakdown of the large carbohydrate molecules to smaller, assimilable sugars. Many fungal cultures, including *T. viride*, *A. niger*, and *Sporotrichum pulverulentum*, have the ability to do so and can therefore use these substrates directly. Yeasts, however, do not produce complex carbohydrate hydrolyzing enzymes and therefore are able to utilize these substrates only after pretreatment. The most common pretreatment methods, grinding and steam explosion, render the complex substrates available for utilization by yeasts. Bacterial species such as *Cellulomonas*, *Bacillus*, and *Brevibacterium* have demonstrated the ability to produce protein on lignocellulosic waste materials.

Since the 1950s there has been considerable interest in the large-scale cultivation of algae from the renewable resources, CO₂, and sunlight. Algae can also be grown using artificial lighting or heterotrophically, in the dark, with organic carbon and energy sources. Single-celled or multicellular, filamentous algal cultures (e.g., *Chlorella*, *Scenedesmus* spp., *Spirulina maxima*) can utilize CO₂ as a source of carbon and energy (Raja et al., 2008). These phototrophic organisms, which also include phototrophic bacteria such as *Rhodospseudomonas*, are cultivated in flat trays containing medium supplemented with additional nutrients. The major disadvantage of growing algae is the requirement for vast stretches of land. Since the process also depends on the availability of light, it may be useful

only in geographical regions where light is abundant. Another disadvantage is the high cost of harvesting algal cultures, which do not reach high cell densities because of limited capacities for the absorption of light. An attempt was made to grow the multicellular, filamentous *Hydrodictyon* on wastewater from the breeding of fish, which were fed a diet of this algal species (Präve et al., 1987).

4.5.3.2 Substrates from nonrenewable resources Nonrenewable petrochemical substrates include gas–oil and the paraffins (ethane, propane, *n*- and isobutane). The normal *paraffin* process developed by the British Petroleum Company was based on the growth of *C. lipolytica* on paraffin. During the 1950s these substrates were considered to be economically feasible for use in the production of biomass protein. By definition, however, these substrates were always limited in their availability, and after the hike in petroleum and natural gas prices in the early 1970s the plants ceased to be profitable, hence had to be shut down. Licensing problems including toxicity, together with the increase in oil prices, stimulated efforts toward SCP production from methanol and ethanol.

Several properties make methanol an attractive candidate for use as substrate: it has a high solubility in water; it can be removed easily when the process is complete; it gives high productivity; and it lacks the explosivity hazards associated with other petroleum-based substrates. Ethanol can be assimilated by a large number of different microorganisms and it technically represents the simplest and neatest variant of the process of SCP production. However, the raw material is obtained either from carbohydrate by fermentation or from ethylene via petrochemical precursors. The combination of the market price of ethylene-derived ethanol and declining soybean prices prevents large-scale commercialization at present.

Methane, another substrate of interest, is available in large amounts in natural gas produced as a result of biological degradation of organic matter. It is, however, necessary to supply a nitrogen source. This may be added as easily assimilable anhydrous ammonia, ammonium salts, or urea. It is essential that the C/N ratio be maintained at 10:1 or less. This favors increased protein content and minimizes the accumulation within the cell of lipids or cell storage substances such as poly- β -hydroxybutyrate (PHB). The other essential nutrient required is phosphorus, which may be added in the form of phosphoric acid. Also required are iron, calcium, magnesium, manganese, potassium, and sodium. If sufficient amounts are not already present in the water supply, these elements may be added in the form of sulfates or hydroxides rather than chlorides, to minimize corrosion problems.

UniBio A/S from Odense, Denmark is one of the few companies currently possessing knowledge in the field of fermentation technologies producing SCP that can be used as feed for animals. The company possesses a patented U-Loop technology, which is the result of more than 30 years of development that has the ability to convert natural gas into a high-concentrated protein product (71%), named UniProtein. The U-Loop technology has been tested at semi-industrial scale in Trinidad & Tobago. UniProtein has been approved as animal nutrition in Europe and moreover the EU Commission regulation number 575 of 16 June 2011 generally approves the use of UniProtein for all fish and animals (<http://www.unibio.dk>).

The fungus *Scytalidium acidophilum* isolated peat moss that can grow at below pH 1 offers advantages of (i) low-cost aseptic conditions (no need to have costly aseptic conditions), (ii) avoiding over 100-fold dilution of the acidic hydrolysates to pH values needed for other microbes, (iii) using a wide variety of sugars or carbohydrate wastes, and (iv) reusing acids again after the biomass is harvested.

4.5.4 Production process

The fermentation process begins with the inoculation of the culture into a fermentor containing the production medium (Nasseri et al., 2011). The culture is then allowed to grow under controlled conditions (temperature, pH, oxygen tension, etc.) to give an optimum yield of biomass protein. Preparation of the medium depends on the nature of the substrate. The solid raw materials (mineral salts, cellulose, starch, etc.) are dissolved or suspended in water. This facilitates sterilization. The concentration of the soluble carbohydrate in most processes is adjusted to 1–5%. The liquid raw materials such as methanol, ethanol, or normal paraffins may be fed directly to the fermentor after a filtration step to purify or sterilize the medium. The concentration of substrates such as methanol is maintained as close to zero as possible to prevent these materials from inhibiting growth. The gaseous raw materials – air, pure oxygen, methane, ammonia, hydrogen, and carbon dioxide – are filter-sterilized under pressure. This is done to eliminate volatile aerosol-like or fine impurities. The oxygen requirement also depends on the raw material being used. Hydrocarbon fermentations require a greater input of oxygen than the carbohydrate-based processes. The pH optima of yeast and fungal cultures are lower than those of the bacterial and algal cultures and they are maintained by phosphoric acid, which also serves as a source of phosphorus. The temperature tends to rise during fermentation as a result of the release of heat by the actively metabolizing cells. Therefore, it becomes necessary to have a cooling system for the process, especially in fungal fermentations, since temperature tolerance limits of fungi are lower than those of bacteria. The spent medium remaining behind after the cells have been harvested may be recycled until the substrate has been completely utilized.

The size of the microorganism being used for the production of biomass protein determines the cost of the recovery process. Bacterial cells are the smallest (1–2 μm). With a density close to that of water (1.003 g/cm³) and cell densities in the (dry weight) range of 10–30 g/L, they require centrifugation and hence are the most expensive to recover. The use of vacuum filters does not offer a greater advantage because the filtration process eventually slows down, when the filters become blocked by the cells. *Flocculation* or *agglomeration* of the cells has proven to be an effective means of concentrating cells before the centrifugation process. Bacterial cells can be aggregated by heating, the addition of ionic or nonionic flocculants, a change in pH or in an electric field; the use of filter aids for recovery contaminates the cell product, however, making it unsuitable for use as feed. The concentrated biomass is heated to inactivate the enzyme systems and then dried. Yeast cultures, too, are thickened to a maximum concentration of solid matter by means of separator centrifuges and decanter centrifuges and then dried. The filamentous fungi are easier to recover than bacteria or yeast cells. A low speed centrifugation process is adequate for the recovery of fungal mycelia. For large volumes, however, *rotary vacuum filters* are employed.

Filtration is also accompanied by spray washing of the mycelium. The better the dewatering process, the cheaper will be the eventual drying costs. Drying is done using belt dryers or ring dryers to produce a flaky, powderlike product. In most cases, a continuous process has been found to be more satisfactory than a batch process; although more effective as a rule, batch processes are labor intensive. Drying of fungal mycelia is much easier and cheaper because these organic materials can withstand stress, whereas bacterial cells tend to collapse. The drying of fungal mycelia is commonly done by heating or by the use of a rotary drum drier. Care should be taken to maintain the nutritional value of any product that is subjected to a drying process.

If individual components (soluble or insoluble protein fractions, total or individual lipid components, nucleotides or nucleic acids, or vitamins) are to be recovered, the dewatered

or crude biomass may be subjected to a breakdown process in the form of an extraction, crystallization, or precipitation procedure.

4.5.5 Nutritional aspects

The three main applications of SCP are its use as protein supplements in human foods, as protein supplements for livestock feeding, and as functional ingredients in foods. In the first two cases, emphasis must be laid on the nutritional properties of the biomass, whereas for functional applications, the functional behavior of the biomass ingredients in the food products has greater importance. With all three, however, the product must be free of toxins or other undesirable metabolites, heavy metals, and pathogens; it must have acceptable sensory properties, and a low viable cell count.

Since "total nitrogen" includes such nonprotein nitrogenous substances as nucleic acids and gives no information about the amino acid profiles, it does not reflect true nutritional value. A comparative study of the amino acid profiles of some biomass proteins with the reference protein established by the Food and Agriculture Organization (FAO) of the United Nations reveals that microbial proteins tend to be deficient in methionine. Among the different protein sources, bacterial proteins have higher levels of methionine than yeasts, fungi, and soybean (Table 4.16). The best approximation of the nutritional value can be obtained by animal feeding studies. The parameters that are studied include determination of *protein digestibility*, *protein efficiency ratio* (PER), *biological value* (BV), and *feed conversion ratio* (kilograms of product consumed to kilograms of weight gained). From such studies, the blue-green alga *Spirulina* was shown to be a good source of protein in experiments with sexually maturing rats and was used as the sole source of protein in their diet (Contrezas et al., 1979).

Although bacteria and molds have been consumed for many centuries, many of them have been shown to produce toxic substances. Moreover, the substrate used for production of the biomass may be toxic and may remain associated with the biomass after processing. Therefore, before a new product can be marketed, a number of tests must be carried out to determine the safety of the product. The U.S. Food and Drug Administration (FDA) and the Protein Evaluations Group of the FAO have developed guidelines for evaluating the safety of products in human and domestic livestock. The nutritional and toxicological testing process can cost millions of dollars, and 10 years may elapse before approval is granted. Some of the common problems that may arise are allergic skin reactions and digestive problems. Many sources of MBP are not digestible because many of these organisms produce compounds such as intracellular polymerized lipids and cellulose. In human feeding studies, consumption of certain MBPs has been shown to cause a wide range of gastrointestinal complaints, from relatively mild symptoms such as bulky stools and flatulence to the more serious symptoms of nausea, vomiting, and diarrhea (Litchfield, 1985). Other pathophysiological reactions noted included peeling skin from the palms of the hands and the soles of the feet.

Microorganisms have a high content of nucleic acids – bacterial cells, for example, contain 6–11% (dry weight basis). The estimated safe intake for a healthy adult is 2 g/day. Taking the example further, we note that human beings lack the enzyme uricase, which breaks down uric acid to allantoin. We note also that consumption of yeast as a source of protein would cause an increase in the serum uric acid levels. Uric acid, being only slightly soluble at physiological pH, may crystallize out in joints, causing gout as well as kidney stone formation. Methods described (Litchfield, 1985) to reduce the nucleic acid content to 1–2% of the tolerance level include (i) lowering the growth rate, thus minimizing the RNA content, (ii) heat-shock incubation for degrading ribonucleases, (i.e., rapid heating

Table 4.16 Comparative analysis of various biomass products

Biomass products*	Bacteria/ methanol	Yeasts/ paraffin	Yeasts/ carbohydrates	Fungi/ carbohydrates	Algae/ CO ₂
Crude protein, %	80	55–60	45–50	35–45	40–60
Nucleic acids, %	10–15	5–8	10	10	6
Fat %	8	9	2–5	2–5	5–9
Minerals, %	7–8	8	5–10	5–10	10–15
Selected aminoacids, g/16 g nitrogen					
Isoleucine	4.5	3	4.5	5	5–6
Alanine	7	6	6	6.5	–
Leucine	7	5.5	6.5	7	8–9
Glycine	5.5	3	5	5	–
Lysine	6	6.5	6.5	6.5	4–5
Phenylalanine	3.5	2.5	3.5	4	4–5
Methionine	2.5	2	1.5	2	2–3
Proline	3.5	2.5	3.5	4	–
Threonine	4.5	3.5	5.5	4	5
Aspartic acid	9	8	8	9	–
Tryptophan	1	0.5	1	1	1
Glutamic acid	10	9	10	2	–
Valine	5	3.5	5	5	6–7
Tyrosine	3	3	3.5	3.5	5
Arginine	4.5	3.5	4.5	5	9–10
Histidine	2.5	2	3	2	1.8
Serine	3.5	3	3.5	4	–

*Various biomass products that are produced by a given group of organisms when growing on a given carbon substrate (e.g., bacteria growing on methanol (bacteria/methanol)).

Source: Author's compiled data.

of the culture to 64 °C to inactivate the fungal proteases and allow the endogenous RNases to hydrolyze the disrupted ribosomal RNA, whereupon the 5' nucleotides produce diffuse into the culture broth and are thus eliminated), and (iii) the use of alkali to extract and coagulate the nucleic acid. The third method has also been demonstrated to improve the consistency, color, and odor of the Pekilo protein biomass when the alkali is neutralized with acid before the biomass is washed. However, allowing the pH to drop below 6 caused reprecipitation of RNA onto the biomass. Livestock are able to tolerate higher levels of nucleic acid than humans. Human consumption of *Alcaligenes eutrophus* was found to cause gastrointestinal disturbances such as nausea and vomiting, while these effects were not observed in animal feeding studies.

MBP can also be used to impart to processed foods various characteristics such as flavor, fat and water binding, dispersing action, whipping and foaming action, or extrusion and spinning properties. There is no information regarding whether these proteins have any better functional properties than the conventionally used soymeal protein concentrates or isolates. Also, since these proteins are still more expensive than the soymeal protein, there has been no attempt to replace the soy protein with MBP.

4.5.6 Economics and new developments

A major economic constraint is the large working capital required for the bacterial biomass process. This is followed by the cost of the raw material, which is estimated to account for

Table 4.17 Economics of the five SCP processes versus soymeal and fish meal

Process	Investment (\$ × 10 ⁶)	Unit product cost (\$/ton)	Raw materials utilities (\$/ton)	Protein content (%)	Protein cost (\$/ton)
Bagasse	78,204	1098	465	60	1830
Molasses	48,770	756	332	52	1454
Methanol	77,200	1264	618	71	1780
Cassava	–	605*	212 [†]	17	3558
Sulfite liquor	14,000	340	290	50	688
Soymeal	–	150	–	49	315
Fish meal	–	330	–	60	530

*Raw materials assumed at 35% of total unit product cost.

[†]Based on raw materials only (utilities not included).

Source: Author's compiled data: 1985 US dollar amounts.

50–70% of the cost of production. Table 4.17 compares five SCP processes with soymeal or fish meal in terms of costs and protein content.

The cost of the raw material can be minimized by coupling the biomass protein, production process with another existing process whose waste products can be utilized as substrates for MBP production. Food-grade yeasts such as *S. cerevisiae* and *K. fragilis* have a good position in the food ingredient markets, but yeasts cannot utilize lactose in whey or agricultural biomass (xylose), which are two promising substrates. Through the use of genetic engineering, the characteristic enzyme systems required can be introduced into yeasts, consequently enabling the use of less expensive, more abundant substrate, which will lead to a decrease in costs.

The techniques of recombinant DNA technology have been employed to increase the efficiency of conversion of a process. *M. methylotrophus*, when grown on methanol and NH₃, is an important source of SCP. This organism lacks the enzyme glutamate dehydrogenase and instead uses the ATP-dependent glutamine synthetase/glutamate synthetase (GS/GOGAT) pathway for the assimilation of NH₃. This results in wastage of methanol. Cloning and expression of the glutamate dehydrogenase gene from *E. coli* resulted in a 4.7% increase in the efficiency of the carbon conversion.

The temperature at which fermentation is carried out has a significant role in determining the economics of a process. The higher the operating temperature, the lower is the cooling cost. Therefore development of thermotolerant strains might help to reduce the cooling cost. With technological advances such as the development of cheaper recovery methods, processes that allow achievement of higher cell densities and productivities, and genetically engineered strains having better conversion efficiency and the ability to use cheaply available substrates, the economics of the process should begin to look more attractive.

Summary

The advantages of using microbes as a source of protein notwithstanding, research in this field is of no particular interest to scientists in the developed countries, where food is in vast excess. Although the field is of great interest to developing countries, the economics of the production process are still not comparable with the conventional protein sources such as soybean meal or fish meal traditionally available in these countries. This balance, however, is likely to change in the future because of increased pressure on conventional

food protein sources as a result of the expanding world population. Agriculture continues to be a major contributor to the economy of the developing countries, but this long-term trend is beginning to decrease. In the near future, even developed countries may turn to the production of protein from biomass.

4.6 Polysaccharides

4.6.1 Introduction

Polysaccharides or gums (trivial name) occur as energy reserves and as structural materials in cell walls and in extracellular capsules. A large number of such polymers obtained from plant tissues have gained commercial importance as industrial gums. The ability of polysaccharides to dissolve in water and to alter rheological characteristics of their aqueous environment, along with their low toxicity, has resulted in the application of these polymers in a wide variety of industrial situations in the food, cosmetics, chemical, and medical fields, waste treatment, and oil industries. World production of guar gum alone sits at about 700,000 tons (www.agrogums.com/blog/2013/06/). India is the main exporter and the demand for this gum continues to go up annually owing to the unique application of fast hydration guar gum powder in various industrial processes. In United States there is a huge demand from the oil and gas industry. Apart from the oil and gas industry, the food industry in United States and United Kingdom has also contributed to the high demand, but the prices of plant-derived gums are expensive; thus, microbial gums have been receiving more attention and several microbial gums are very popular. The global hydrocolloid market dominated by algal and plant polysaccharides such as starch, galactomannans, pectin, carrageenan, and alginate is expected to reach 3.9 billion US dollars by 2012 (Phillips and Williams, 2002). Besides these traditionally used plant and algal gums, considerable progress of microbial polysaccharides has been made in discovering and developing new microbial extracellular polysaccharides (exopolysaccharides, EPSs) – four EPSs, namely, xanthan, pullulan, curdlan, and levan, as biopolymers with outstanding potential for various industrial sectors. Much effort has been devoted to the development of cost-effective and environmentally friendly microbial production processes such as investigating the potential use of cheaper fermentation substrates.

Carrageenan, *agar*, and *alginate* are often produced from red or brown algae, but there are inherent disadvantages in the production of polysaccharides from plant and algal sources: (i) the chemical composition of polysaccharides varies in response to metabolic requirements, which in turn, reflect changes in the environment, such as seasonal variations, aging cycles, and time of harvesting; (ii) modification and degradation of the product by harsh treatment during processing (alkali treatment, acid precipitation, bleaching, etc.) introduce undesirable degradation, odor, or color to the finished products; and (iii) plant products are subject to variable supply, depending on harvesting and climatic conditions, and the presence of disease or pollution. The production of polysaccharides from microbial sources offers controllable polymer synthesis from materials in constant supply, yielding products that possess unique physical and chemical properties, improved functional characteristics, and low BOD. Therefore, there will be a great demand for new, cheaper, safe, and esthetically acceptable sources of microbial polysaccharides. Microbial polysaccharides are still relatively expensive to produce, but their long-term market prospects are very promising. The major consumers and industrial applications of gums are listed in Table 4.18, and the market size and value for the major gums in the United States are shown in Table 4.19. The application of recombinant DNA technology to the production

Table 4.18 Some consumer and industrial applications of gums

Application	Function	Sales ($\times 10^6$)
<i>Consumer</i>		
Food	Gellant, stabilizer, food processing, clarification of beverages, and so on.	347
Cosmetics	Dispersant, stabilizer, gellant, and so on.	47
Other	Coagulant for protein, precipitations and recoveries, and so on.	82
<i>Industrial</i>		
Paper	Adhesive or binding, finishing, and so on.	185
Petroleum	Gellant, lubricants, and so on.	150
Textiles	Adhesive or binding, finishing	73
Other	Matrix for gel chromatographies, immobilization, and so on; membranes for separation or filtration; bulking agents for drugs; insecticides, fertilizers, and so on.	54

Source: Adapted from Baird and Pettitt 1991.

Table 4.19 Sales of major food-approved polysaccharides

Gums	Sales ($\times 10^6$)
<i>Thickeners</i>	
Starch	125
Guar	12–30
Carboxymethylcellulose	30
Cellulose derivatives (methyl, hydroxymethyl, microcrystalline, etc.)	3.5
Xanthan	44–60
Locust bean gum (carob)	12.5
Gum arabic	30
<i>Cellants</i>	
Agar	10
Gelatin	60
Sodium alginate	30–47
Carrageenan	22
Pectin	20

Source: Adapted from Baird and Pettitt 1991.

of microbial polysaccharides could lead to structural manipulation at the genetic level and to the possibility of unlimited development of new biopolymers (Lu et al., 2007).

4.6.2 Microbial polysaccharides

Several microbial polysaccharides are now produced commercially by many species of bacteria, as well as some algae and fungi. Microbial polysaccharides consist of three main types: *intracellular polysaccharides*, which may provide mechanisms for storing carbon or energy for the cell, *structural polysaccharides*, which are components of cell structures (e.g., lipopolysaccharides), and *extracellular polysaccharides*, or *EPSs*.

Table 4.20 Microbial polysaccharides related to the food industry

Biopolymer	Microorganism	Substrate	Composition	Molecular weight
Alginate	<i>Azotobacter</i>	Sucrose	D-Mannuronic acid	1.5×10^6
	<i>Pseudomonas</i>	Sucrose	L-Guluronate	–
Cellulose	<i>Acetobacter</i>	–	–	–
	<i>Agrobacterium</i>	–	–	–
Chitosan	<i>Alcaligenes</i>	Glucose (β -1,4)	D-Glucose	2×10^6
	<i>Mucorale</i>	Glucose	D-Glucosamine	1.7×10^4 to 1.3×10^5
Curdlan	<i>Alcaligenes</i>	–	–	–
	<i>Agrobacterium</i>	Glucose (α -1,3)	D-Glucose	$10^4 - 10^8$
Dextran	<i>Cellulomonas</i>	–	–	–
	<i>Lacobacillus</i>	Sucrose	D-Glucose	$10^4 - 10^8$
Gellan	<i>Leuconostoc</i>	(α -1,6, α -1,3)	–	–
	<i>Streptococcus</i>	–	–	–
Pullulan	<i>Pseudomonas</i>	Glucose, Rhamnose,	D-Glucose, D-rhamnose, D-glucuronate	5×10^6
	<i>Sphingomonas</i>	Glucuronic acid	–	–
Xanthan	<i>Aureobasium</i>	Sucrose (α -1,6, α -1,4)	D-Glucose	2.5×10^5 to 1×10^7
	<i>Xanthomonas</i>	Lactose, carbohydrate	D-Glucose, D-mannose, D-glucuronate	5×10^6
Levan	<i>Bacillus</i> ,	Sucrose, Glucose	Fructose	2.5×10^6
	<i>Zymomonas</i>	–	–	5×10^7

Source: Author's compiled data.

Advances in the use of microorganisms to produce industrially useful polysaccharides may be made by effecting the following improvements: (i) increasing the rate and extent of polysaccharide formation, (ii) modifying the polysaccharide produced, (iii) altering the surface properties of the producer microorganism to simplify cell separation in downstream processing, (iv) eliminating enzyme activities that may modify the polysaccharide in unwanted ways, and (v) transferring the genetic determinants of polysaccharide synthesis to more amenable host process organisms. Table 4.20 summarizes the production of polysaccharides by various microorganisms using a wide variety of substrates, including glucose, fructose, sucrose, lactose, hydrolyzed starch, methanol, and different hydrocarbons. In view of their unique and novel chemical and physical properties, microbial polysaccharides are being used as gelling agents, emulsifiers, stabilizers, binders, coagulants, lubricants, film formers, thickeners and suspension agents.

4.6.3 Fermentation process

Like any microbial process, EPS production requires a cheap carbon source, sterilizable fermentors, sterile air, and growth media. The success of the fermentation process relies heavily on environmental influences. Indeed, control of the fermentation is found at the environmental level as well as the equipment design and operational levels. The composition of the medium affects the process greatly. In the case of xanthan and alginate production, the organisms need limited carbon conditions if greatest yields are to be obtained. *Pseudomonas* species do not carry any particular carbon limitations.

EPS composition can be manipulated by changing the growth medium. For example, the concentration of pyruvate residues in xanthan gum can be varied up to 7.5% by the use of certain growth media or organism strains. Temperature as well as other operation parameters (e.g., pH, DO, CO₂) affect the process. In the case of xanthan gum, when the pH of the fermentation medium falls to 5.5, the fermentation stops. To ensure good fermentation yields, pH control must be maintained around the optimum 7.0. Specific rates of synthesis can vary depending on whether the process is batchwise or continuous. In batch fermentation, for example, the culture quickly becomes oxygen-limited by the high viscosity of the broth. Because the polymers are generally released into the medium, problems of stirring and aeration of the medium are introduced. Furthermore, the ongoing fermentation is accompanied by extreme rheological changes (from low viscosity Newtonian to highly viscose non-Newtonian systems); thus, the problems of mixing, heat transfer, and mass transfer must not be overlooked.

The steps in the recovery of an EPS product include separation, concentration, isolation, and purification (to deactivate undesirable contaminants such as enzymes). Modification of the product's chemical properties can occur as in the case of the cellulase enzyme in xanthan gum. Finally, the polysaccharide undergoes the testing procedures demanded by national or international regulatory agencies before any new product can be approved for food use. Continuous fermentation has been primarily used to study the physiology of polysaccharide synthesis but offers the promise of continuous high productivity by a simple nutrient limitation. Using a suitable starting substrate as well as a nitrogen source and minerals (Ca, P) and a particular organism, fermentation takes place. Temperature, pH, and dissolved oxygen concentration are monitored. Nitrogen is often used as the limiting nutrient (C/N = 10:1), but other limiting nutrients such as sulfur, magnesium, potassium, or phosphorus may be used. The particular nutrient limitation employed often determines the character of the polymer, in terms of its viscosity characteristics and degree of acylation. For example, phosphorus limitation can reduce the extent of phosphorylation and even alter the ratio of monosaccharides in the final product, whereas potassium limitation tends to reduce polysaccharide synthesis because of an inhibition of nutrient uptake. Separation is achieved by using solubility differences of the same polymer in various organic solvents (alcohols, acetone), causing the polysaccharide to precipitate. Other methods may also be available, but economics will dictate which one will be used.

4.6.4 Bacterial polysaccharides

Bacterial polysaccharides represent a small fraction of the current biopolymer market; with successful application of the new genetic engineering technologies, however, potentials for development of novel and improved products are high indeed. Commercial preparations of several microbial polysaccharides including alginate, curdlan, dextran, and xanthan gum, are known and mostly used in industries, but of all the bacterial polysaccharides, xanthan gum is the most used, and its consumption in 1983 was estimated to be 4% of the total. With new fermentation process developments and additional organisms being used, other microbial polysaccharides will become more competitive in the future. More recently, a number of investigations have examined genes involved in the biosynthesis of alginate, xanthan gum, yeast cell wall D-glucans, and zooglucan, as well as polyhydroxybutyrate.

4.6.4.1 Xanthan *Xanthan gum* has been produced commercially since 1967 by means of the growth of *Xanthomonas campestris* on glucose, sucrose, starch, corn sugar, distillers' solubles, or acid whey. Xanthan gum is widely used as a substitute for egg whites in salad

dressings, sauces, and fillings. It is also often added to low-fat or nonfat dairy products such as some types of nondairy ice cream to bring a “fatty feel” to the food.

Total global production in 2012 is expected to be in excess of 110,000 tons. China became the world’s largest producer of xanthan gum in 2005 and now produces and exports about two thirds of the world’s supply. Other major producers and exporters are the United States, France, Austria and Japan (<http://spendmatters.com/2012/09/24/xanthan-gum-another-possibly-sticky-situation>). Fierce competition during the global financial crisis of 2008/09 drove xanthan gum prices down. The price of xanthan gum in China has now increased by over 25% each year due to the increased costs of energy, labor, and corn.

Xanthan gum has increasingly been used as a less-expensive alternative to guar gum in many food-based applications, but it is also widely used in the oil industry to thicken drilling mud for extracting more crude oil and natural gas. This polyanionic polymer is composed of D-glucose, D-mannose, and D-glucuronic acid in a molar ratio of 2:2:1 with two types of carboxyl group (acetate and pyruvate) and an allulose backbone. The repeating unit is a pentasaccharide consisting of a β -1,4-linked glucose backbone with branches consisting of a mannose- β -1,4-glucuronic acid- β -1,2-mannose- α -1,3-linked trisaccharide. It is produced with yields of 25 g/L in batch culture using commercial D-glucose as a carbon source. Continuous production of xanthan has been investigated but not commercially implemented. The newly developed lactose-using bacteria produced 30 g/L of xanthan gum for every 40 g/L of whey powder.

The major advantages of xanthan gum as a commercial polymer include high yield of production, high viscosity solutions at low gum concentrations, high pseudoplastic flow behavior, and stability over wide ranges of pH, temperature, and salt concentration. This polymer was approved for use in the food industry since 1969, and it is used as a viscosifier, suspending agent, emulsion stabilizer, and settling agent. Specific food applications include uses in fruit-flavored beverages, canned foods, bakery fillings, ice cream, dry mixes, instant frozen foods, salad dressings, processed cheeses, flavored beverages, sauces, and gravies. The synergistic interaction between xanthan and plant galactomannans such as locust bean or guar gum makes possible applications in animal feeds (e.g., canned pet food) because viscosity increases are much higher than would be expected with the individual gums.

The fermentation process for the production of microbial xanthan is shown in Figure 4.11. There is limited fundamental research to elucidate the genetic and biochemical basis for overproduction of this gum, but recent studies on cloning of genes involved in pathogenicity of *X. campestris* demonstrate the feasibility of recombinant DNA technology for manipulating xanthan biosynthetic genes (Lu et al., 2007).

4.6.4.2 Alginate Alginates have been traditionally produced by brown seaweeds such as *Laminaria* spp., but this source of supply is subject to considerable variability and to the ravages of pollution. The production of alginate by microbial fermentation offers a readily controllable source and supply. *Azotobacter vinelandii* and *Pseudomonas aeruginosa* are two bacteria that can produce alginate-like heteropolysaccharides of β -D-mannuronic and α -L-guluronic acids. Microbial alginate differs from the algal alginate in that a fraction of the D-mannuronic acid residues are *o*-acetylated. The biosynthetic pathway of alginate from *Azotobacter* and *Pseudomonas* has been partially elucidated, and as a result several genes involved in alginate biosynthesis have been cloned. The control of the activity of C-5 epimerase, which converts D-mannuronic acid (M) to L-guluronic acid (G) would permit control of the M/G ratio of bacterial alginate. Other alternative would be to isolate epimerase and then use the enzyme to upgrade low G alginates. Mannuronan C-5 epimerases are produced extracellularly by *A. vinelandii*. The process has been commercialized using *A. vinelandii* in batch culture under conditions of carbon excess, yielding

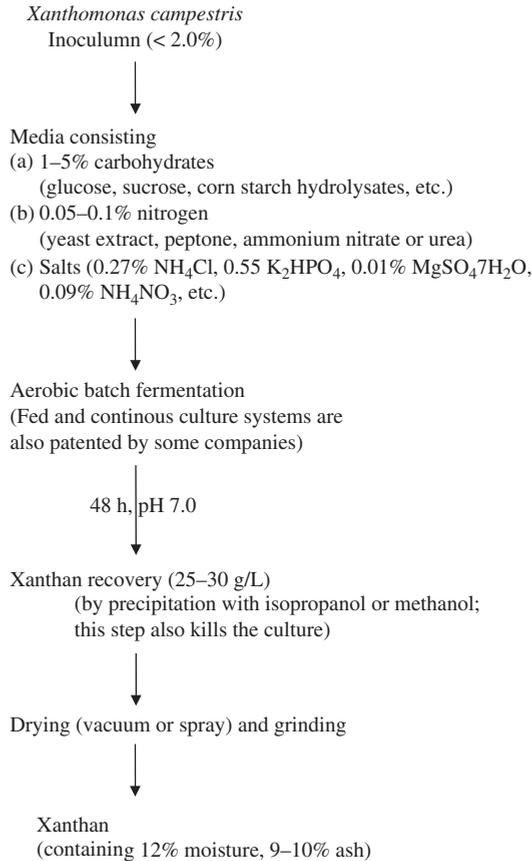


Figure 4.11 Fermentation process for the production of microbial xanthan.

5–7 g/g dry cell. The alginate production in continuous culture has also been studied, and the yield from sucrose could increase to 50%, compared to 25% in batch culture.

Alginate production is responsive to control by manipulation of the nutrient supply of *A. vinelandii*. Reduction of the phosphate concentration in the medium enhanced the formation of extracellular polysaccharide, yielding a product with a higher average molecular weight, which in turn resulted in higher viscosity. Alginates from plant sources are predominantly used commercially for water holding, gelling, stabilizing, and emulsifying properties: to stabilize yogurts, to control the formation of ice crystals in ice creams, bakery fillings, and icings, or in puddings and dessert gels. Alginate polysaccharides in the presence of multivalent cations such as calcium result in thermoirreversible gels and thus have been used for the immobilization of microorganisms.

4.6.4.3 Exopolysaccharide (EPS) and dextrans Some of LAB produce EPS, that normally plays a major role as a natural texturizer in the industrial production of yogurt, cheese, and milk-based desserts. EPS produced by LAB have received increasing attention, mainly because of their health benefits on immune stimulation, antimutagenicity, the antitumor

activity, and probiotic effect of fermented dairy products (Harutoshi, 2013). EPS are polysaccharides either secreted by the cell or produced on the outer cell by extracellular enzymes. EPS produced by the cell secretion or by extracellular enzymes from LAB are divided into two classes, homo- and hetero-EPS. Homo-EPS are composed of one type of monosaccharide, whereas hetero-EPS consist of regular repeating units of 3–8 different carbohydrate moieties synthesized from intracellular sugar nucleotide precursors. While homo-EPS are made from sucrose using glucansucrase or levansucrase, the synthesis of hetero-EPS involves four major steps, sugar transportation, sugar nucleotide synthesis, repeating unit synthesis, and polymerization of the repeating units (de Vuyst et al., 2001). The major physiological functions of EPS are biological defenses against various stresses such as phage attack, toxic metal ions, and desiccation, but some are likely to use EPS as an energy source and prebiotic growth factors.

Prebiotics are defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and activity of one or a limited number of bacterial species already resident in the colon, and thus improving host health.

Dextrans are α -D-glucan or glucose homopolysaccharides of fairly high molecular weight that have an α -1,6-linked main chain with α -1,3-linked branches. The extracellular slime produced from sucrose by lactic acid bacteria, *Lc. mesenteroides* is a common problem in sugar, where slime coats vessels and equipment, slows filtration, and upsets crystallization of the sugar. The same dextrans are used in the food, pharmaceutical, and chemical industries as food stabilizers, blood plasma extenders, anticoagulants (sulfated dextrans), and adsorbents (DEAE dextran). Dextrans are synthesized by other bacterial species such as *Lactobacillus*, *Streptococcus*, and *Aerobacter*. The synthesis of the polymer is an extracellular process involving the enzyme dextransucrase, which catalyzes the reaction:



where upon fructose is fermented by the bacterium.

To obtain high dextran concentration, acids produced are normally neutralized by alkali. The high molecular weight polymer is solvent-precipitated and then degraded, either enzymatically (using *exo*- or *endo*-dextranses), by mild acid hydrolysis, or by heat, to generate a product with the correct molecular weight range. Alternatively, low molecular weight primer polysaccharides can be used to initiate polymerization to produce dextrans having the desired degree of polymerization. Using a low molecular weight dextran primer (10,000–25,000) and sucrose (10% w/v, pH 5, 15 °C), a large proportion of the product (50%) has a molecular weight range of 50,000–100,000, which does not require further fractionation for clinical use. The enzyme has been immobilized on a laboratory scale, but not yet commercially. If the latter can be achieved, dextrans could be produced directly and cost-effectively by controlled hydrolysis. Despite problems related to dextran isolation and purification, a great deal of development is expected for dextran production in the future. Because dextrans produced by oral LAB play an important role in the etiology of dental diseases, extensive genetic studies have been conducted. Optimizing conditions of dextran synthesis by the bacterium *Lc. mesenteroides* grown in molasses are described (Vedyashkina et al., 2005).

4.6.4.4 Gellan gum *Gellan gum* is the generic name for the extracellular polysaccharide secreted by *Pseudomonas elodea* (American Type Culture Collection, ATCC, 31461) when subjected to aerobic submerged fermentation. Gellan, a polysaccharide of glucose, rhamnose, and glucuronic acid residues with 3–4.5% *o*-acetyl groups, occurs in three different forms: native, low acetyl, and low acetyl/clarified. The low acetyl form, produced from

the native polymer by heating at pH 10, produces firm and brittle gels upon heating and cooling. Gel strength is a function of gum/salt ratio and the nature of the cations, where divalent cations such as Ca^{2+} and Mg^{2+} are desirable for maximum gel strength rather than monovalent cations. A deacylated product may be obtained by alkali treatment (80 °C, pH 10, 10 min) followed by alcohol precipitation. Clarified products freed from bacterial cell debris may be obtained by filtering the hot fermentation liquor.

Kelco, now CP Kelco Div. of JM Huber is virtually the only producer of gellan gum. A few sources exist in China but are small and little found in the market. Pure gellan gum is one of the most expensive hydrocolloids. Its cost in use, however, is competitive with much lower-priced hydrocolloids.

Gellan gum is currently used in microbiological media, under the trade name Gelrite, and it is superior to agar in terms of improved clarity, strong gel strength at half the concentration, reduced toxicity, and high resistance to enzymatic degradation. It has versatility as a broad spectrum gelling agent for preparing either as cold setting/thermosetting or as thermoreversible/thermoirreversible gels.

4.6.4.5 Curdlan *Curdlan* is a β -1,3-linked homopolymer of glucose, formed by *A. faecalis* var. *myxogenes* in 10% ethylene glycol as the sole carbon source. Under alkaline conditions, the polymer is disordered and the solution has a low viscosity. During fermentation, this polymer is obtained by removing the bacterial cell as follows:

fermentation suspension \rightarrow acid neutralization \rightarrow centrifugation \rightarrow gel suspension
(alkaline conditions: disorderd) (fermentation wash: ordered) (gel centrifuged off) (in water)

This bacterium produces two structurally distinct polysaccharides: a water-soluble acidic heteroglycan and a water-insoluble neutral homoglycan. The heteroglycan contains succinyl groups and is called *succinoglycan*. Aqueous suspensions of the homoglycan gel upon heating and the polymer is called *curdlan*. The resultant gel strength depends on the temperature used, being nonreversible above 54 °C, constant from 60 to 80 °C, and increasing over the range of 80–100 °C. The molecular structure changes from a single- to a triple-stranded helix above 120 °C. A series of mutants of this bacterium was obtained by treatment of various mutagenic agents, and a high-yielding curdlan producer, mutant 10C3K-u, was obtained.

This polysaccharide is used as a gelling agent in cooked foods such as sausages and starch-based jellies, as a binding agent in spaghetti and hamburgers, or as a gelling agent for sweet jellies and desserts. Other industrial uses include artificial food for fish or silkworms, additive for tertiary oil recovery, support for immobilized enzymes, and binding agent in tobacco products.

4.6.4.6 Pullulan The yeast, *A. pullulans*, has been used in the production of *pullulan*, a polymer composed of α -1,6- and α -1,4-maltotriose or occasionally maltotetrose units, which may contain 90–900 maltotriose units. Pullulan has also been described in the species of *Arthrobacter*, *Beijerinckia*, and methane- or methane-utilizing bacteria. Pullulan is produced commercially in Japan using *Azotobacter pullulans*. By selection of strain and fermentation conditions, pullulan can be produced in various viscosity grades. Carbohydrate, ammonium salt, some organic nitrogen, and mineral salts are added to meet nutritional requirements. The polysaccharide yield is enhanced if the supply of nitrogen is somewhat restricted. The mean molecular weight of pullulan can be reduced by keeping the pH in the range of 5.5–6.5 and the phosphate concentration between 0.1% and 0.5%. The mean molecular weight value is high early in the fermentation process and declines thereafter.

The main interest in this polysaccharide is due to its capacity to form strong resilient films and fibers. Edible films of this gum are currently used as an edible package for granular food and as a packaging film for hams. The intermediate grades are used as a binder in foods, especially in diet foods, and the finest grades are used in the production of packaging film. One property of the film is its low oxygen-permeability when cross-linked with other materials. As an adjunct to tofu (soybean curd), it improves curd precipitation when $MgCl_2$ is used alone. Other uses have been proposed in the fields of cosmetics, pharmaceuticals, and water-based paints. Pullulan is resistant to hydrolysis by α -amylase but is degraded by pullulanase.

4.6.5 Other polysaccharides

All microorganisms are potential sources of new polysaccharides. Relatively few polysaccharides have been studied extensively, and thus there is still a great deal of scope for the development of new commercial polysaccharides. Examples of polysaccharides that have potential application in foods include baker's yeast glucan (BYG), bacterial cellulose, microbial chitosan, and other EPSs from Gram-negative organisms such as *Zoogloea ramigera* and XM6 secreted by *Enterobacter*. BYG, the cell-wall material, extracted from *S. cerevisiae* is composed primarily of glucose and mannose. BYG could be added to foods to stimulate the mouthfeel of fats and/or oils. *Acetobacter xylinum* produces cellulose as microfibrils; it can be used in gelled food products and dietetic/low calorie foods, or formed and dried to produce films.

The biosynthesis of microbial chitin by two enzymes, chitin synthetases I and II, has recently been worked out in *S. cerevisiae*. Only chitin synthetase II appears to be responsible for chitin synthesis *in vivo*; the other form may be nonessential (disruption of the gene for I did not prevent the production of chitin). Levan, which is the polymer of fructose, was produced by *Zymomonas mobilis* and *Alcaligenes viscosus* grown in sucrose and lactose, respectively. EPSs and polyhydroxyalkanoates by *Archaea* are also of significant biotechnological importance as their biopolymers possess unique properties that offer insights into their biology and evolution (Poli et al., 2011).

Summary

Although both the composition and the amount of EPS produced by microorganisms are genetically determined traits, they are highly influenced by media components and culture conditions. EPSs synthesis is generally favored by the presence of carbon source in excess, concomitant with limitation by another nutrient (e.g., nitrogen, oxygen). The structure, composition, and viscosity of EPSs depend on several factors, such as the composition of the culture medium, carbon and nitrogen sources and precursor molecules, mineral salts, trace elements, type of strain, and fermentation conditions such as pH, temperature, oxygen concentration, and agitation. Microorganisms used as industrial or technical producers of extracellular polysaccharides are mainly pathogenic bacteria, but *Xanthomonas*, *Leuconostoc*, *Pseudomonas*, and *Alcaligenes* which produce xanthan, dextran, gellan, and curdlan, respectively, are the most industrially used. Actually, the EPSs produced by LAB, which are already accepted as GRAS (generally recognized as safe) represent the most suitable polymers for the food industry. They are widely employed in the dairy industry since the *in situ* production of their EPSs improves the texture of fermented dairy products and also confers health benefits as a result of their immunostimulatory, antitumoral or cholesterol-lowering activity. Dextran (produced by LAB such as *Lc. mesenteroides* and the

mesophilic dental pathogen *Streptococcus mutans*), xanthan gum (the EPS from the plant pathogen *X. campestris pv. campestris* bacterium), gellan (produced by the nonpathogenic bacterium *P. elodea*), and curdlan (produced by the alkaline-tolerant mesophilic pathogen *A. faecalis*) are some examples of commercial microbial polysaccharides that entered the market. Because of the pathogenicity of the commercial EPS-producing strains, in recent years significant progress has been made in discovering and developing novel and functional EPSs from extremophilic producer strains. Currently, despite the vast number and biodiversity of the extremophilic producers of EPS, these nontoxic and biodegradable polymers represent only a small fraction of the current polymer market. These few marketable EPSs derived from extremophiles belong only to the bacteria domain. The high production costs and the poor physicochemical properties (if compared with those of industrial EPSs from plant such as guar gum, cellulose, pectin and starch, and from seaweed as alginate and carrageenan), make the microbial EPSs not suitable for profit-generating enterprises. The fermentation media that can represent almost 30% of the cost for a microbial fermentation usually are made of expensive nutrients such as yeast extract, peptone, and salts. In order to maximize the cost-effectiveness of the process, recent works shifted to using multicomponent feedstock systems, and the synthetic media were replaced by cheaper alternatives: molasses were successfully used for fermentative production of commercial polysaccharides such as curdlan, xanthan, dextran, and gellan, and the use of spent malt grains, apple pomace, grape pomace and citrus peels for xanthan production by solid state fermentation, the use of olive mill wastewater in xanthan production are some examples. Besides the use of cheaper substrates, the reduction of production costs may involve the improvement of product yields by optimizing fermentation conditions or developing higher yielding strains (e.g., by mutagenesis or genetic manipulation), and by optimizing downstream processing. Moreover, the interest for the development of microbial EPSs could be related to their use in high-value market niches, such as, cosmetics, pharmaceuticals and biomedicine, where traditional polymers fail to comply with the required degree of purity or lack some specific functional properties.

The microbial polysaccharides have the advantages of controlled cost, continuous supply, and diverse chemical structure for varied functionality. The market for new microbial polysaccharides in food applications should continue to grow because of the continuous replacement of traditional plant-derived gums, unique and constant functionality of the microbial products, and the constancy of a supply high in purity. Indeed, xanthan gum already has displaced other thickener gums such as gum arabic, the premium stabilizer for salad dressings, and gellan has the potential to replace carrageenan, agar, pectin, algin, and gelatin in many applications. In addition, new microbial polysaccharides may come to replace gum arabic, which is in uncertain supply, and even the unique functionality of carrageenan in dairy products, which require a specific interaction with proteins. The challenges facing gum research in the food industry include the development of (i) bulking agents for artificially sweetened food products, (ii) products having enhanced cholesterol-reducing activity, possibly delivered by means of soluble fiber, (iii) products suitable for the partial replacement of fat, and (iv) gels with improved performance and organoleptic properties.

Opportunities exist to reduce the cost of existing and newly developed microbial gums by optimization of media and fermentation conditions. The isolation and characterization of polysaccharide biosynthetic genes may open up new opportunities in strain improvement. Manipulation of genes to control polysaccharide structure and function could ultimately lead to the ability to design and produce unique gum structures for specific functional applications, which will provide novel biopolymers with diverse functional properties.

Lactose-utilizing *X. campestris* was constructed to produce xanthan gum from whey as a cheap carbon source. Another potential application using specific enzymes is the structural modification of plant or microbial polysaccharides. The use of mannanan-C-5 epimerase changes D-mannuronic acid to L-guluronic acid, which results in an increase in the guluronic acid content of alginate. This modification increases the gel strength significantly. α -Galactosidase has also been used to remove the α -1,6-galactose side chains from guar to produce a product with a similar galactose level, to duplicate the useful gel properties of the higher cost locust bean gum isolated from a perennial plant (Grant Reid et al., 1988).

PHB is a biodegradable and thermoplastic polyester consisting of repeat units of β -hydroxybutyrate monomers or heteropolymer of various β -hydroxy fatty acid monomers. PHB has been recognized as an energy-reserve material accumulated by various microorganisms, and some species such as *Alcaligenes eutrophus* and *Azotobacter beijerinckii* may accumulate PHB as intracellular granules up to 70% of their dry weight. To render them useful for commercial polymer production, the molecular weight of the polymer must be of the order 200,000–300,000.

Bibliography

- Adams, R. M., S. Yeast, S. E. Mainzer, K. Moon, A. L. Palombella, D. A. Estell, S. D. Power, and B. F. Schmidt. 1994. Characterization of two cold-sensitive mutants of the β -galactosidase from *Lactobacillus delbrueckii* subsp. *bulgaricus*. *J. Biol. Chem.* 269: 5666–5672.
- Adjakly, M., M. Ngollo, J. P. Boiteux, Y. J. Bignon, L. Guy, and D. Bernard-Gallon. 2013. Genistein and daidzein: different molecular effects on prostate cancer. *Anticancer Res.* 33: 39–44.
- Aoki, H., I. Uda, K. Tagami, Y. Furuta, Y. Endo, and K. Fujimoto. 2003. The production of a new temph-like fermented soybean containing a high level of α -aminobutyric acid by anaerobic incubation with *Rhizopus*. *Biosci. Biotechnol. Biochem.* 67: 1018–1023.
- Arora, G., F. Cormier, and B. H. Lee. 1995. Analysis of odor-active volatiles in cheddar cheese headspace by multidimensional CG/MS/Sniffing. *J. Agri. Food Chem.* 43: 748–752.
- Azarnia, S., D. St-Gerais, C. Champagne, and B. H. Lee. 2010a. Effect of encapsulated recombinant aminopeptidase of *Lactobacillus rhamnosus* on the accelerated Cheddar cheese. *Food Biotech.* 24: 135–149.
- Azarnia, S., B. H. Lee, V. Yaylayan, and K. N. Kilcawley. 2010b. Proteolysis development in enzyme-modified Cheddar cheese using natural and recombinant enzymes of *Lactobacillus rhamnosus* S93. *Food Chem.* 120: 174–178.
- Azov, V., G. Shelef, and R. Moraine. 1982. Carbon limitation of biomass production in high-rate oxidation ponds. *Biotechnol. Bioeng.* 24: 579–584.
- Bacus, J. 1984. Utilization of Microorganisms in Meat Processing, p. 170. Research Studies Press, Letchworth, UK.
- Baird, J. K. and D. J. Pettitt. 1991. Biogums used in food and made by fermentation. In *Biotechnology and Food Ingredients*, I. Goldberg and R. Williams, Eds., pp. 223–263, Van Nostrand Reinhold, New York.
- Battat, E., Y. T. Peleg, A. Bercovitz, J. S. Rokem, and I. Goldberg. 1990. Optimization of L-malic acid production by *Aspergillus flavus* in a stirred fermenter. *Biotech. Bioeng.* 37: 1108–1116.

- Beddows, G. C. 1985. Fermented fish and fish products. In *Microbiology of Fermented Foods*, Vol. 2, B. J. B. Wood, Ed., Elsevier Applied Science, New York.
- Benkerroum, N. 2013. Traditional fermented foods of North African countries: technology and food safety challenges with regard to microbiological risks. *Compreh. Rev. Food Sci. Food Saf.* 12: 54–89.
- Berovic, M. and M. Legisa. 2007. Citric acid production. *Biotechnol. Ann. Rev.* 13: 303–343.
- Björkroth, J. and J. Koort, Eds., 2011. LACTIC ACID BACTERIA: taxonomy and biodiversity. In *Encyclopedia of Dairy Sciences*, 2nd ed. pp. 45–48. Elsevier Science, Amsterdam.
- Broadbent, J. R., H. Cai, R. L. Larsen, J. E. Hughes, D. L. Welker, V. G. De Carvalho, T. A. Tompkins, Y. Ardö, F. Vogensen, A. De Lorentiis, M. Gatti, E. Neviani, J. L. Steele. 2011. Genetic diversity in proteolytic enzymes and amino acid metabolism among *Lactobacillus helveticus* strains. *J. Dairy Sci.* 94: 4313–4328.
- Carminati, D., Giraffa, G., Quiberoni, A., Binetti, A., Suarez, V., and Reinheimer, J. 2010. Advances and trends in starter cultures for dairy fermentation Chapter 10. In *Biotechnology of Lactic Acid Bacteria: Novel Applications*. F. Mozi, R. R. Raya, and G. M. Vignolo, Eds., Wiley-Blackwell Publisher.
- Chandan, R. C. and A. Kilara, Eds., 2013. *Manufacturing Yogurt and Fermented Milks*, 2nd ed. 496 pages. Wiley-Blackwell, Hoboken.
- Contrezas, A., D. Z. Herbert, B. G. Grubbs, and I. L. Cameron. 1979. Blue green alga, *Spirulina*, as the sole dietary source of protein in sexually mating rats. *Nutr. Rep. Int.* 19: 746–752.
- Crueger, W. and A. Crueger. 1990. *Biotechnology: A Textbook of Industrial Microbiology*, 2nd ed. Sinauer Associates, Inc., MA.
- Davies, F. L. and B. A. Law, Eds. 1984. *Advances in the Microbiology and Biochemistry of Cheese and Fermented Milk*, Elsevier Applied Science, London.
- Deng, Y., S. Li, Q. Xu, M. Gao, and H. Huang. 2012. Production of fumaric acid by simultaneous saccharification and fermentation of starchy materials with 2-deoxyglucose-resistant mutant strains of *Rhizopus oryzae*. *Bioresour. Technol.* 107: 363–367.
- Evans, C. G. T., R. G. Yeo, and D. C. Ellwood. 1979. Continuous culture studies on the production of extracellular polysaccharides by *Xanthomonas juglandis*. In *Microbial Polysaccharides*, W. C. Berkeley, G. W. Gooday, and D. C. Ellwood, Eds., Academic Press, New York.
- Fadda, S., C. López, and G. Vignolo. 2010. Role of lactic acid bacteria during meat conditioning and fermentation: peptides generated as sensorial and hygienic biomarkers. *Meat Sci.* 86: 66–79.
- Feeney, R. E. and Y. Yeh. 1993. Antifreeze proteins: properties, mechanism of action and possible applications. *Food Technol.* 47: 82–89.
- Fox, P. F. 1987. *Cheese: Chemistry, Physics and Microbiology*, Vol. 1. Elsevier Applied Science, London.
- Fukara, M., Y. S. Park, and K. Toda. 1992. Improvement of acetic acid fermentation by molecular breeding and process development. *J. Appl. Bacteriol.* 73: 446–454.
- Fukao, M., K. Oshima, H. Morita, H. Toh, W. Suda, S. W. Kim, S. Suzuki, T. Yakabe, M. Hattori, and N. Yajima. 2013. Genomic analysis by deep sequencing of the probiotic

- Lactobacillus brevis KB290 harboring nine plasmids reveals genomic stability. *PLoS One* 8(3): e60521.
- Gasson, M. J. and W. M. de Vos, Eds. 2013. *Genetics and Biotechnology of Lactic Acid Bacteria*, p. 300, Springer, The Netherlands.
- Giraffa, G., N. Chanishvili, and Y. Widyastuti. 2010. Importance of lactobacilli in food and feed biotechnology. *Research Microbiol.* 161, 480–487.
- Grant Reid, J. S., M. Edwards, and I. C. M. Dea. 1988. Enzymatic modification of natural seed gums. In *Gums and Stabilizers for the Food Industry 4*, G. O. Phillips, D. J. Wedlock, and P. A. Williams, Eds. pp. 391–398, IRL Press, Washington, DC.
- Goldberg, I., Y. Peleg, and J. S. Rokem. 1991. Citric, fumaric, and malic acids. In *Biotechnology and Food Ingredients*, I. Goldberg and R. Williams, Eds., pp. 349–374, Van Nostrand Reinhold, New York.
- Gu, C., Y. Zhou, L. Liu, T. Tan, and D. Li. 2013. Production of fumaric acid by immobilized *Rhizopus arrhizus* on net. *Bioresour. Technol.* 131: 303–307.
- Habibi Najafi, M. B. and LEE, B. H. 1996. Bitterness in cheese: a review. *Crit. Rev. Food Sci. Nutr.* 36: 397–411.
- Haileselassie, S. S., B. H. Lee, and B. F. Gibbs. 1999. Purification and identification of potentially bioactive peptides from enzyme-modified cheese. *J. Dairy Sci.* 82: 1612–1617.
- Hakim, A., J. B. Nguyen, K. Basu, D. F. Zhu, D. Thakral, P. L. Davies, F. J. Isaacs, Y. Modis, and W. Meng. 2013. Protein and its implications for ice binding crystal structure of an insect antifreeze. *J. Biol. Chem.* 288: 12295–12304.
- Hamer, G. 1979. Biomass from natural gas. In *Microbiol. Biomass-Economic Microbiology*, Vol. 4, A. H. Rose, Ed., pp. 315–360. Academic Press, New York.
- Hammes, W. P., D. Haller, and M. G. Ganzle. 2003. Fermented meats. In: *Handbook of Fermented Functional Foods*, E. R. Farnworth, Ed., pp. 390, CRC Press.
- Harutoshi, T. 2013. Chapter 22. Exopolysaccharides of lactic acid bacteria for food and colon health applications. In *Biochemistry, Genetics and Molecular Biology “Lactic Acid Bacteria – R & D for Food, Health and Livestock Purposes”*, M. Kongo, Ed., ISBN 978-953-51-0955-6, In Tech, Texas, USA.
- Hill, L., L. H. Woodruff; J. C. Foote, and M. Barreto-Alcoba. 2005. Esophageal injury by apple cider vinegar tablets and subsequent evaluation of products. *J. Amer. Dietetic Assoc.* 105: 1141–1144.
- Hirsjärvi, S., S. Dufort, G. Bastiat, P. Saulnier, C. Passirani, J. L. Coll, and J. P. Benoît. 2013. Surface modification of lipid nanocapsules with polysaccharides: from physico-chemical characteristics to in vivo aspects. *Acta Biomater.* 9: 6686–6693.
- Horii, M. 2008. Tempeh. In *Advanced Science on Natto*, K. Kiuchi, T. Nagai, and K. Kimura, Ed., pp. 234–237, Woodhead Publishing Ltd, Tokyo, Japan.
- Huang, T. C. and Teng, D. F. 2004. *Soy Sauce: manufacturing and biochemical changes*. In *Handbook of Food and Beverage Fermentation Technology*. Marcel Dekker.
- Hutkins, R. W. 2006. *Microbiology and Technology of Fermented Foods*. Blackwell publishing, Oxford.
- Lee, J. M., D. C. Lee, ong Chul, and S. M. Kim. 2013. The effects of koji and kistidine on the formation of histamine in anchovy sauce and the growth inhibition of histamine degrading bacteria with preservatives. *Amer. J. Adv. Food Sci. Technol.* 1: 25–36.
- Jiménez, J. J., J. Borrero, D. B. Diep, L. Gútiéz, I. F. Nes, C. Herranz, L. M. Cintas, and P. E. Hernández. 2013. Cloning, production, and functional expression of the bacteriocin

- sakacin A (SakA) and two SakA-derived chimeras in lactic acid bacteria (LAB) and the yeasts *Pichia pastoris* and *Kluyveromyces lactis*. *J. Ind. Microbiol. Biotech.* 40: 977–973, June.
- Kilcawley, K. N., M. G. Wilkinson, and P. F. Fox. 2005. A novel two-stage process for the production of enzyme-modified cheese. *Food Res. Intern.* 39: 619–627.
- Klaenhammer, T. R., R. Barrangou, B. L. Buck, M. A. Azcarate-Peril, and E. Alterman. 2005. Genomic features of lactic acid bacteria affecting bioprocessing and health. *FEMS Microbiol. Rev.* 29: 391–409.
- Kongkiattakajorn, J. 2013. Chapter 4. Potential of Fermented Sausage-Associated Lactic Acid Bacteria to Degrade Biogenic Amines During Storage. In *Lactic Acid Bacteria – R & D for Food, Health and Livestock Purposes*, M. Kongo, Ed., 658 pages, InTech Publ.
- Koskella, B. and S. Meaden. 2013. Understanding bacteriophage specificity in natural microbial communities. *Viruses* 5: 806–823.
- Kosikowski, F. V. and V. Mistry. 2013. *Cheese and Fermented Milk Foods*, p. 1050, New England Cheesemaking Supply, Inc.
- Kuda, T. and M. Miyawaki. 2010. Reduction of histamine in fish sauces by rice bran nuka. *Food Control*, 21: 1322–1326.
- Le Loir, Y. et al., 2005. Protein secretion in *Lactococcus lactis*: an efficient way to increase the overall heterologous protein production. *Microb. Cell Fact.* 4, 2–15.
- Lee, B. H. 1991. Bioconversion of starch wastes. In *Bioconversion of Waste Materials To Industrial Products*, A. M. Martin, Ed., pp. 265–291, Elsevier Applied Science, New York.
- Litchfield, J. H. 1985. Bacterial biomass. In *Comprehensive Biotechnology*, Vol. 3, M. Moo-Young, Ed., pp. 463–481, Pergamon Press, London.
- Liu, G., H. Wang, M. W. Griffiths, and P. Li. 2011. Heterologous extracellular production of enterocin P in *Lactococcus lactis* by a food-grade expression system. *Europ. Food Res. Technol.* 233: 123–129.
- Lopez-Kleine, L. and V. Monnet. 2011. Proteolytic systems. In *Encyclopedia of Dairy Science*, 2nd ed., J. W. Fuquay, P. F. Fox, and P. L. H. McSweeney, Eds., Academic Press.
- Lotfy, W. A., K. M. Ghanem, and E. R. El-Helow. 2007. Citric acid production by a novel *Aspergillus niger* isolate: II. Optimization of process parameters through statistical experimental designs. *Bioresour. Technol.* 98: 3470–3477.
- Lüche, F. K. 1994. Fermented meat products. *Food Res. Intern.* 27: 299–308.
- Lüche, F. K. and R. Earnshaw. 1991. Starter cultures. In *Food Preservations*, N. S. Russell and G. W. Gould, Eds., pp. 215–234, Blackie, Glasgow.
- Lu, G. T., Z. F. Ma, J. R. Hu, D. J. Tang, Y. Q. He, J. X. Feng, and J. L. Tang. 2007. A novel locus involved in extracellular polysaccharide production and virulence of *Xanthomonas campestris* pathovar *campestris*. *Microbiology* 153: 737–746.
- Mah, J. H. and H. J. Hwang. 2009. Inhibition of biogenic amine formation in a salted and fermented anchovy by *Staphylococcus xylosus* as a protective culture. *Food Control* 20: 796–801.
- Mahony, J. and D. van Sinderen. 2012. Structural aspects of the interaction of dairy phages with their host bacteria. *Viruses* 4: 1410–1424.
- Manitchotpisit, P., C. D. Skory, S. W. Peterson, N. P. J. Price, K. E. Vermillion, and T. D. Leathers. 2012. Poly(β -L-malic acid) production by diverse phylogenetic clades of *Aureobasidium pullulans*. *J. Ind. Microbiol. Biotechnol.* 39: 125–132.

- Martinez, F. A. C., E. M. Balciunas, J. M. Salgado, J. M. D. González, A. Converti, and R. P. S. Oliveira. 2013. Lactic acid properties, applications and production: a review. *Trends Food Sci. Technol.* 30: 70–83.
- Matsuda, S., T. Miyazaki, Y. Matsumoto, R. Ohba, Y. Teramoto, N. Ohta, and S. Ueda, 1992. Hydrolysis of riboflavones in soybean cooked syrup by *Lactobacillus casei* ssp. *rhamnosus* IFO3425. *J. Ferment. Bioeng.* 74: 301–304.
- Matthias, U. 2009. *Bacterial Polysaccharides: Current Innovations and Future Trends*. Caister Academic Press.
- Mehaia, M. A. and M. Cheryan. 1986. Lactic acid from acid whey permeate in a membrane recycle bioreactor. *Enzyme Microbiol. Technol.* 8: 289–292.
- Mital, B. K. and S. K. Garg. 1995. Anticarcinogenic, hypocholesterolemic, and antagonistic activities of *Lactobacillus acidophilus*. *Crit. Rev. Microbiol.* 21: 175–214.
- Mujtaba, I. M., E. A. Edreder, and M. Emtir. 2012. Significant thermal energy reduction in lactic acid production process. *Appl. Energy* 89: 74–80.
- Nasseri, A. T., S. Rasoul-Amini, M. H. Morowvat, and Y. Ghasemi. 2011. Single cell protein: production and process. *Am. J. Food Technol.* 6: 103–116.
- Park, S. Y., B. F. Gibbs, and B. H. Lee, 1995. Effects of crude enzyme of *Lactobacillus casei* ssp. *casei* LLG on the water soluble peptides of enzyme modified cheese. *Food Res. Int.* 28: 43–49.
- Park, Y. W., F. W. Haenlein, S. H. Kim, and S. J. Oh. 2013. 16. Fermented Milk and Yogurt. In *Milk and Dairy Products in Human Nutrition: Production, Composition and Health*. John Wiley & Sons, Ltd, New York.
- Peng, Y., X. Yang, and Y. Zhang. 2005. Microbial fibrinolytic enzymes: an overview of source, production, properties, and thrombolytic activity in vivo. *Appl. Microbiol. Biotechnol.* 69:126–132.
- Perdigon, G., S. Alvarez, M. Rachid, G. Agüero, and N. Gobbato. 1995. Symposium: probiotic bacteria for humans: clinical systems for evaluation of effectiveness. *J. Dairy Sci.* 78: 1597–1606.
- Peters, I. D., C. L. Hew, and P. L. Davies. 1989. Biosynthesis of antifreeze proprotein in *E. coli*. *Protein Eng.* 3: 145–151.
- Phillips, G. O. and P. A. Williams. 2002. *Gums and Stabilisers for the Food Industry* 11. p. 384. Royal Society of Chemistry.
- Poli, A., P. D. Donato, G. R. Abbamondi, and B. Nicolaus. 2011. Synthesis, production, and biotechnological applications of exopolysaccharides and polyhydroxyalkanoates by *Archaea*. *Archaea* 2011: 1–13. doi:10.1155/2011/693253.
- Prasanna, P. H. P., A. S. Grandison, and D. Charalampopoulos, D. 2013. Microbiological, chemical and rheological properties of low fat set yoghurt produced with exopolysaccharide (EPS) producing *Bifidobacterium* strains. *Food Res. Intern.* 51: 15–22.
- Präve, P., U. Faust, W. Sittig, and D. A. Sukatsch, Eds. 1987. *Fundamentals of Biotechnology*, pp. 602–622, VCH Publishers, New York.
- Rehm, B. H. A. 2009. *Microbial Production of Biopolymers and Polymer Precursors: Applications and Perspectives*. Caister Academic Press.
- Ramesh, C. and A. K. Chandan. 2013. *Manufacturing Yogurt and Fermented Milks*. John Wiley & Sons, Inc.
- Raja, R., S. Hemaiswarya, N. A. Kumar, S. Sridhar, and R. Rengasamy. 2008. A perspective on the biotechnological potential of microalgae. *Crit. Rev. Microbiol.* 34: 77–88.

- Reddy, N. R., and M. D. Pierson. 1994. Reduction in antinutritional and toxic components in plant foods by fermentation. *Food Res. Intern.* 27: 281–290.
- Robinson, R. K., Ed. 1991. *Therapeutic Properties of Fermented Milks*. Elsevier Applied Science, New York.
- Rodríguez Couto, S. and M. A. Sanromán. 2006. Application of solid-state fermentation to food industry – A review. *J. Food Eng.* 76: 291–302.
- Rouse, S., D. Field, K. M. Daly, P. M. O'Connor, P. D. Cotter, C. Hill, and R. P. Ross. 2012. Bioengineered nisin derivatives with enhanced activity in complex matrices. *Microbial Biotechnol.* 5: 501–508.
- Rouhi, M., S. Sohrabvandi, and A. M. Mortazavian. 2013. Probiotic fermented sausage: viability of probiotic microorganisms and sensory characteristics. *Crit. Rev. Food Sci. Nutr.* 53: 331–348.
- Sakurai, K., S. Yamazaki, M. Ishii, Y. Igarashi, and H. Arai. 2013. Role of the glyoxylate pathway in acetic acid production by *Acetobacter aceti*. *J. Biosci. Bioeng.* 115: 32–36.
- Settanni, L. and A. Corsetti. 2008. Application of bacteriocins in vegetable food biopreservation. *Int. J. Food Microbiol.* 121: 123–138.
- Sodeck, G., J. Modl, J. Kominek, and W. Saalzvrunn. 1981. Production of citric acid according to the submerged fermentation process. *Process Biochem.* 16: 9–11.
- Sonomoto, K. and A. Yokota. Eds. 2011. *Lactic Acid Bacteria and Bifidobacteria: Current Progress in Advanced Research*, pp. 286, Caister Academic Press, UK.
- Soukoulis, C., P. Panagiotidis, R. Koureli, and C. Tzia. 2007. Industrial yogurt manufacture: monitoring of fermentation process and improvement of final product quality. *J. Dairy Sci.* 90: 2641–2654.
- Steinkraus, K. H., Ed. 2005. *Industrialization of Indigenous Fermented Foods*, 2nd ed., Marcel Dekker, New York.
- Steele, J., J. Broadbent, and J. Kok. 2013. Perspectives on the contribution of lactic acid bacteria to cheese flavor development. *Curr. Opin. Biotech.* 24: 135–141.
- Sutherland, I. W. 1982. Biosynthesis of microbial exopolysaccharides. *Adv. Microbial Physiol.* 24: 79–150.
- Szczepankowska, A. K., R. K. Górecki, P. Kołakowski, and J. K. Bardowski. 2013. Chapter 2. Lactic Acid Bacteria Resistance to Bacteriophage and Prevention Techniques to Lower Phage Contamination in Dairy Fermentation. In *Lactic Acid Bacteria - R & D for Food, Health and Livestock Purposes*, M. Kongo, Ed., 658 pages, InTech Publ.
- Toldrá, F. 2007. *Handbook of Fermented Meat and Poultry*, 576 pages, Wiley-Blackwell.
- Tomoo, K., K. Mikiya, F. Takashi, and K. Takayuki. 2009. Acetic acid upregulates the expression of genes for fatty acid oxidation enzymes in liver to suppress body fat accumulation. *J. Agric. Food Chem.* 57: 5982–5986.
- Tyagi, R. D., D. Kluepfel, and D. Couillard. 1991. Bioconversion of cheese whey to organic acids. In *Bioconversion of Waste Materials to Industrial Products*, A. M. Martin, Ed., pp. 313–333, Elsevier Applied Science, New York.
- Vedyashkina, T. A., V. V. Revin, and I. N. Gogotov. 2005. Optimizing the conditions of dextran synthesis by the bacterium *Leuconostoc mesenteroides* grown in a molasses-containing medium. *Appl. Biochem. Microbiol.* 41: 361–364.
- de Vuyst L., F. de Vin, F. Vaningelgem, and B. Degeest. 2001. Recent developments in the biosynthesis and applications of heteropolysaccharides from lactic acid bacteria. *Int. Dairy J.* 11: 687–707.

- Wang, T. T. and B. H. Lee. 1997. Plasmids in *Lactobacillus*. Crit. Rev. Biotech. 17: 227–272.
- Weimer, B. C. 2007. Improving the Flavour of Cheese. Woodhead Publishing Series in Food Science, Technology and Nutrition, Vol. 142, 600 pages, Elsevier, Amsterdam.
- Whitman, W. B., M. Goodfellow, P. Kämpfer, H. J. Busse, M. E. Trujillo, W. Ludwig, K. I. Suzuki, A. Parte, Eds. 2012. The Actinobacteria. In Bergey's Manual of Systematic Bacteriology. Vol. 5, pp. 1750, Springer.
- Wu, P., C. Cai, X. Shen, L. Wang, J. Zhang, Y. Tan, W. Jiang, and X. Pan. 2014. Formation of ethyl carbamate and changes during fermentation and storage of yellow rice wine. Food Chem. 152: 108–112.
- Wu, A. H., E. Lee, and C. Vigen. 2013. Soy isoflavones and breast cancer. Am. Soc. Clin. Oncol. Educ. Book 33: 102–106.
- Xu, Q., L. Liu, and J. Chen. 2012. Reconstruction of cytosolic fumaric acid biosynthetic pathways in *Saccharomyces cerevisiae*. Microb. Cell Fact. 11: 24.
- Yakabe, T., T. Shimohata, and A. Takahashi. 2013. *Lactobacillus brevis* KB290 enhances IL-8 secretion by *Vibrio parahaemolyticus*-infected Caco-2 cells. J. Microbiol. Biotechnol. 23: 118–124.
- Zaman, M. Z., F. A. Bakar, S. Jinap, and J. Balar. 2011. Novel starter cultures to inhibit biogenic amines accumulation during fish sauce fermentation. Intern. J. Food Microbiol. 145: 84–91.
- Zendo, T. 2013. Screening and characterization of novel bacteriocins from lactic acid bacteria. Biosci. Biotechnol. Biochem. 77: 893–899.
- Zhang, L., X. Zhang, C. Liu, C. Li, S. Li, T. Li, D. Li, Y. Zhao, and Z. Yang. 2013. Manufacture of Cheddar cheese using probiotic *Lactobacillus plantarum* K25 and its cholesterol-lowering effects in a mice model. World J. Microbiol. Biotechnol. 29: 127–135.

5

Other Organism-Based Processes and Products

5.1 Enzymes

5.1.1 Introduction

Enzymes, which are protein catalysts synthesized by living systems, are important as synthetic and degradative catalysts. With the help of a multitude of enzymes, microorganisms are able to carry out the huge number of stepwise chemical reactions necessary for the growth and maintenance of cells (metabolism). Many microorganisms synthesize additional, often complex substances, which play no part in the growth process, the so-called secondary metabolites such as flavor and fragrances. Most of the chemical reactions taking place in living cells are completed in milliseconds or less, within a relatively narrow range of physical conditions. This rapid rate of metabolism is due to the existence of biological catalysts, namely enzymes. Enzymes not only make essential contributions to cellular activities, but also find many applications in biotechnology, especially in the food processing industry, for manufacturing cheese, beer, wine, bread, sweeteners, and so on, and in the chemical and pharmaceutical industries for synthesizing amino acids and antibiotics.

While the presence of natural enzymes is advantageous in curing food products such as cheese and meat to give desirable textures and flavors, natural enzymes may produce undesirable reactions, such as rancidity from lipases and browning reactions due to polyphenol oxidases. Sometimes natural enzymes in foods are used as an index of the pasteurization of milk or cheese (by the detection of phosphatase or catalase) or to indicate the presence of peroxidase in vegetable products (as evidence of incomplete blanching). Recognition of the functions and the usefulness of enzymes in bringing about desirable changes in foods has led to the large-scale production of commercial enzymes. Of the more than 4000 enzymes known from animal, plant, or microbial sources, fewer than 20 are industrially produced on a large scale for the production of foods and intermediates. The majority of enzymes are hydrolases such as amylases, cellulases, pectinases, and proteases, which degrade polymeric substances to simple molecules. The world market for total enzymes is predicted to \$7 billion in 2013 (<http://www.reportlinker.com/p0148002/World-Enzymes-Market.html>),

among which global food enzyme market is expected to reach \$2.3 billion by 2018. Enzymes are employed in a diverse array of applications in industries and scientific research, ranging from the degradation of various natural substances in the starch processing, detergent and textile industries, to the manipulation of DNA/RNA in biotechnology research (Li et al., 2012). Continued strong demand for specialty enzymes, animal feed enzymes, and ethanol production markets will drive advances. Specialty clinical enzymes, nucleases and polymerases, along with various other enzyme types are expected to the more industrially concentrated lipases, carbohydrases, and proteases. Through 2013 world enzyme demand will average annual gains of 6.3% per year, led by pharmaceutical and biocatalyst enzymes. Among the industrial enzyme markets, animal feed and ethanol production will both achieve above average advances, while the food and beverage market will grow at a healthy pace. Novozymes (Denmark) is the key player that accounts for little over two-fifth of the global enzyme market, thereafter DSM (The Netherlands), DuPont (USA), and Roche (Germany) are the competitive companies, which together account for almost one-third of the market. Asia-Pacific, particularly China is the fastest growing market for food and feed enzymes. As illustrated in Figure 5.1, the global enzyme market was dominated by the food and beverage industry, among which growth came mostly from baking enzymes and other smaller applications such as fat and oil processing. However, the growth will be moderate in developed regions such as North America and Western Europe and much faster growth is expected in the Asia/Pacific and other developing regions. The Asia/Pacific region will undergo a rapid increase in enzyme demand due to the high population in China, Japan, and India, reflecting the size and strength of these country's economics. It is different with the world enzyme demand patterns, with the food and beverage processing occupying the largest market share. During 2008–2013, consumption of feed enzymes in China has grown at an average annual rate of about 7.5% and increased to 46.03×10^3 tons in the year 2013 (Li et al., 2012; Sarrouh et al., 2012). The fast growth over the past decade has also been seen in a wealth of other industries spanning from organic synthesis in pharmaceutical industry to diagnostics enzyme with expanded access to medical care in developing countries. Meantime, the detergent industry, once the largest sector in the global enzyme market, declined somewhat due in part to the pricing pressures from the main detergent manufactures after the turn of the century. The bioenergy production enzyme demand was limited by the new legislative mandates for

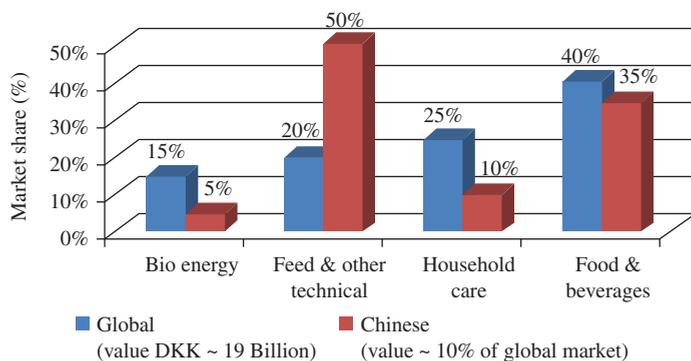


Figure 5.1 Global versus Chinese enzyme market in 2010. *Source:* Adapted from Li, S, et al. 2012. Technology prospecting on enzymes: application, marketing and engineering. *Comput. Struct. Biotechnol. J.* 2:1–11, e201209017, <http://dx.doi.org/10.5936/csbj.201209>. (See insert for color representation of this figure.)

grain-based ethanol, but biofuels derived from cellulosic raw materials will be in favor of demand growth over a long time.

5.1.2 Production of enzymes

5.1.2.1 Sources and characteristics of enzymes Commercial enzymes used by industries may be obtained from plant, animal, and microbial sources. Malt amylase, proteases, papain, ficin, and bromelain are from plant sources, while proteases, amylase, lipases from pancreas; pepsin and rennet from stomach mucosa; and catalase from liver are from animal sources. Today microorganisms have become a major source of commercial enzymes, but enzymes from animal and plant material are still used for special applications. Enzymes are very important in the food-processing industry for ingredient production and texture modification. Some of the major industrial applications include production of corn syrup, beverage clarification, brewing, baking, meat tenderization, and low lactose milk preparation. The reasons for employing microbial enzymes for industrial processes are (i) the existence of different types of enzyme activity, (ii) the rapidity and stability of production through the inexpensive, reproducible, and safe microbial fermentation route, and (iii) the improvements in yield that are obtained much more easily with genetic or protein engineering than from plant or animal cultures. The ability of microorganisms to produce enzymes that are active and stable under extreme conditions of pH and temperature may be responsible in certain industrial processes for improvements in product yield and economics. Use of enzymes instead of chemical synthesis to produce new products or improve product quality has advantages, such as lowering manufacturing costs and complexity. Enzymes have a very high specificity and stereospecificity, properties that may be useful in chemical and pharmaceutical catalysts. However, these specificities may not necessarily be desirable for the production of primary product analogues or food products, which require broad substrate specificity. The other advantage of enzymatic over chemical catalysis is of course the high catalytic rates obtainable under mild reaction conditions.

Several aspects must be considered in the selection of a microorganism as the source of an enzyme:

1. The strain must be able to give high yields of enzyme within the shortest possible fermentation time.
2. If possible, extracellular enzymes should be used because they are easier to produce and isolate, whereas intracellular enzymes must go through expensive disintegration processes.
3. A *food-grade microorganism* (GRAS, *generally recognized as safe*) that does not produce any toxic substances is preferred, to facilitate obtaining regulatory agency approval.
4. A production strain should grow on an inexpensive medium containing cheap substrates, since raw material costs are the major expenses in fermentation.

It is a reflection of these concerns that most food enzymes are produced by *Bacillus* and *Aspergillus* species, which are capable of secreting the enzymes and of growing in cheap media. Enzymes are sold on the basis of activity rather than weight or volume, and thus the stability of an enzyme preparation during storage is of prime importance. Industrial enzymes, except for those used in the amino acid and antibiotic industries, are rarely pure, but the impurities need not interfere with the activity of the enzyme. Although enzymes come into contact with foods, most of these catalysts do their work, and then are inactivated during cooking, baking, or pasteurization.

5.1.2.2 Development of new enzymes New sources of enzymes and novel enzymes produced by microorganisms are continuously being sought, and the improvement of strains is a major task for microbiologists and biochemists. Commercially successful enzyme production must be preceded by assurance of demand for the product, satisfactory technical properties of the process contemplated, and economic feasibility of the enterprise. Most new enzymes fail to satisfy at least one of these aspects. It may be possible to change the properties of enzymes by chemical modification of the molecule, but for economical and technical reasons, chemical methods have not been used on industrial enzymes. The search for new enzymes has, therefore, consisted of selecting new microorganisms by traditional microbiological methods using enrichment cultures and selective media. With the exception of the food industry, which utilizes live cells for fermentation, only a few commercial fermentation processes use wild strains isolated directly from nature.

Mutants that are specifically adapted to the fermentation process are often used in the production of enzymes and other secondary metabolites. Mutation alters the control function, inducing synthesis of the product to continue at a higher rate. Microorganisms used for commercial production of primary metabolites are rarely modified at only one genetic site. It is often necessary to alter several control sites to achieve overproduction of the desired compound. Through an extensive strain development program, yield increases up to 100 times or more may usually be attained. For instance, since a constitutive mutant for xylose eliminates the need for an expensive inducer, xylose has been used for the production of glucose isomerase. Objectionable by-products, such as antibiotics from the culture of *Bacillus* strains, could be eliminated by using mutants. Undesirable side enzymatic activity (transglucosidase) in glucoamylase of wild strains has also been eliminated. Genetic recombination, in which genetic information from each of two genotypes is combined in a new genotype, is another effective means of industrial strain development. However, relatively few industrially important organisms exhibit sexual reproduction as such. One of the most significant approaches to strain development can be anticipated from the use of *in vitro* recombinant DNA technology.

5.1.2.3 Fermentation process Both surface (solid) cultures and submerged (liquid) cultures are used to produce microbial enzymes. Cultures of microorganisms are carried out on the surface of solid media such as wheat bran, whose high content of nutrients includes minerals and salts. This technique is still used for the production of amylase, protease, and lipase from *Aspergillus* and *Mucor* species, as well as for pectinase and cellulase from *Aspergillus* and *Penicillium* species. The culture of fungi is carried out either by the tray process, in which the substrate is spread in a thin layer in incubation rooms, or by the drum process, in horizontally rotating drums. After cultivation of fungi with spores, the mycelia are extracted with water or salt solution in countercurrent mode, and the concentrated enzyme solution is precipitated. Handling costs and control of infection, temperature, humidity, and aeration are the major difficulties in this process.

Submerged culture methods using fermentors today dominate in the production of enzymes, since they present less risk of infection and offer reduced handling costs and higher yields. Submerged culture usually takes place in mechanically stirred tanks with capacities of 10,000–100,000 L in batch operation. Equipment and techniques are most often adapted from antibiotic fermentations. Depending on the enzyme and the microorganisms employed, the main fermentation, especially for extracellular enzymes, lasts from about 30–150 h. Continuous fermentation on the industrial scale has been used only for the production of glucose isomerase. Continuous fermentations have so far found only limited application for the production of industrial enzymes, mainly because of the instability of the production strains and the difficulty of media sterilization.

Though continuous methods are rarely applied, the batch process is extended by fed-batch fermentation (i.e., substrate is fed in increments, as in the fermentation processes).

The formation of enzyme and many secondary metabolites is often subject to catabolite repression by high concentrations of glucose. In addition to the influences of nutrient medium and size and age of inoculum, operational parameters such as pH, aeration, and agitation must be taken into account to optimize the production of enzymes. Addition of surface-active agents may lead to increased excretion of extracellular enzymes.

5.1.2.4 Isolation of enzymes In the case of extracellular enzymes, the majority of enzyme is found in the culture supernatant, and thus recovery methods are simple unit operations such as centrifugation, or filtration, vacuum evaporation, and precipitation of proteins. To recover intracellular enzymes, which are concentrated inside the biomass, mechanical and physical methods must be used to disintegrate intact cell walls. Autolysis may be used, but on an industrial scale physical methods such as the high pressure homogenizer or agitator bead mill are preferred. The cost of isolating and purifying intracellular enzymes for commercial processes may dampen the profitability of their use to the point of unattractiveness. However, the advantages of isolated enzymes must be balanced against cost, depending on the nature of the conversion process. When the cells are broken, the enzyme can be purified as in the case of extracellular enzymes; but the process is normally more difficult because of the presence of cell debris and nucleic acids from the broken cells. The precipitation of proteins may be achieved by ammonium sulfate or sodium sulfate. With thermostable enzymes, proteins are denatured at elevated temperatures and supernatant can be precipitated after the aggregates have been discarded. Organic solvents such as alcohols (ethanol, methanol, isopropanol) are the main precipitants used on the industrial scale. Several polymers (e.g., polyethylene glycol, polyethylene imine) have also been used in industry.

Nucleic acids impart high viscosity to an enzyme solution and interfere with protein fractionation and chromatographic separations; thus it is desirable to remove these components before attempting the purification of intracellular enzymes. Magnesium salts, streptomycin sulfate, protamine sulfate, and polyethylene imine are among the components used as precipitants. Ultrafiltration may be used advantageously to concentrate the enzyme solution rather than vacuum evaporation, but the membranes are easily clogged by precipitates. Many chromatographic and partition processes are available for further purification, but few technical enzymes except for medical and genetically modified organisms (GMO) enzymes are subjected to chromatographic purifications. The precipitate may be dried by freeze drying, vacuum drying, or spray drying depending on the thermostability of the enzyme. During drying, stabilizers such as sugars, enzyme substrates, cofactors, and reducing agents are added to stabilize enzymes. In the past, all enzymes were sold as dusty powders of very small particle size, but today most enzymes are prepared as dustless granulates to avoid exposing people to enzyme dust and to improve storage stability. General schemes for the production of extracellular and intracellular enzymes from microorganisms are shown in Figure 5.2.

5.1.3 Applications

Large-scale applications of the enzymes are given in Table 5.1. Most of the enzymes are hydrolases, indicating that enzyme technology so far has been concentrated on simple hydrolytic processes, while the more complicated enzymatic reactions, which require cofactors, have not been successful for large-scale production. There is also no demand for

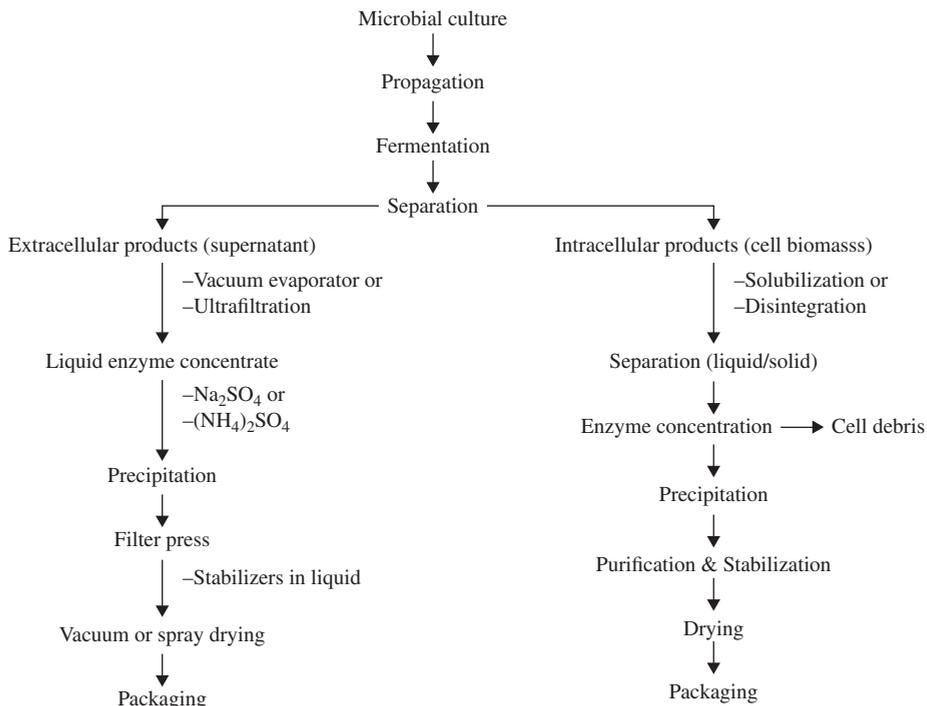


Figure 5.2 General scheme for enzyme production and downstream processing.

the application of such complicated enzymes in the food industry. On the basis of sales volume (500 tons/year) in the 1990s, detergent enzymes such as alkaline proteases represent the greatest single item in enzyme production. Protein dirt and stains that adhere to textile fibers can be better dissolved if proteolytic enzymes are added to the detergent. The alkaline serine protease from *Bacillus licheniformis* and *Bacillus amyloliquefaciens* is the preferred enzyme for this purpose, but detergent enzymes have limited applications in foods. In this chapter, only food enzymes obtained from microorganisms are discussed, in their order of economic importance within the food industry.

5.1.3.1 Starch products processing Three of the enzymes used in the greatest volume in the starch industry are bacterial α -amylase, glucoamylase (amyloglucosidase), and glucose isomerase. These enzymes, which act on starch, have the most numerous applications. Various amylases from plant, animal, and microbial sources have been used for many years, but basically there are three types of amylase that hydrolyze starch: α -amylases, β -amylases, and glucoamylases. The terms *liquefying* and *saccharifying* denote the two major types of enzyme action. α -Amylases are endoamylases; they cleave at random the α -1,4-glycosidic bonds of amylose and amylopectin of starch molecules to yield oligosaccharides of varying length, thereby liquefying starch rapidly, but also producing extensive saccharification upon prolonged action. Since, however, α -amylases cannot hydrolyze the α -1,6-branching linkages in starch, the end products are limited dextrans, together with smaller amounts of glucose and maltose.

Table 5.1 Applications of important commercial food enzymes

Enzymes	Source	Production*	Applications
<i>Starch Processing</i>			
α -Amylase	<i>Bacillus licheniformis</i> , <i>Aspergillus</i> spp.	300	Starch liquefaction, alcohol production
β -Amylase	Plant (malt)	10,000	Maltose production, alcohol production
Glucoamylase	<i>Aspergillus</i> spp.	300	Starch saccharification, brewing, baking
Glucose isomerase	<i>Bacillus coagulans</i>	50	High fructose corn syrup sweeteners
Invertase	<i>Saccharomyces cerevisiae</i>	–	Invert sugar, sugar confectionery
Pullanase	<i>Klebsiella</i> spp.	–	Debranching of starch, brewing
<i>Dairy Processing</i>			
Rennet	Stomach of calves	2	Cheese manufacture (milk coagulation)
Microbial rennet	<i>Mucor miehei</i>	10	Cheese manufacture (milk coagulation)
Lipase/esterase	Fungal, bacterial, animal	–	Cheese ripening, milk fat modification, sausage ripening
Protease/peptidase	<i>Aspergillus niger</i>	–	Cheese ripening
Lactase	<i>Kluyveromyces</i> , <i>Aspergillus</i> spp.	10	Lactose hydrolysis
Catalase	Bovine liver, <i>Pyrobaculum calidifontis</i>	2	Milk sterilization, bleaching
<i>Fruit/Vegetable Processing</i>			
Pectinase	<i>Aspergillus</i> spp.	15	Extraction/clarification of fruit juices
Cellulase	<i>Trichoderma</i> , <i>Aspergillus</i> spp.	–	Fruit and vegetable processing

*1991 world production, in tons of pure enzyme protein.

Thermostable α -amylases, used in high temperature liquefaction, are derived from *B. amyloliquefaciens* (optimum 70 °C), and *B. licheniformis* (92 °C). *B. licheniformis* amylases can be used at temperatures as high as 110 °C for short periods of time in the presence of calcium ions. β -Amylases are exoamylases that cleave α -1,4-glucosidic bonds; these are saccharifying enzymes, producing maltose from the nonreducing end of the starch chain. Glucogenic β -amylases are also able to hydrolyze α -1,6-glucosidic bonds in branched oligosaccharides, whereas maltogenic β -amylases (cereal β -amylase) are unable to bypass these branch points. When β -amylase acts on the branched amylopectin fraction of starch, the action of β -amylase ceases when it reaches an α -1,6-linkage, leaving the so-called β -limited dextrins.

Glucoamylases, frequently called *amyloglucosidase*, are mainly formed by fungi such as *Aspergillus niger* or *Rhizopus* species. They hydrolyze α -1,6 and α -1,3 linkages at a slower rate than α -1,4 linkages; thus, they are saccharifying enzymes, producing only glucose by progressive hydrolysis of glucose units from the nonreducing ends of starch chains. Since glucoamylase has low activity toward branching points (α -1,6 linkages), it may be advantageous to use a debranching enzyme, such as pullulanase, in the beginning of the hydrolysis process. Glucoamylases always contain slight transglucosidase activity. Transglucosidase catalyzes the reverse reaction, which leads to polymerization of glucose to maltose and isomaltose in the sugar reaction. This may considerably lower the glucose yield of the saccharification process, and thus it is important that the glucoamylase be pure.

Immobilized glucoamylase isolated from *Bacillus coagulans* has been developed for commercial use.

Glucose isomerase is primarily a xylose isomerase, and thus xylose must be added for induction of this enzyme. Xylan or wheat bran may be used to replace xylose, which is expensive. Since glucose isomerase is formed intracellularly in most strains, many commercial processes are carried out with immobilized cells or by the addition of partly broken cells. A major process for high fructose corn sweeteners is achieved by the continuous column processes. More detailed processes on corn syrup, high fructose corn syrup (HFCS), and so on are discussed in connection with sweeteners (see Section 6.2.2.3).

Some important commercial applications of amylases and other enzymes are summarized in Table 5.1. Amylases have been used in many food-processing industries as well as in other applications. The first industrial application of the fungal amylase Takadiastase was for an important pharmaceutical digestive enzyme, and this still continues to be a major use.

5.1.3.2 Dairy products processing In the dairy industry, microbial acid proteases from the thermophilic fungi *Mucor miehei* and *Mucor pusillus* have to a large extent replaced the expensive animal rennet (chymosin) obtained from calves. The specificity of acid proteases is similar to that of chymosin, and only minor differences in flavor and texture were detected in cheeses that require a long maturation time. One common problem of the microbial proteases is thermostability. Since about 90% of the proteolytic activity is recovered in the pasteurized whey, the residual activity interferes with the formulation of the whey into dairy products and baby foods. The thermostability of microbial rennet was lowered considerably by mild oxidation of the enzyme, and microbial rennets have been on the market for more than 10 years. The total sales of rennet in 1988 were estimated to be about \$100 million, of which 30% was accounted for by microbial rennets.

An alternative approach has been the use of recombinant DNA technology to clone and express the calf chymosin gene into microorganisms such as *Escherichia coli* and *Kluyveromyces fragilis*. It was proved in cheese trials that recombinant chymosin was exactly identical to the calf rennet. Thus, the microbial product provides a virtually unlimited source of synthetic rennet and it will reduce the need to slaughter suckling calves. The general scheme shown in Figure 4.8 can be applied to the production of chymosin using recombinant *Kluyveromyces lactis*.

Lactase, or β -galactosidase (EC 3.2.1.23), catalyzes the conversion of lactose to glucose and galactose. Lactose is the major constituent of milk and whey, where it accounts for 36% and 75% on a dry weight basis, respectively. Lactose has a low sweetness, forms crystals at high concentrations, causes environmental pollution (as a waste by-product of cheese manufacture), and should be avoided by people whose systems are lactose intolerant. These problems may be overcome by the use of lactase to hydrolyze lactose in whey and in milk. Commercial lactases are usually derived from yeasts, (*K. lactis* or *K. fragilis*) or fungi (*A. niger* or *Aspergillus oryzae*). Yeast lactases function in the range of pH 6–7, but fungal enzymes show pH optima near 4–5. For this reason, yeast lactases are used for the hydrolysis of lactose in milk or neutral sweet cheese whey, and fungal enzymes are more suitable for hydrolysis of acid whey. Properties and applications of commercial lactases are summarized in Table 5.2.

The major applications for lactase are found in milk and milk powder (to improve the product for lactose-intolerant individuals or to increase the sweetness of milk-based drinks), concentrated milk products (to prevent lactose crystallization), fermented milk products (to increase fermentation rate) and whey (as a food ingredient in baking, confectionery, ice cream products or an additive to animal feeds). In a lactose hydrolysis process, milk is pasteurized, and then incubated with lactase for about 4 h. Before packaging for

Table 5.2 Some properties and applications of commercial lactases

Source	Optimum pH	Optimum temperature, °C	Applications
<i>Kluyveromyces lactis</i> (soluble form)	6.5	40–50	Whey hydrolysates for animal feeds
<i>Kluyveromyces fragilis</i> (soluble form)	6.7	35–45	Lactose-hydrolyzed whey syrup used as food ingredients
<i>Aspergillus oryzae</i> (soluble form)	5.0	55	Lactose-hydrolyzed milk
<i>Bacillus</i> spp. (immobilized)	6.5	60	Galacto-oligosaccharides as bifidobacteria growth factor

further treatment, the milk is again pasteurized or sterilized by ultra high temperature (UHT) treatment. Low temperature hydrolysis at about 7 °C under refrigeration to retard microbial contamination is also used, but hydrolysis time is much longer (≈ 20 h) than at ambient temperature. Most of the lactase processes use the batch system with the soluble enzyme, but immobilized lactase systems have also been developed. Immobilized lactase systems have not yet made significant progress because they are expensive compared with soluble systems and because of the problem of microbial contamination.

Lipases (glycerol ester hydrolases, EC 3.1.1.3) hydrolyze triglycerides to di- or mono-glycerides and fatty acids. Lipases hydrolyze the ester bonds at the interface between the aqueous phase (in which the enzyme is soluble) and the insoluble substrate phase. Because of the reversibility of the lipase reaction, hydrolysis and synthesis of glycerides occur when lipases are incubated with oils. This causes an exchange of fatty acid groups between triglyceride molecules, giving interesterification products. Lipases are extracellularly produced from fungi (*Aspergillus*, *Mucor*, *Rhizopus*, *Penicillium*, *Geotrichum*) and yeasts (*Torulopsis*, *Candida*). Bacterial lipases from *Pseudomonas*, *Achromobacter*, and *Staphylococcus* have also been investigated, but commercial lipases are manufactured only from *Aspergillus*, *Mucor*, *Rhizopus*, and *Candida*. In most cases, lipase production is induced by the addition of substrates such as oils and fats. In some cases, however, fats (glycerol) actually repressed enzyme production from *Geotrichum candidum*. The preferred method for production of microbial lipase is by semisolid culture. Lipase may be recovered as a by-product of the production of microbial rennet by *M. miehei* by adsorption on clay minerals. Some properties and applications of commercial lipases are described in Table 5.3.

Table 5.3 Some properties and applications of commercial lipases

Source (specificity)	Optimum pH	Optimum temperature, °C	Applications
Pancreas (1,3-specific)	8.0	50	Digestive enzymes Interesterification of fats
<i>Aspergillus niger</i> (1,3-specific)	7.0	45	Manufacture of cheese and butter flavors
<i>Mucor miehei</i> (1,3-specific)	7.5	50	Acceleration of cheese ripening; enzyme-modified cheese
<i>Rhizopus delemas</i> (1,3-specific)	5.6	35	Soap manufacture
<i>Candida cylindracea</i> (nonspecific)	7.5	50	–

Lipases and esterases (from the bovine or caprine abomasum) are normally used for flavor development in specialized European hard cheeses such as Romano, Provolone, and Feta, as well as processed cheese, or to accelerate cheese ripening. Lipase-modified butter fat releases butyric, caproic, caprylic, capric, and long-chain fatty acids. These flavors are utilized in confectionery (toffees and caramels) and in coffee whiteners to give a creamy flavor. Not all microbial lipases are specific, and this type catalyzes reactions at all the positions of glycerol. These are used as catalysts for the interesterification of triglyceride mixtures, producing a random distribution of fatty acid groups. Some lipases show regiospecificity; that is, they catalyze reactions selectively at either the 1- or the 3-position of glycerol, which is very useful in commercial applications. Microbial lipases also show stereospecificity in reactions with esters of many types, but no true stereospecificity in reactions with triglycerides has been detected to date. The reaction systems used for interesterification of triglycerides consist of a lipase catalyst and a limited amount of water dispersed in organic solvents (nonaqueous media) such as hexane. By restricting the amount of water in the reaction system, hydrolysis is limited, and interesterification becomes the dominant reaction.

Catalase (hydrogen peroxide oxidoreductase, EC 1.11.1.6) is an oxidoreductase that decomposes hydrogen peroxide to water and oxygen. Catalase has been found in almost all aerobic organisms, in some anaerobes, and in plant and animal cells. Catalases have been purified from many sources and resemble one another with respect to molecular weights ($\approx 225\text{--}270$ kDa). Catalase has been used in food, pharmaceutical, and textile processing to remove hydrogen peroxide in bleaching. Biologically this enzyme plays an important role in cellular metabolism, namely, in freeing a living cell from the toxicity of hydrogen peroxide. The principal application of catalase in the food industry is in connection with cold sterilization of milk by hydrogen peroxide. In many undeveloped countries where cooling facilities are not available on farms, heat pasteurization is not technically feasible. Thus to preserve milk, 1 mL of 33% hydrogen peroxide is added at the farm to each liter of milk, followed by another milliliter of at the dairy plant, where the milk is heated to 50 °C for 30 min. After cooling to 35 °C, catalase is normally added to destroy the residual peroxide. In the United States and Europe, hydrogen peroxide treatment instead of heat treatment of milk is permitted for cheese making. It is very effective in reducing the population of pathogenic microorganisms.

5.1.3.3 Fruit and vegetable processing Pectinases have been used on a trial-and-error basis to clarify fruit juices and fruit juice concentrates. Since the 1960s, the structure of the enzyme substrates and the mechanism of enzyme mechanism have been unraveled, and these enzymes have been used directly in fruit juice processing. The pectic substances include a multitude of complex and variable compounds, with polymers made up of a linear chain of α -1,4-linked galacturonic acid having more than 50% of its carboxyl groups esterified with methanol (called *high methoxyl pectin*). These highly esterified pectins yield semisolid gels with sugar and acid of the kind that are present in jellies and jams.

The pectic enzymes are classified on the basis of their action toward the galacturonan part of pectin molecules. Two main groups are pectin methylesterases, which hydrolyze the methyl ester linkages, and pectin depolymerases (polygalacturonase, pectin lyase, pectate lyase). Pectin esterases remove methoxyl groups from methylated pectin, yielding the formation of methanol and low methoxyl pectin. In addition, pectin esterases act preferentially on methoxyl groups of esterified galacturonic acid molecules. Polygalacturonase exists in two forms (endo and exo), and both act randomly, only on glycosidic linkages between galacturonic backbones. Exopolygalacturonase releases small fragments from

the reducing end of polysaccharide chains and thus does not reduce greatly the viscosity, but the endo type can cause rapid depolymerization, and so reduce viscosity.

Pectin lyase acts on glycosidic bonds between galacturonic acid molecules that are esterified or methylated. Pectate lyase splits glycosidic linkages between nonmethylated galacturonic acid molecules low in methoxyl pectin or pectic acid. Pectin enzymes occur widely in many plants and microorganisms. The commercial pectinases are derived from fungi, namely, *A. niger* or *Aspergillus wentii* and *Rhizopus* species. Both surface and submerged cultures are used, and fed-batch cultures of *A. niger* in 2% sucrose and 2% pectin run normally for 60–80 h at pH 3–4 and 37 °C. Mixed pectinases are used primarily to clarify fruit juices and grape must, to macerate vegetables and fruits, and to extract olive oil. Cellulases and hemicellulases derived from *A. niger*, *Trichoderma viride*, and *Trichoderma konongii* have been used to treat citrus products prior to concentration and for the extraction of flavor materials.

5.1.3.4 Technical and other enzymes

Food and feed digestive enzymes Enzymes can be used to improve the nutritional quality of food for humans and animals. The full utilization of the potential nutritive value in legume- and soy-based foods is limited by the presence of non-digestible flatulence sugars such as raffinose and stachyose, causing gassing discomfort. As mammals do not produce the enzyme, α -galactosidase, this enzyme is used to convert stachyose and raffinose to simple sugars that are adsorbed by the human digestive tract, thereby preventing the flatulence often caused by legumes (Yamaguishi et al., 2009). People without enough α -galactosidase can also experience a very serious condition known as *Fabry's disease*, which can later lead to kidney malfunction and an increased risk of heart disease (Waldek et al., 2009).

Other feed enzymes are mostly for (i) NSP (nonstarch polysaccharide) such as xylanase, cellulase, glucanase, mannanase, and pectinase; (ii) phytase; and (iii) protease. Another enzyme important to reduce phosphorous in animal feed is phytase. Poultry and hog feed grains contain phosphorous which is bound to phytic acid. In this form, the phosphorous is not available to the animals and is excreted in the animal's waste. Since these animals, similar to humans, need phosphorous for bone growth and other biochemical processes, the feed suppliers normally add extra phosphorous to the diet. A specific enzyme, phytase releases the bound phosphorous, making it digestible to the chicken or hog. Phytase added to the feed eliminates the need for compensating levels of phosphorous and thus dramatically reduces the phosphorous content of the animal waste.

The beneficial effects of using xylanase (hemicellulase) enzyme preparation in major plant cell walls of barley, wheat, rye diets are due to its effect on reducing digesta viscosity by hydrolysis of xylan, decrease of their viscosity and also release xylose-oligosaccharides that will increase intestinal microflora, that will improve protein digestibility (Khattak et al., 2006). The overall effect is to improve feed utilization and develop a healthier digestive system for monogastric animals.

Enzymes for biofuels production Ethanol fuels can be derived from renewable resources from agricultural crops (corn, sugar cane, and sugar beet or from agricultural by-products such as whey from cheese making and potato processing waste streams. Ethanol can be used as a 100% replacement for petroleum fuels or as an extender. Ethanol can also be utilized in petroleum fuels as a replacement for the toxic oxygenate, methyl *t*-butyl ether (MTBE). Enzymes such as α -amylase, glucoamylase, invertase, and lactase hydrolyze starch, sucrose, and lactose into fermentable sugars. The sugars are then fermented with yeast to produce ethanol.

The shift from food crop feedstocks to waste residues, native switchgrass, and seaweed offers significant opportunities. The current best available technology for conversion of cellulosic biomass employs an acid hydrolysis of the biomass into sugars. The enzymatic alternative, using cellulase and hemicellulase, avoids the use of strong acids and results in a cleaner stream of sugars for fermentation and fewer by-products. Although a few companies around the globe are already producing cellulosic ethanol, cellulosic ethanol commercialization faces significant challenges. Capital and operating costs are expected to remain higher than those of corn ethanol producers, and even they are currently struggling with low margins. The pretreatment of cellulosic biomass and the chemistry/physics are formidable obstacles working against the success of cellulosic ethanol. It is unlikely mass-produced more cheaply than corn ethanol, and that financial troubles of industries are well-documented lately (Energy Trends Report, March 19, 2013).

Technical enzymes – textiles, leathers, and pulp/papers Textile processing has benefited greatly on both environmental and product quality aspects through the use of enzymes. Before the weaving of yarn into fabric, the warp yarns are coated with a sizing agent to lubricate and protect the yarn from abrasion during weaving. The main sizing agent used for cotton fabrics has been starch because of its excellent film-forming capacity, availability, and relatively low cost. Before the fabric can be dyed, the applied sizing agent and the natural noncellulosic materials present in the cotton must be removed. Before the discovery of amylase enzymes, the only alternative to remove the starch-based sizing was extended treatment with caustic soda at high temperatures, and this resulted in inefficiency and in degradation of the cotton fiber. Traditionally, to get the look and feel of stonewashed jeans, pumice stones were used. Using cellulase enzymes, the jeans industry can now reduce and even eliminate the use of stones. Enzymes give the manufacturer a newer, easier set of tools to create new looks (Cavaco-Paulo and Gubitz, 2003). The pumice stones used to “stonewash” the denim clothes can also over abrade or damage the garment. By using enzymes, the manufacturer can give consumers the look they want, without damaging the garment.

The conventional way to remove hair from hides for leather tanning (dehairing, bating) is to use harsh chemicals such as lime and sodium sulfide. These chemicals completely dissolve the hair and open up the fiber structure. With enzyme-assisted dehairing, it is possible to reduce the chemical requirements and obtain a cleaner product and a higher area yield with fewer chemicals in the wastewater. Since the enzyme does not dissolve the hair as the chemicals do, it is possible to filter out the hair, thus reducing the chemical and biological oxygen demand of the wastewater. Additionally, the hides and skins contain proteins and fat between the collagen fibers that must be all or partially removed before the hides can be tanned. To make the leather pliable, an enzymatic treatment before tanning is necessary to selectively dissolve certain protein components that are called *bating*. Traditionally, dog or pigeon dung was used as the bating agent, but the leather industry is using bacterial proteases and pancreatic trypsin.

Various types of chemical products are used to provide properties such as printability, color, strength, softness, and durability to paper and paperboard. In helping papermakers to reduce their chemical load such as chlorine oxidants to bleach pulp and make white paper, enzymes can be used to reduce the use of harsh chemicals. Hemicellulase enzymes such as xylanase can enhance the bleaching efficacy, allowing a dramatic reduction in the consumption of chlorine. The enzymatic treatment opens up the pulp matrix allowing better penetration of the bleaching chemicals and better extraction or washout of lignin and the associated dark brown compounds. The basic technology to recycle waste paper is a relatively straightforward process by repulping and cleaning, and made into new paper. The

majority of the fillers and binders used in the original paper can be easily extracted during reprocessing, but the residual printing inks and adhesives are the most difficult to remove. Rather than using the traditional chemicals, enzymes such as cellulase and hemicellulase are able to hydrolyze some of the linkages that entrap the ink.

Medical and pharmaceutical applications The medical and pharmaceutical enzymes cover a wide range of applications. In contrast to industrial uses where production is on a much larger scale, medical and pharmaceutical enzymes generally require small quantities of highly purified enzymes. Enzymes and enzyme-generated products are administered to patients in very small doses to avoid possible side effects. The first applications are on biosensors on analytical tests to detect and measure amounts of glucose in blood. The amount of glucose in the blood and urine is a crucial indicator in the diagnosis of diabetes. It is normally detected by the enzyme glucose oxidase, which is impregnated onto a strip of paper, and a biosensor. The enzyme catalyses the reaction between glucose and oxygen to form gluconic acid. The biosensor then uses the amount of gluconic acid produced to indicate the quantity of glucose and oxygen in the blood indicated by a color change. Enzymes are also very important when diagnosing diseases found in the liver. By testing the blood for alternate enzyme activity, liver damage can be confirmed. Enzymes are vitally important in preventing excessive blood clotting and reducing the tendency for platelets and red blood cells to “clog.” Trypsin and chymotrypsin can be used in fibrinolysis; this is a process that dissolves blood clots. One use is in the case of thrombosis; this is when blood clots form in damaged blood vessels. If these clots are carried to a small artery they may block the heart leading to a heart attack or a stroke can be caused. This can be treated by enzymes such as trypsin and protease. Digestion of the insoluble fibrin clot takes place and the enzymes convert to amino acids, thus freeing the trapped blood cells and eliminating the clot. In addition to aiding the prevention of clotting, the enzyme protease can be used as a debriding agent to clean wounds and accelerate the healing process.

Another example is the lactose-intolerant people who require the lactase enzyme to hydrolyze lactose. Enzymes can also be used to aid digestion where they are used to supplement amylase, lipase and protease produced mainly by the pancreas. Other enzyme application in medicine is the production of antibiotics, in particular, penicillin. The major pharmaceutical products produced using enzyme technology are medical enzymes such as penicillin G acylase to make semisynthetic antibiotics, amine transaminase to synthesis sitagliptin for the treatment of type 2 diabetes mellitus (T2DM) (Bornscheuer et al., 2012). Only a small number of enzyme application in medicine has been mentioned, but many significant advantages are in the treatment of genetic defects, the development of artificial organ function, neoplasm, anti-inflammatory reagents, drug manufacture, the removal of chiral components, etc. Some important therapeutic enzymes are listed in Table 5.4 (<http://www.lsbu.ac.uk/water/enztech/medical.html>).

A major potential therapeutic application of enzymes is in the treatment of cancer, among which asparaginase has proved to be particularly promising for the treatment of acute lymphocytic leukaemia. As tumor cells are deficient in aspartate-ammonia ligase activity, which restricts their ability to synthesize the normally nonessential amino acid L-asparagine, they are forced to extract it from body fluids. The action of the asparaginase does not affect the functioning of normal cells, which are able to synthesize enough for their own requirements, but reduce the free exogenous concentration and thus induces a state of fatal starvation in the susceptible tumor cells. The enzyme is administered intravenously and it is effective only in reducing asparagine levels within the bloodstream, showing a half-life of about a day (in a dog). This half-life may be increased 20-fold by use of polyethylene glycol-modified asparaginase.

Table 5.4 Few important therapeutic enzymes in medicine

Enzyme	EC number	Reaction	Use
Asparaginase	3.5.1.1	L-Asparagine $\text{H}_2\text{O} \rightarrow$ L-Aspartate + NH_3	Leukaemia
Collagenase	3.4.24.3	Collagen hydrolysis	Skin ulcers
Glutaminase	3.5.1.2	L-Glutamine $\text{H}_2\text{O} \rightarrow$ L-Glutamate + NH_3	Leukaemia
Hyaluronidase*	3.2.1.35	Hyaluronate hydrolysis	Heart attack
Lysozyme	3.2.1.17	Bacterial cell wall hydrolysis	Antibiotic
Rhodanase†	2.8.1.1	$\text{S}_2\text{O}_3^{2-} + \text{CN}^- \rightarrow \text{SO}_3^{2-} + \text{SCN}^-$	Cyanide poisoning
Ribonuclease	3.1.26.4	RNA hydrolysis	Antiviral
β -Lactamase	3.5.2.6	Penicillin \rightarrow penicilloate	Penicillin allergy
Streptokinase‡	3.4.22.10	Plasminogen \rightarrow plasmin	Blood clots
Trypsin	3.4.21.4	Protein hydrolysis	Inflammation
Uricase§	1.7.3.3	Urate + $\text{O}_2 \rightarrow$ allantoin	Gout
Urokinase¶	3.4.21.31	Plasminogen \rightarrow plasmin	Blood clots

*Hyaluronoglucosaminidase.

†Thiosulphate sulfurtransferase.

‡Streptococcal cysteine proteinase.

§Urate oxidase.

¶Plasminogen activator.

5.1.4 New developments and protein engineering

The most important future research on enzymes would include (i) the use of nonnatural catalytic properties of enzymes, (ii) elucidation of molecular mechanisms of enzyme inactivation, (iii) enzyme catalysis in organic solvents and other extreme environments, and (iv) use of protein engineering to tailor catalytic or other properties of enzymes. Enzyme immobilization, enzyme catalysis in organic solvents, and recombinant DNA technology are discussed elsewhere. The application of recombinant DNA technology and protein engineering will significantly improve the production of commercial enzymes in the near future, and already many recombinant enzymes (chymosin, amylase, lipase, protease, phytase, etc.) are appeared on the market, but it is not easy to know which are the wild type or recombinant ones, as most of them are purified and no declaration would be required.

Protein engineering is a science in which many disciplines, such as biology, protein chemistry, structural chemistry, and enzymology attempt to define and exploit the relationship between enzyme structure and catalytic function. Because current enzymes and microorganisms used in food processing are not ideal catalysts, it would be desirable to redesign enzymes to obtain enhanced stability, improved catalytic properties, improved enzyme activity, and so on, to suit the specific requirements of food processes. The technique of protein engineering, more specifically termed *in vitro mutagenesis* or *site-directed mutagenesis*, involves the alteration of one or a small number of known amino acids in a protein through the use of short oligonucleotide primers coding for the change that permits modification of catalytic or other properties. This approach may also be applied to other proteins to modify antigenicity, digestibility, nutritional value, and so on.

The site to be mutagenized is first subcloned as a single-stranded M13 phage template. A synthetic oligonucleotide coding for the change is annealed, and then the rest of the sequence is filled in by a DNA polymerase. The final double-stranded form can be transformed into a recipient strain, the mutant clones are identified, and then the mutant fragment is introduced into the original construction. When oligonucleotide-directed mutagenesis fails to predict the changes in properties due to amino acid replacement

by a specific mutation, it is often more satisfactory to randomly mutagenize the whole structural gene or a portion thereof by chemical mutagenesis – for example, by means of sodium bisulfite, hydroxylamine, or methoxylamine. The success of this method depends on screening techniques used to identify the mutations that result in the desired properties. Both approaches should be used in parallel, since regions of the molecule identified by random mutagenesis may serve as targets for further rounds of site-specific modification. Eventually site-directed mutagenesis will replace tedious traditional mutation procedures, but this technique requires recombinant DNA technology and also needs detailed knowledge of the three-dimensional structure of the enzyme. Since, a great deal of research effort is required to obtain such information, this technique is an expensive undertaking, and only improvements worthy enough to justify heavy research and development (R&D) expenditures should be begun. Computer-aided molecular modeling can provide a three-dimensional image of structures, allowing their manipulation in real time and the visualization of molecular parts.

Some protein engineering techniques are listed in Figure 5.2. In one example of potential commercial success, a detergent protease, *subtilisin* from *Bacillus subtilis*, was modified by site-directed mutagenesis (Wells and Estell, 1988). A surface methionine residue at position 222 is sensitive to oxidation by bleaches in the detergent powders. The modification that allows the enzyme to be used in detergents was achieved by substitution of the met-222 residue. Changing a single amino acid, aspartic acid to serine at position 99 in *Bacillus amyloliquefaciens*, also led to an alteration in the pH optimum.

Those enzymes derived from extremophilic microorganisms are especially desirable for improving laundry processes in hot water cycles and/or at low temperatures for washing colors and darks. They are also useful for industrial processes where high temperatures are required, or for bioremediation under harsh conditions (e.g., in the arctic). Recombinant enzymes (engineered proteins) are being sought using different DNA technologies such as site-directed mutagenesis and DNA shuffling. Excellent review on protein engineering can be found elsewhere (Figure 5.3) (Bornscheuer et al., 2012; Woodley, 2013).

Despite the large number of successful demonstrations of protein modification in model systems, only three examples of food enzymes, namely, glucose isomerase, β -galactosidase and papain, have been described so far. The three-dimensional structures for the enzymes characteristic of *Streptomyces*, *Actinoplanes*, and *Arthrobacter* have been determined; the active site of histidine has been identified, and its mode of substrate and analogue binding

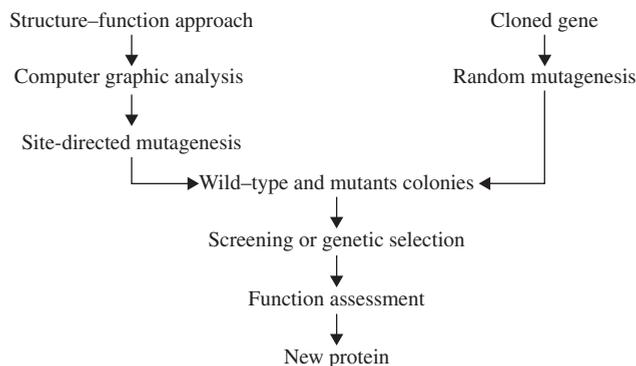


Figure 5.3 A schematic classification of protein engineering techniques.

has been deduced. The objectives were to improve thermostability and enzyme activity at a lower pH, as well as to modify surface lysine residues. The substitution of arginine for lysine at position 253 significantly improved the resistance of this enzyme to thermal denaturation and also produced an improved half-life of the enzyme under the HFCS process conditions (Henrick et al., 1987).

Replacing a glutamic acid residue with lysine was also achieved by inserting the *xylA isomerase* gene into a kanamycin-resistant plasmid followed by *in vitro* mutagenesis. Glucose isomerase: insights into protein engineering was also studied for increased thermostability (Hartley, et al. 2000).

The enzyme papain was also modified to alter the function of its activity. Moreover the properties of fermented food products have been improved by metabolic pathway engineering. In one case, increased shelf life for yogurt was achieved by engineering a cold temperature and low pH-sensitive β -galactosidase from *Lactobacillus delbrueckii bulgaricus* (Adams et al., 1994).

5.1.5 Economics

With the exception of glucose isomerase, a majority of food enzymes are extracellular hydrolytic enzymes that degrade polymers such as starch, proteins, pectins, and cellulose. The cost of the enzyme process relative to other processes is the single most important factor in determining whether enzymes will be selected for use in food processing. To be acceptable in this application, enzymes must provide an economic advantage over other methods. Economic considerations also include the cost of producing the enzyme from animal, plant, or microbial sources, purification of the enzyme, standardization of enzyme activity, and possibility to store in a stable form. Key factors driving the market growth include new enzyme technologies endeavoring to reduce costs and increase productivity. Other factors propelling market growth are the production of enzymes related to textile, animal feed, detergent, bioethanol, cosmetics, and pharmaceutical industries.

Summary

Major enzymes can be divided into four areas: (i) technical enzymes (33% market) for starch, textile, leather, pulp/paper, and fuel ethanol; (ii) detergent enzymes (32% market): detergent and household enzymes; (iii) food enzymes (23% market): baking, brewing, beverages alcohol, juice, wine, dairy, oils/fats, and (iv) feed enzymes (12% market), but a fast-growing (~10%) segment of feed additives, particularly in Asia (China, India), due to growing meat (poultry and swine) consumption. Other applications of enzymes for the industries of biomedical, organic synthesis, and cosmetics can be found elsewhere (Li et al. 2012). Presently, immobilized proteins/enzymes are used routinely in the medical field, such as in the diagnosis and treatment of various diseases (Liang et al., 2000).

Enzyme technology is going through a phase of maturation. This is evident in the development of the theory of enzyme function and how this relates to the primary structure of enzymes through the evolution and configuration of their three-dimensional structure, as seen in the ever-broadening range of enzymatic applications. There still remains much room for the development of useful processes and materials based on this understanding. New enzymatic processes such as immobilization are being developed, and new applications are being devised for currently available commercial enzymes. Enzymes also work in organic solvents. New enzymes are being produced by classical methods and by redesigning the enzymes by means of protein engineering to produce better enzymes.

Genetic engineering offers great promise for the future. Recently cell-free biology, which uses crude cell lysates and capitalizes on both synthetic biology and chemical engineering tools, provides another growth area for biocatalysis. Also a future possibility is to create artificial metalloenzymes, composed of a metal complex and host protein in combining the advantages of a catalytic metal complex with those of an enzyme. However, it is important to recognize that protein engineering methods take a long time to develop and R&D costs are expensive.

5.2 Sweeteners

5.2.1 Introduction

Although historically the sense of sweet taste has directed humans to nutritive substances, in modern times most Western concerns about obesity and related illnesses are partly attributed to excess calorie consumption. Consequently, an industry has developed around the production of sweeteners that are low in calories or noncaloric altogether. The sweeteners that provide calories to humans, called *high nutritive sweeteners*, are sucrose, fructose, corn syrups, molasses, other carbohydrates, and polyhydric alcohols. Those that do not provide calories to humans (saccharin, cyclamates, aspartame, acesulfame-K, etc.) are categorized as high-intensity sweeteners. Other high-intensity sweeteners that have limited application in foods at this time are stevioside and thaumatin. Four low-calorie sweeteners that provide functionality beside low sweetness in foods, and for which approved products are maltitol, lactitol, isomalt, fructo- and galacto-oligosaccharide, and other oligosaccharides. Biotechnology has had its impact on developments in the nutritive sweeteners market, which was derived from cane and beet crops as well as glucose syrups developed through enzyme technology. Alternative nonnutritive sweeteners may be produced by plant culture, enzymatic synthesis, or recombinant DNA technology. The properties and recent development of nonnutritive sweeteners are well discussed (Shankar et al., 2013).

5.2.2 Nutritive sweeteners

5.2.2.1 Invert sugar Invert sugarcane and beet sugar can be converted to a 1:1 mixture of glucose and fructose either by acid or by enzyme (invertase) hydrolysis. Sweeter than sucrose, invert sugar is used in the confectionery industry to prevent the crystallization of sucrose; it can be used in cream centers, fudge, marshmallows, caramels, chewy candies, and so on. Invert sugar has stability at low pH, enhances the flavors of fruits, and acts as a humectant. Invert sugar is also used in pharmaceutical products such as cough syrup for enhancement of flavor, consistent viscosity, and shelf life. However, the market has decreased, because of the development of HFCS. Invertase is currently produced by baker's yeast, *Saccharomyces cerevisiae*, and is available in powder, liquid, and immobilized forms.

5.2.2.2 Dextrose and corn syrups Dextrose and corn syrups, hydrolysis products of starch, are called corn sweeteners in the United States. In Europe, where they may be made from potato or wheat starch as well, they are known as *glucose syrups*. Dextrose is the common name for D-glucose, which is liberated by the complete enzymatic hydrolysis of starch. Corn and related syrups are described mainly by the term *dextrose equivalent (DE)*, which is defined as the percentage of reducing sugar calculated as glucose on a

dry weight basis. This is an indicator of the degree of hydrolysis. Corn syrups provide sweetening and viscosity, control humectancy, and impart a desirable texture to food, beverages, and confectionery products. Until 1960, dextrose was produced commercially by acid hydrolysis of starch at elevated temperature; as a result of degradation products, however, the dextrose yield was about 86%. Formation of acid reversion products during liquefaction limited the dextrose yield. Higher dextrose yields were achieved by the enzyme process in 1960. This process utilized a thermostable bacterial α -amylase derived from *B. subtilis* for starch thinning and resulted in dextrose yields of 95–97%. In this process, starch (30–40 wt%) is thinned at 85 °C for 1 h, followed by 5–10 min at 120–140 °C. Then a second enzyme is added at 85 °C to complete the liquefaction step. The resulting 10–15 DE hydrolysate is then saccharified with glucoamylase. Elimination of the heat treatment step was achieved by the discovery of extremely thermostable α -amylases derived from *Bacillus stearothermophilus* and *B. licheniformis*, which are capable of efficient starch hydrolysis above 100 °C. In this process, starch slurry (30–40 wt%) is liquefied continuously with a steam injection heater at pH 6.0–6.5 and 103–107 °C for 5–10 min. A second stage of liquefaction is then achieved at about 95 °C for 1–2 h to complete the liquefaction (Figure 5.4). α -Amylase continues to liquefy starch up to 1 h (*B. licheniformis* amylase) or 2 h (*B. stearothermophilus* amylase) at 105 °C with little decrease in activity.

Dextrose syrup is finally produced by the action of glucoamylase during saccharification (Figure 5.5). Glucoamylase is derived from strains of *A. niger* or *Rhizopus* species by surface fermentation, but the enzyme properties of *Rhizopus* are somewhat inferior to those of *Aspergillus*. The saccharification of liquefied starch hydrolysate is usually carried out by batch or continuous process in a large reactor (58–61 °C, pH 4.0–4.5) in 1–4 days. The dextrose yield from starch slurry (30–35 wt%) is about 95–96%. If the saccharification reaction is extended beyond the normal time, the level of dextrose decreases and isomaltose and maltose are produced as a result of a reverse reaction by glucoamylase. The dextrose yield may be increased by conducting saccharification at a lower solid level (10–12 wt%) and by using enzymes such as pullulanase and transferase to enhance the action of glucoamylase, resulting in increases of dextrose yield by 0.5–1%. The high dextrose hydrolysate is clarified by centrifugation or filtration to eliminate insoluble matters. Decolorization and removal of trace impurities may be achieved by carbon treatment or ion-exchange resins. The decolorized syrup is then evaporated to 50–55% solids and may be given a second carbon treatment. Commercial dextrose

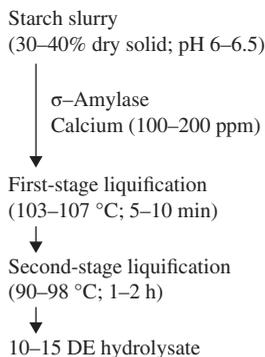


Figure 5.4 Production of 10–15 DE syrup by thermostable α -amylase.

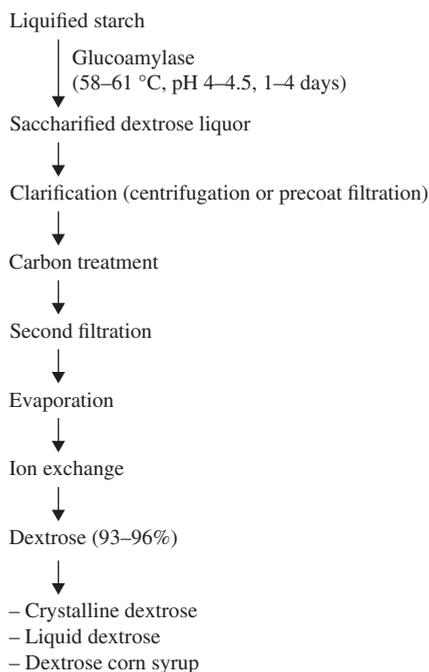


Figure 5.5 Process flowchart for dextrose production.

products include crystalline monohydrate or anhydrous dextrose, liquid dextrose, and dextrose corn syrup. The expensive dextrose crystallizers and high production costs have caused dextrose prices to increase to levels above those of HFCS and corn syrup. Worldwide prices in 1988 were about \$1.12/kg for anhydrous dextrose, \$1.21/kg for USP (United States Pharmacopeia)-grade anhydrous dextrose, \$0.51 for monohydrate dextrose and \$0.21 for corn syrup. Dextrose syrups can be directly used as food ingredients and sweeteners in the food-processing industry – for example, in soft drinks in conjunction with sucrose, fruit juices and thirst quenchers – and in the confectionery industry to supply softness and crystallization controls. Dextrose syrups may also be used in canning, in ice cream to minimize crystallization, in baking as a humectant, in fermentation, in jam and jellies, in pickles and sauces, and in meat products.

5.2.2.3 High fructose corn syrups (HFCS) If the starch slurry contains more than 0.3% total protein, the syrup produced has a greater potential to develop color as a result of the Maillard reaction despite treatment with carbon or ion-exchange resin. Such a development was a necessary precursor to the introduction of HFCS or isoglucose (glucose–fructose mixtures). HFCS is basically a concentrated solution containing mainly fructose and dextrose with lesser quantities of higher molecular weight saccharides. HFCS containing 42% fructose is used in most food products that contain a liquid sweetener. The development of second-generation HFCS or ultra high fructose glucose syrup (UHFGS) containing 55% fructose greatly extended the use of HFCS in many applications. HFCS is

produced by partial enzymatic isomerization of dextrose hydrolysates, followed by refining and concentration. The commercial production of glucose isomerase from *Arthrobacter cloacea* or *Streptomyces rubiginosus* was a major milestone in the corn sweetener industry. The isomerization process involves the direct addition of an isomerase enzyme, which converts the glucose substrate in a batch system. The fructose/glucose syrup (42%/58%) is regarded as being as sweet as sucrose, and thus the term *isosweet* was used for the first commercial 42 DE HFCS material. Three commercial HFCS products contain 42%, 55% and 90% fructose.

Enzymatic conversion of dextrose to fructose with glucose isomerase was first reported in 1957 and was patented in 1960 by the Corn Products Company in the United States. Japanese developments of *Streptomyces* glucose isomerase moved the technology to the first commercial production of HFCS in 1967 in the United States by a batch process. Before the product could become a serious competitor to sucrose and invert sugar, however, a number of problems had to be dealt with, including high enzyme cost, the formation of colored products, the production of nonmetabolizable sugar (D-psicose), and enzyme inhibition by minerals, especially calcium ions. In 1972, a continuous system that allowed reuse of the enzyme was initiated using enzyme immobilization. This continuous process for HFCS production using glucose isomerase was the first large-scale industrial use of immobilized enzymes. The process resulted in a significant reduction of production costs, to levels competitive with the production of sucrose.

The early process involved the immobilization of heat-treated whole cells or cell-free enzymes on various supports such as adsorption on insoluble matrices (ion exchange and cellulose) or entrapment in insoluble matrices (cellulose, collagen, alumina, etc.). The Novo Industry system of covalent bonding and the Corning Glass Company are currently licensed. A schematic diagram of the HFCS process is shown in Figure 5.5.

The first syrup, marketed by the Corn Processing Company, was called *Isomerase 30* (first 30% fructose; later, 42% fructose). HFCS (42%) is produced from dextrose hydrolysate that has been clarified, carbon-treated, ion-exchanged, and evaporated to 40–50 wt% (dry basis). Magnesium is needed as a cofactor to maintain isomerase stability and to prevent inhibition by trace amounts of residual calcium. The typical immobilized glucose isomerase process operates with the enzyme encapsulated in 1–2 mm particles in a packed-bed column reactor (1.5 m diameter \times 4–5 m height). Hydrolysate is normally passed through a fixed bed of immobilized isomerase at a controlled flow rate. Process conditions are generally 55–65 °C, pH 7.5–8.5 and 1 h or less reaction time. Such a system would contain up to 4000 kg of enzyme and would produce about 5×10^5 kg of fructose (dry weight) per day.

Although HFCS 42 is useful in some applications, many users prefer HFCS with a higher level of fructose. Thus, the second-generation (55 wt%) HFCS, which aims at direct replacement of the liquid invert sugar used in the food and beverage industry, appeared in 1976. To produce HFCS 55, fructose is removed by chromatographic separation from HFCS 42, and the enriched fructose (90% plus 10% glucose) is blended with HFCS 42 until the fructose level is about 55%, producing HFCS 55. Raffinate containing about 87% glucose, with 5% fructose and 8% oligosaccharides, is recycled to isomerization columns for production of HFCS 42. To remove contaminants, HFCS 55 is purified by means of a column of activated charcoal, then concentrated by vacuum drying to 70–86% dry solids as an essentially noncrystallizable syrup. In addition, further enriched product, containing more than 90% fructose, is produced by fractionation (crystallization). This highly enriched product is called *very enriched fructose corn syrup* (VEFCS) or UHFS. The 55% HFCS may replace up to 100% sucrose, especially in the soft drink industry, while the 90% HFCS is limited to low calorie foods because of its high sweetness (Figure 5.6).

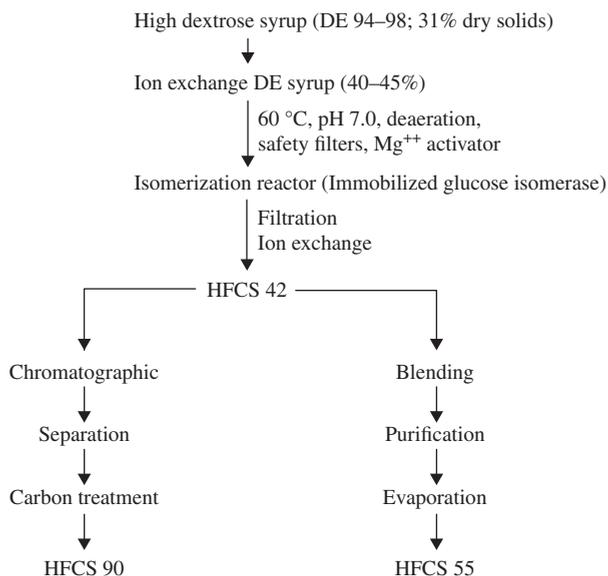


Figure 5.6 Schematic diagram of the production of HFCS 55 and HFCS 90 from high dextrose syrup.

5.2.3 High-intensity sweeteners

5.2.3.1 Saccharin and cyclamates The discovery of intensive sweeteners was a matter of chance, though major research efforts were carried out in some cases. The first of the intensive sweeteners was saccharin, discovered by Fahlberg in 1878. This compound, which exhibited a sweetness potency significantly (300 times) greater than that of sucrose, was an unexpected result of the oxidation of α -toluenesulfonamide. However, it was not derived from the application of biotechnology. At higher concentration, the taste is sour and bitter, and thus saccharin was often used in combination with other nonnutritive sweeteners such as cyclamate. This blend was very popular among diabetics and the obese, until the appearance of the safety issue of saccharin and the cyclamate ban of 1969 for possible bladder cancer in rats. Saccharin and aspartame blends are currently in use. The risk of saccharin is now recognized to be more a function of dose than an all-or-none phenomenon. Saccharin is extremely stable in any food applications and is a very cheap sweetener; the wholesale price per pound was \$2.75 in 1989. It is marketed under the brand names Sweet'N Low[®], Sugar Twin[®], and Necta Sweet[®] and is mostly used in soft drinks, baked goods, jams, canned fruit, candy, salad dressing, dessert toppings, and chewing gum besides tabletop sweetener. The major advantages of saccharin are that the sweetening power is not reduced when heated, and it does not get metabolized in the gastrointestinal (GI) tract, thereby not affecting the insulin levels (Mukherjee and Sarkar, 2011). Acceptable daily intake (ADI) of saccharin is set at 5 mg/kg body weight for adults and children (about 8.5 tabletop sweetener packets each day), and it is unlikely that consumption will exceed these limits.

Cyclamate is the generic term for the cyclohexylsulfamates, discovered in 1937 at the University of Illinois. Cyclamate is almost invariably used as either the sodium or calcium salt, each of which is a noncaloric, white crystalline powder. The cyclamates readily dissolve in water and are extremely stable for use in all food applications. Cyclamate is about 30 times as sweet as sugar. A 10:1 blend of cyclamate and saccharin provides a

synergistic sweetening effect, while masking the off-taste problems inherent in the use of either product separately. Cyclamate is still used in more than 50 countries in tabletop sweeteners, soft drinks, salad dressings, confections, and processed fruits. In the United States, cyclamate is currently used only in foods destined for export. Cyclamates were first approved in 1951 for the use of diabetics and others who need to restrict the intake of sugar. In 1958, they were approved as one of the GRAS substances by the US Food and Drug Administration (FDA). In 1969, however, on the basis of a study of the effects on rats of a cyclamate–saccharin blend, the FDA declared cyclamate as a carcinogen causing bladder tumors and banned its use in all foods and beverages sold in the United States. It is now generally accepted that cyclamates are not carcinogens, but the major concern from the standpoint of toxicity is related to the biological activity (hypertension and testicular atrophy) of cyclohexylamine, a major cyclamate metabolite. In Canada, cyclamates are approved for tabletop and a few other minor applications. At the price of \$0.54/pound, the use of sodium cyclamate in 1990 represents a very substantial saving over sucrose.

5.2.3.2 Aspartame Aspartame is a dipeptide methyl ester of aspartic acid and phenylalanine, known as *L-aspartyl-L-phenylalanine-1 methyl ester (APM)*. APM was accidentally discovered in 1965 by James Schlatter, a chemist for Searle & Company, while working on ulcer treatment drugs. Upon licking his finger to pick up a piece of paper, Schlatter detected a sweet taste. Aspartame is about 180–200 times as sweet as sucrose and has a clean taste, without the bitterness or metallic aftertaste characteristic of other intense sweeteners. In its flavor and taste profile, aspartame is indistinguishable from sucrose. It exhibits sufficient solubility for all food applications. However, it has hydrolytic instability in most food and beverage products, and it is not suitable for baking or other applications requiring extended periods of high temperature.

Aspartame is rapidly metabolized by the body into methanol and its two component amino acids (phenylalanine and aspartic acid). Since phenylalanine is a metabolic product, individuals who cannot metabolize it – that is people who have the genetic disease phenylketonuria, can be at risk. Intake of both amino acids greater than dietary requirements, and toxic methanol, may cause safety issues. However, the safety of aspartame has been studied more extensively than that of any known food additive, and in 1983 the FDA approved as the ADI up to 50 mg/kg body weight based on clinical studies.

Selected enzymes such as proteases, particularly thermolysin (a metalloproteinase from *Bacillus thermolyticus*) have been used in the synthesis of aspartame, instead of conventional chemical synthesis. This enzymatic synthesis allows the selective production of natural α -aspartame, which is sweet, and not β -aspartame, which is bitter. In the preferred aspartame synthesis, the aspartic acid is carbobenzoxyated to produce the benzyloxy-carbonyl-L-aspartate, and the phenylalanine is esterified to produce the phenylalanine methyl ester. The enzymatic coupling of these two compounds takes place at neutral pH in an aqueous or biphasic system to produce benzyloxycarbonyl-L-aspartyl-L-phenylalanine methyl ester, which precipitates together with D-phenylalanine methyl ester. The addition compound is further separated and racemized before being recycled. The hydrogenation of the separated benzyloxycarbonyl-L-aspartyl-L-phenylalanine methyl ester then splits off the benzyloxycarbonyl group, leaving only the sweet product, aspartame.

The gene aspartame may also be produced in microorganisms by recombinant DNA technology. The gene, a dodecanucleotide that specifies aspartylphenylalanine, was chemically synthesized, cloned into a plasmid, and expressed. This gene was produced as multiple repeating units of the aspartame dipeptide by cloning adjacent to a controllable tryptophan promoter. *Neotame*, the newest artificial sweeteners made by Nutrasweet is about 40 times sweeter than aspartame (8000 times sweeter than sugar) and is metabolized like aspartame.

5.2.3.3 Alitame *Alitame* (aspartyl alanine) is a dipeptide about 2000 times as sweet as sucrose developed by Pfizer and about 10 times sweeter than aspartame. It has high solubility in water, a clean taste, and better stability than aspartame. Alitame is currently marketed in some countries under the brand name *Aclame*. Unlike aspartame, alitame does not contain phenylalanine, and can thus be used by people with phenylketonuria. Alitame has approved for use in Mexico, Australia, New Zealand, and China. Similar two other dipeptide sweeteners (PS 99 and PS 100), esters of α -amino dicarboxylic acids and α -amino esters, were patented by General Foods in 1988. They are 2000–2500 times sweeter than sucrose. They are reported to have better stability at low pH and better heat stability than other dipeptide sweeteners. They have a clean sugar sweetness with no aftertaste.

5.2.3.4 Acesulfame-K Acesulfame-K was another accidental discovery, made while work was being carried on the reactions of fluorosulfonylisocyanate with acetylenes in the laboratory of Hoechst AG in 1967. This product, 5,6-dimethyl-1,2,3-oxathiazin-4(3*H*)-one-2,2-dioxide, was found to be sweet. After screening many similar products, the preferred product was found to be 6-methyl-1,2,3-oxathiazin-4(3*H*)-one-2,2-dioxide, which was deemed to have the best taste properties and the most commercial feasibility. The initial generic name, acesulfame, was later changed to acesulfame potassium salt, shortened to acesulfame-K, and is marketed by Hoechst under the brand name Sunette. Acesulfame-K is similar in structure to saccharin but is half as sweet. The sweetness is about 200 times that of sucrose at the 3% sucrose level, and some off-tastes (bitter, metallic) may be detected at higher concentrations. It has excellent stability, even at a pH of 3, and a storage temperature of 40 °F. Beverages containing this sweetener can be pasteurized, and the product is quite stable in baked goods. Combinations with other sweeteners such as sugar alcohol are useful in many applications, particularly in products requiring bulking agents for texture or viscosity. Despite inadequate and poor-quality toxicity tests, this sweetener was approved by the FDA in 1988 for use in dry beverage mixes, tabletop sweeteners, desserts and puddings, chewing gum, and dry products. No safety problems have been revealed in more than 50 international studies, and the FDA set an ADI of 15 mg/kg body weight.

5.2.3.5 Sucralose *Sucralose* discovered in 1976 was approved in 1998 by FDA for use as a sugar substitute in 15 food and beverage categories as the brand name, Spenda[®]. It has a taste profile similar to sugar and has no unpleasant aftertaste. It has excellent safety profile to be used for all population including pregnant and nursing mothers.

They are 600 times sweeter than table sugar, and it is made by selective substitution of chlorine for hydroxyl group on a sucrose core. It is found in more than 4000 products in the United States and used as a sweetener in more than 80 countries today. Sucralose taste is very similar to the table sugar; unlike table sugar, it does not promote tooth decay. Thus, it has been approved by the FDA for the nonpromotion of dental carries. As the safety tests showed no carcinogenic and no genotoxicity, it is considered to be safe for long-term use. However, sucralose may be a causative agent in triggering migraine headaches and in increased incidence of *inflammatory bowel disease* (IBD) (Qin, 2011, 2012).

5.2.3.6 Tagatose *Tagatose* is a new, naturally occurring sweetener present in only small amounts in fruits, cacao, and dairy products. It is a ketohexose also known as *D-tagatose*, that has a clean neutral taste, and it browns very well in baked goods. *D*-Tagatose has been successfully used in chocolate and chewing gum. Tagatose produces a lower glycemic response and virtually zero calories. This sweetener not only has physical attributes identical to that of sucrose, but also has the sweetness that is comparable to sucrose. *D*-Tagatose

was found to contain no toxic, carcinogenic, or teratogenic compounds or negative effects upon consumption. The brand name is PreSweet. Humans can easily tolerate up to 75 g of D-tagatose as long as it is equally distributed over three meals and could be used safely in products to assist with weight loss as well as diabetes. Although, the trials are still under way, human studies have suggested that tagatose could be the new potential antidiabetic drug because of its effects on postprandial hyperinsulinemia and hyperglycemia in individuals with T2DM (Lu et al., 2008).

Tagatose is commercially produced from galactose through an enzymatic process using β -galactosidase for hydrolysis of lactose which is hydrolyzed to glucose and galactose. The galactose is isomerized under alkaline conditions to D-tagatose by calcium hydroxide. The resulting mixture can then be purified and solid tagatose produced by crystallization. An enzymatic process from whey for D-tagatose with the brand name Nutrilatose (Italy) was considerably cheaper than the chemical process. In 2013, PepsiCo and Yoplait were interested in using tagatose and Damhert was considering as a long-term plan to build a tagatose plant in Belgium that could turn out 10,000 tons of tagatose per annum.

A new, simple method for the production of D-is yet to be discovered. A recombinant *Pichia pastoris* yeast strain secreting β -D-galactosidase from *Arthrobacter chlorophenolicus* was constructed and a recombinant L-arabinose isomerase from psychrotolerant bacterium *Arthrobacter* sp. 22c was tried (Wanarska and Kur, 2012). The combination of the recombinant yeast strain and the enzyme in one reaction mixture containing whey permeate resulted in the simultaneous hydrolysis of lactose. Other works on cloning and expression of D-arabinose isomerase from *Geobacillus pallidus* into *E. coli* and its application in D-tagatose conversion were also published (Zhang et al., 2013). The cloning and analysis of two totally different C4-epimerases (L-ribulose-5-phosphate 4-epimerase and C4-epimerase) from other microorganisms have also been suggested.

5.2.3.7 Thaumatin/stevioside and monk fruit Thaumatin is a mixture of sweet proteins extracted from the fruit of the West African plant, *Thaumatococcus daniellii*. Its sweetening powers had been known to native West Africans for centuries, but it was not introduced to the Western world until the early nineteenth century. Thaumatin is currently marketed under the Tate & Lyle trademark as Talin. Thaumatin is a single chain of 207 amino acids with eight disulfide bonds and a molecular weight of 22 kDa; it consists of two major protein components, designated thaumatin I and thaumatin II. These basic proteins, having a pI point of about 12, are extremely soluble in water. In addition, they are stable under heat treatment at 100 °C for a few hours and at a pH below 5.5.

On a molar basis, thaumatin is one of the most potent sweeteners ever found. Thaumatin has about 100,000 times the sweetness of sucrose on a molar basis potency, but exhibits about 2000–2500 times the sweetness of sucrose (10% reference) on a weight basis. The onset of sweetness is relatively slow, and a lingering aftertaste of licorice is observed. This sweetness profile differs so significantly from that of sucrose that thaumatin has only limited use in food applications as a sole sweetening source. It might be useful in multiple sweetener systems. Thaumatin has the effect of enhancing the flavor of foods in conjunction with monosodium glutamate (MSG) and nucleotides, and the San-Ei Chemical Company has introduced a food-enhancing product, called *Neo-San Mark* containing MSG, food acids, amino acids, 5'-nucleotides, and thaumatin. Thaumatin was approved in the United States as a flavor adjunct in chewing gum (not as a sweetener) and has been approved in many other countries. Great Britain approved the use of thaumatin in 1983 for drinks, dietary products, and foods except for baby foods.

Because thaumatins are derived from plants, the supply is uncertain and product quality is variable, and thus biotechnological alternatives are being investigated. Several

laboratories at the University of Kent (UK), Unilever (the Netherlands), and Beatrice Foods (USA) have undertaken to produce this protein by using genetically engineered microorganisms. Both *S. cerevisiae* and *K. lactis* could produce thaumatin intracellularly and could also secrete thaumatin after the addition of secretion signals to the recombinant vector. Thaumatin was also cloned into *E. coli* (Verrips et al., 1990).

Another plant extract, stevioside, is a glycoside composed of three glucose moieties and a diterpenoid aglycone from the leaves of *Stevia rebaudiana*. This diterpenoid glycoside is known as *stevioside*, and it is about 300 times as sweet as sucrose. Stevioside may simply be extracted from the dry leaves with water, clarified and crystallized. It is available in three different forms: crude extract, 50% purity, and 90% purity. The structure of stevioside was determined in the 1960s, and since that time at least eight sweet glycosidic analogues have been characterized. The most important one is rebaudioside A because of its improved taste profile obtained by incubation with starch and glucosyl transferase. Natural rebaudioside A and enzyme-modified natural diterpenoid glycosides are approved for use in foods in Japan, South Korea, China, Brazil, and Paraguay. It can be widely used in food, beverages, medicine, daily-used chemical, brew and cosmetics, and so on and can save 70% of cost than cane sugar. It is approved by the Ministry of Public Health and Ministry of Light Industry of China and the FDA of United States as a sweetener.

Stevioside is similar to sucrose in its sweetness profile, but it exhibits substantial bitter and cooling flavor attributes. Thus, stevioside is not likely to find many applications in foods unless it is used in combination with other sweeteners. It has a synergistic effect with aspartame, cyclamate, and acesulfame-K, but not with saccharin. Stevioside is very stable in acid solutions and at alkaline pHs up to 9. Research showed some effects on protecting people from hypertension, diabetes mellitus, obesity, heart diseases, tooth decay, and so on. Lo Han Kuo (or monk fruit), an ancient Chinese fruit is about 200 times sweeter than sugar. It is a better low calorie natural sweetener and it received the FDA GRAS status in 2009. The monk fruit extract is available commercially as a creamy, white color, natural, powdered concentrate. Commercial products are Fruits-Sweetness™ or PUREFRUIT™. The brand name Nectresse (a blend of monk fruit, erythritol, sugar, and molasses) most closely approximates sugar and can be used for baking, biscuit, drinks, and so on.

5.2.4 Low calorie sweeteners

Low calorie sweeteners of commercial significance – sugar alcohols (maltitol, lactitol, isomalt, xylitol, manitol, sorbitol), fructo-oligosaccharide (FO) – are used to deliver all the functionality of sucrose (i.e., sugar macronutrient substitutes), but none of them is noncaloric.

Maltitol, a disaccharide alcohol, is prepared commercially from starch by enzymatic hydrolysis, chromatographic separation, and hydrogenation of maltose. It has a sweetness potency about 0.9 times that of sucrose and may reduce about 40% calorie (2.4 cal/g), compared with fully nutritive sweeteners (4 cal/g). It is currently approved for food applications in many countries, although the petition submitted to the US FDA by Towa Chemical Industries in Japan has not been approved. Lactitol is also a disaccharide alcohol and is obtained commercially by the hydrogenation of lactose, which is available as a by-product of the dairy industry. *Lactitol* exhibits a sweetness potency similar to that of sucrose and a bioavailable calorie value of about 2.0 cal/g. Lactitol is not metabolized in either the small or the large intestine, but eventually microbial fermentation converts it into organic acids and gases. *Isomalt* is a 1:1 mixture of the disaccharide alcohols 1-*O*- α -D-glucopyranosyl mannitol and 1-*O*- α -D-glucopyranosyl sorbitol. Isomalt

is prepared commercially from sucrose in a two-step process generally approved for food applications in most countries: first the enzymatic production of isomaltulose, and then hydrogenation to isomalt. Isomalt may give a 50% reduction of calories. *Xylitol* is a natural sweetener that is marketed globally including Canada and the United States and is accepted by the FDA and the WHO, and the American Dental Association. Xylitol contains 75% less carbohydrates and 40% less calories than sugar, has a myriad of oral health benefits including the prevention of tooth decay, and is safe for diabetics. Its sweetening power was comparable to that of sucrose and is higher than that of sorbitol and mannitol. Xylitol is manufactured from xylose through a chemical hydrogenation using nickel as a catalyst; it has some disadvantages, including a high energy requirement, extensive purification steps, and a high cost of product. The microbial production of xylitol has been examined as an alternative to the chemical process, which on an industrial scale is time consuming mainly due to sterilization and inoculum development. The enzymatic production of xylitol from lignocellulosics is an attractive and promising alternative to the chemical process, potentially eliminating the major disadvantages of conventional processes (Rafiqul and Sakinah, 2013). As the use of xylitol has been limited by the lack of a reliable, low-cost, high-quality supplier, using a combination of steam and enzymes, Xylitol Canada (<http://www.marketwatch.com/investing/stock/xyltf>) has been able to produce xylose with significantly less waste from hardwood through the cellulosic xylose process.

Other polyols (sugar alcohols) such as *mannitol*, *erythritol*, *sorbitol*, and xylitol found in fruits and vegetables are produced by certain microorganisms, thus biotechnological production by lactic acid bacteria (LAB) and other microbes have also been investigated. While heterofermentative LAB may naturally produce mannitol and erythritol under certain culture conditions, sorbitol and xylitol have been only synthesized through metabolic engineering processes (Ortiz et al., 2013).

FO sweetener is a mixture of FOs, which may be found in onions, asparagus, Jerusalem artichoke, and wheat. It contains ketose, nystose, and 1- β -fructofuranosylnystose in the ratio of 28:60:12. Meiji Seika Company in Japan has done most of the development work on this sugar and has given it the brand name Neosugar. The sweetness potency of FO is about 0.4–0.6 times that of sucrose. FO is prepared from sucrose by treatment with fructosyltransferase derived from *A. niger* and other microorganisms. The constituent sugars are not hydrolyzed by digestive enzymes but are fermented in the large intestine, specifically by probiotic bacteria such as bifidobacteria. Other galacto-oligosaccharides derived from lactose by lactase-containing galactosyltransferase activity and galactomanan-oligosaccharides produced by hydrolysis of guar gum or locust bean can also be used as probiotics (Baek and Lee, 2009). The term *probiotic* has been used to indicate microorganisms or substances that contribute to an ideal microbial balance in the digestive tract. Thus, probiotics include not only bacterial cultures (viable microbial products) but also yeast, fermentation products, nutrients, and/or metabolites that would contribute to the reduction or elimination of pathogenic organisms and the proliferation of a desirable gut microflora. There is considerable interest in the use of oligosaccharides as physiologically active ingredients called *functional foods* and *nutraceuticals* that will be discussed in Part II.

5.2.5 New developments

Since the discovery of aspartame in 1965, a substantial amount of research effort has been devoted to understanding a taste perception and to introducing other stable and safe dipeptide sweeteners. Takeda Chemical Industries in Japan introduced an aspartame analogue that exhibits a clean, sweet taste with 200 times more potency than aspartame,

but is less stable than aspartame. Pfizer has developed the sweetener Alitame, which exhibits a clean flavor profile similar to that of aspartame but has better hydrolytic stability. Several other dipeptide esters have also been synthesized by other companies, and these substances have been reported to be 1000–2000 times more potent than sucrose and also to be much more stable than aspartame. The Nutrasweet Company has recently discovered a series of sweeteners derived from substituted β -amino acids with potencies up to 20,000 times the sweetness of sucrose. Biotechnology will become increasingly important in the future development of intense sweeteners, since protein-based sweeteners will depend on enzymes to catalyze the product and to improve taste and hydrolytic stability. Genetically engineered hosts may also be able to overproduce sweeteners and their components.

Summary

Until the discovery of nonnutritive saccharin, carbohydrate-based nutritive sweeteners were mostly used. However, the high-intensity (nonnutritive) sweetener and low-calorie sweetener markets are currently growing rapidly, in step with the development of the low-calorie food industry. The high-intensity sweetener market is currently dominated by aspartame and saccharin. However, it is expected that in the near future new sweeteners such as cyclamates, Alitame, sucralose, and few others, not yet approved for sale in the United States, will compete with the existing products in the market. A number of extremely potent sweeteners will also reach the market in the future. The production costs of other low-calorie sweeteners such as maltitol, isomalt, lactitol, and FO are currently high, and most of these substances cause substantial laxative effects. Further research is needed in developing sugar macronutrient substances that are noncaloric and nonlaxative. Furthermore, biotechnology has opened the way to new nonnutritive sweeteners such as aspartame and thaumatin, which may be produced more efficiently using recombinant DNA technology than by means of the traditional process.

5.3 Flavors and amino acids

5.3.1 Introduction

During the past decades, there has been a significant trend away from artificial to natural flavors. This movement, on the part of food manufacturers and consumers alike, is partly due to national legislation, which is trying to limit the use of artificial flavors in processed foods and beverages, and also to an increasing public bias against any food that is chemically or synthetically produced. Since, in addition, chemical synthesis is accompanied by the formation of mixtures of isomers, natural flavors of many types are beginning to find wider use in a variety of food and drink products. Such natural flavors are usually basic building blocks that can serve as the raw materials for conversion or composition of more sophisticated and complex flavors. Flavors and fragrances find wide application in the food, feed, cosmetic, chemical, and pharmaceutical sectors. Many flavor compounds on the market still are produced via chemical synthesis or via extraction from plant and animal sources; however, a rapid switch toward the bioproduction and use of flavor compounds of (micro) biological origin – bioflavors – is observed. Up to now, certain plant and animal sources remain an important source of bioflavors, but these bioactive compounds are often present in minor quantities, making isolation and formulation very expensive, or they are found only in exotic (plant) species. The other bioroute for flavor synthesis is based on de

novo microbial processes (fermentation) or on bioconversions of natural precursors using microbial cells or enzymes (biocatalysis).

Natural flavors include products obtained through microbial or enzymatic processes as long as the precursor/raw material be natural and obtained via physical or bioprocesses and that the precursor and product can be found in nature or are part of traditional foods. Physical processes for obtaining natural flavors are extraction, distillation, concentration, crystallization, etc., that is, from animal sources (e.g., beef, chicken, seafood) or plant sources (e.g., spices, mushroom, citrus, fruits, mints). Products that occur in nature but are produced via a chemical (a non-natural) process are called *nature identical*; this mode of production is no longer accepted as consumer-friendly.

International Flavors & Fragrances Inc. (IFF), a leading global creator of flavors and fragrances for consumer products and Evolva Holding SA, Reinach (Switzerland), have recently entered into pre-production phase to develop and scale-up natural vanillin for commercial application through a cost-effective, natural, and sustainable microbial route (<http://www.evolva.com/media/press-releases/2013/2/5/iff-and-evolva-enter-pre-production-phase-natural-vanillin>). Other companies such as Givaudan, Firmenish, Allelix, Amyris, and Isobionics are also interested in their microbial platforms to produced key flavor components (Bomgardner, 2012). There is another positive review on the supercritical fluid extraction of plant flavors and fragrances (Capuzzo et al., 2013).

5.3.2 Microbial flavors

The production and release of odor-active compounds is a well-known phenomenon to microbiologists, and indeed, there are several advantages of using microorganisms for the production of flavor components. They propagate in a short period of time, produce flavors of natural origin, and have inexhaustible reservoir of strains. They can easily be manipulated phenotypically or genetically, and they supply complex flavor characteristics that cannot be duplicated economically by pure chemicals. There are two principal routes for the production of microbial flavors: biosynthesis (also known as *de novo synthesis*) and biotransformation-based processes. A *biosynthetic process* yields chemical compounds from growing, metabolizing cells (fermentation or secondary metabolism), whereas *biotransformation* is defined as the use of microbial cells to perform specific modifications or interconversions of chemical structures. Although there are numerous examples of bacterial *de novo* synthesis of flavor compounds, the relatively low yield obtained via this route often results in uneconomical processes. In contrast, bioconversions that rely on abundant and relatively inexpensive sources of precursors yield higher levels of product and therefore offer a more attractive industrial process from an economic viewpoint. Microorganisms or enzymes can be used to produce either complex, multicomponent flavor systems or individual flavor compounds. Enzyme-modified cheeses, lipolyzed milk fats, and blue cheese flavor are examples of complex, multicomponent flavor systems being produced by enzymes and microbial fermentation. A few examples of specific microbial flavor molecules have been described in some detail (Christen and Lopez-Mungufo, 1994; McNeil et al., 2013).

5.3.2.1 Methyl ketones When young cheese is ripened with the mold *Penicillium roqueforti*, the typical blue cheese flavor is produced. Proteolytic and lipolytic enzymes of the mold play a major role in the development of the final taste. The enzymes are excreted into the milk, where the protease hydrolyzes the casein, thereby producing both flavor precursors and substrates for mold growth. The lipase produces free fatty acids, which

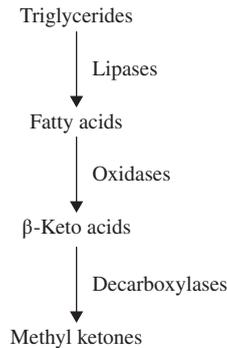


Figure 5.7 Oxidative formation of methyl ketones by *Penicillium roqueforti*.

are the characteristic dairy flavors. However, fatty acids are also toxic to this mold, and the degree of toxicity depends on the concentration of the acids, their chain length, and the pH of the medium. Figure 5.7 shows the formation of methyl ketones through oxidation and decarboxylation. Methyl ketones arise as the result of two reactions, namely, the β -oxidation of fatty acids to β -keto acids, followed by decarboxylation, which produces C_5 - C_{11} 2-alkanones, the key flavors of the blue-type cheeses. In the manufacturing of blue cheese flavor, fermentation broth is thermally inactivated and then spray dried. Both the conidiospores of *P. roqueforti* and the mycelia of the fungus have been used to produce methyl ketones from fatty acids. A continuous reactor system using immobilized spores has also been studied. Methyl ketones are produced on a commercial scale in Britain by Stafford Speciality Ingredients, using a strain of *A. niger* (Janssens et al., 1992).

More recently, recombinant *E. coli* was able to overproduce up to 700-fold of saturated and monounsaturated aliphatic methyl ketones in the C_{11} to C_{15} (diesel) range; this group of methyl ketones includes 2-undecanone and 2-tridecanone, which are of importance to the flavor and fragrance industry and also have favorable cetane numbers (Goh et al., 2012).

5.3.2.2 Diacetyl and acetaldehyde *Diacetyl* imparts the buttery attributes found in cultured dairy products such as sour cream and buttermilk.

Diacetyl, when used in artificial butter flavoring (as used in many consumer foods) may be hazardous when heated and inhaled over a long period. Workers in manufacturing of artificial butter flavoring have been diagnosed with bronchiolitis obliterans, a rare and serious disease of the lungs. In 2007, the Flavor and Extract Manufacturers Association recommended reducing diacetyl in butter flavorings. Diacetyl may exacerbate the effects of β -amyloid aggregation, a process linked to Alzheimer's disease (Swati et al., 2012). Manufacturers of butter-flavored popcorn began removing diacetyl as an ingredient from their products. Lung disease may be caused by the chemicals in microwave popcorn.

Diacetyl formation in *Lactococcus lactis diacetylactis* is associated with citrate utilization and has been found linked to a plasmid. A recombinant DNA approach provides a potential solution for poor diacetyl production resulting from the loss of plasmid-encoded citrate permease activity in starter cultures. Incorporation of citrate permease genetic determinants into the chromosome, a process called integration, has been suggested as a means of stabilizing this trait. The cloning of the lactococcal citrate permease and the development of functional integration vectors for lactococci now make chromosomal integration of citrate permease a technical possibility. Figure 5.8 shows the steps involved

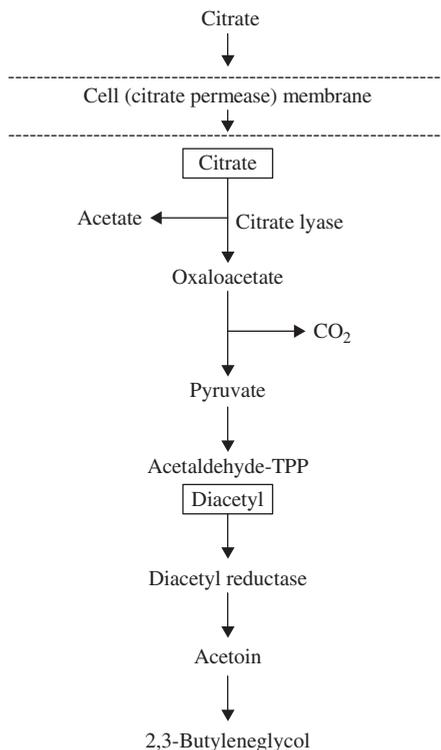


Figure 5.8 Production of diacetyl from citrate.

in the production of diacetyl from citrate. α -Acetolactic acid is the precursor of diacetyl, hence a key component in its biosynthesis. One proposed method for increasing the production of diacetyl consists of increasing the level of its precursor by means of genetic manipulation to reduce the amount of α -acetolactate decarboxylase.

Another important, low molecular weight carbonyl flavor compound is *acetaldehyde*, which is a major flavor of yogurt and some fruit flavors, such as orange. Acetaldehyde occurs naturally in coffee, bread, and ripe fruit, and is produced by plants as part of their normal metabolism. It is also produced by oxidation of ethylene and is the cause of hangovers from alcohol consumption; it is synthesized in the liver by the enzyme alcohol dehydrogenase. Ethanol is converted to acetaldehyde using alcohol dehydrogenase with cofactor nicotinamide adenine dinucleotide (NAD) regeneration or immobilized cells of *Candida utilis* or *P. pastoris*. Acetaldehyde may be a carcinogen in digestive tract cancers (Salaspuro, 2009). The safety issues also solicited input regarding exposure and health effects of acetoin, acetaldehyde, acetic acid, and furfural.

5.3.2.3 Lactones Lactones are associated with pleasant odor impressions, such as fruity, coconut-like, buttery, sweet, and nutty. These flavor materials have been isolated from major foods such as fruits, vegetables, nuts, meat, and dairy and baked products. *T. viride*, a soil fungus, generates a strong coconut aroma on a simple growth medium. Chemically,

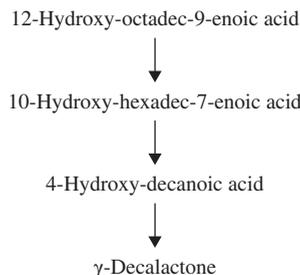


Figure 5.9 Oxidative conversion of ricinoleic acid into γ -decalactone by *Candida lipolytica*.

seven steps are needed for the synthesis of this aroma. *Sporobolomyces odorus* is a yeast that produces *de novo* up to 1.6 mg/L of *decalactone*, resulting in the fruity, fatty aroma typical of peaches. Fermentative production has been described with the yeast *Candida lipolytica* using *ricinoleic acid* (12-hydroxyoctadecyl-9-enoic acid), which is a major component ($\approx 80\%$) of castor oil. This yeast lipolyzes castor oil glycerides. The liberated ricinoleic acid is subsequently metabolized via β -oxidation and eventually converted to 4-hydroxydecanoic acid, which is excreted up to 6 g/L (Janssens et al., 1992). Final conversion to γ -decalactone is achieved by boiling the crude, acidified fermentation broth (Figure 5.9).

5.3.2.4 Terpenes Terpenes are often the most important components responsible for the characteristic odors of essential oils. They are composed of repeating isoprene units. Most of the terpene-producing microorganisms are fungi. The *Ceratocystis* genus has undergone intensive investigation for the production of the most important terpenes and their sensorial characteristics. At present, *de novo* synthesis is not considered to be cost-effective compared with standard extraction methods. The most significant potential of bacterial cultures in the production of terpenes is their ability to carry out biotransformation, converting abundant and inexpensive terpene precursors to higher-value derivatives. Pseudomonads in particular have been noted for their ability to metabolize terpenes. Terpenes are excellent substrates for stereospecific bioconversions. L-Menthol, one of the most important terpene alcohols, is widely used in the flavor and perfume industries. Natural L-menthol is produced by crystallization from peppermint oils extracted from mint plants, the genus *Mentha*; but there has been a world shortage of high grade peppermint oil. Many microbial esterases preferentially hydrolyze asymmetric L-menthyl esters while leaving the D-menthyl esters untouched.

5.3.2.5 Esters Esters are responsible for fruity-flavored taste and aroma defects in many foods such as Cheddar cheese, but they are desirable in beer and wine. Natural fruity esters have been used in the flavor formulations to increase the taste and aroma of foods such as yogurt, ice cream, fruit-flavored beverages, chewing gum, and candy. Ethyl acetate, ethyl butyrate, ethyl isovalerate, and ethyl-hexenoate are produced by various species of *Pseudomonas*, *Lactococcus*, and *Lactobacillus*, and the yeasts *Hansenula anomala* and *C. utilis*. *C. utilis* is the most promising for industrial application of ethyl acetate production from ethanol. Under optimal conditions, at pH 7.0 in phosphate buffer and with an initial ethanol concentration of 10 g/L, an ethyl acetate concentration of approximately 4.5 g/L

was obtained after a 5-h incubation period. Butyric acid and ethyl butylate are produced microbiologically by the American company, Hercules, Inc. (Janssens et al., 1992). The cost of microbial ethyl butylate amounts to about \$180/kg, while its synthetic counterpart costs only \$4/kg. The ester-synthesizing capabilities of certain microbial esterase-lipase enzymes derived from *A. niger* and *M. miehei* have also been investigated. Nonaqueous systems (organic solvents such as *n*-hexane) were examined to improve the ester yield and to simplify the isolation and purification steps. Also immobilized lipase mediated transesterification was tried to produce flavor esters (Garlapati and Banerjee, 2013).

5.3.2.6 Pyrazines Pyrazines are heterocyclic, nitrogen-containing compounds, often associated with roasted and nutty flavors in heated foods. This attribute suggested the use of pyrazines in microwave foods that do not generate these compounds during the heating process. The two principal examples of bacterially produced pyrazines are tetramethylpyrazine (TMP) and 2-methoxy-isopropylpyrazine (MIPP). TMP was first reported to be produced by *B. subtilis* and later by *Corynebacterium glutamicum*. A subsequent report provides an example of the use of standard mutagenic techniques to isolate an overproducing strain. Following treatment with the chemical mutagen *N*-methyl-*N*-nitroso-*N'*-nitrosoguanidine (MNNG), a mutant was isolated that accumulated up to 3 g/L TMP over a 5-day period. It was postulated that a deficiency in reductoisomerase activity caused a blockage in the mutant's isoleucine–valine biosynthetic pathway (Romero, 1992). This allowed for the accumulation of acetoin, the proposed precursor for TMP. One type of pyrazine, methoxyalkylpyrazine, is found in a variety of foods including bell peppers (specifically, 2-methoxy-3-isobutylpyrazine), potatoes, green beans, and green peas. 2-Methoxy-3-isopropylpyrazine reportedly is produced by two *Pseudomonas* species, *Pseudomonas taetolens* and *Pseudomonas perolens* (Gatfield, 1988). Of particular note is the isolation of a mutant following MNNG treatment that produced 375 times more MIPP than the parental strain (Romero, 1992).

5.3.2.7 Aromatic compounds Two aromatic aldehydes of commercial significance are benzaldehyde and vanillin. Benzaldehyde, noted for imparting bitter almond and cherry flavors, has been identified as a product of microbial secondary metabolism. Benzaldehyde can be derived from the degradation of the glucoside precursors mandelonitrile and amygdalin from cherry and almond, respectively. A similar pathway was studied in microorganisms, where benzaldehyde is formed as a metabolic intermediate during the degradation of mandelate. The genetic determinants from *Pseudomonas putida* for the five enzymes in the pathway have been cloned, and the DNA sequences of mandelate racemase, (*S*)-mandelate dehydrogenase, and benzoylformate decarboxylase were determined. One potential method for benzaldehyde production could take advantage of the ability of various pseudomonads to utilize mandelate as the sole carbon source. An examination of the pathway suggests that inactivation of the two benzaldehyde dehydrogenases through various mutagenic techniques would result in the accumulation of benzaldehyde.

Vanillin is the principal flavor component of vanilla and is of particular importance to industry because of its widespread use in a number of flavor formulations. The increasing worldwide demand for vanillin is largely supplied by the vanillin extracted from pulped wood. Alternative sources of vanillin, which have the advantage of being considered natural, can be produced via microbial biotransformation. As with the mandelate degradation pathways, bacterial cultures have been identified that can degrade eugenol, forming vanillin in the process. Bacterial degradation of eugenol was first described using a species of *Corynebacterium*, with the proposed pathway involving vanillin as an intermediate (Figure 5.10). A patent has recently been granted for the bacterial production of vanillin

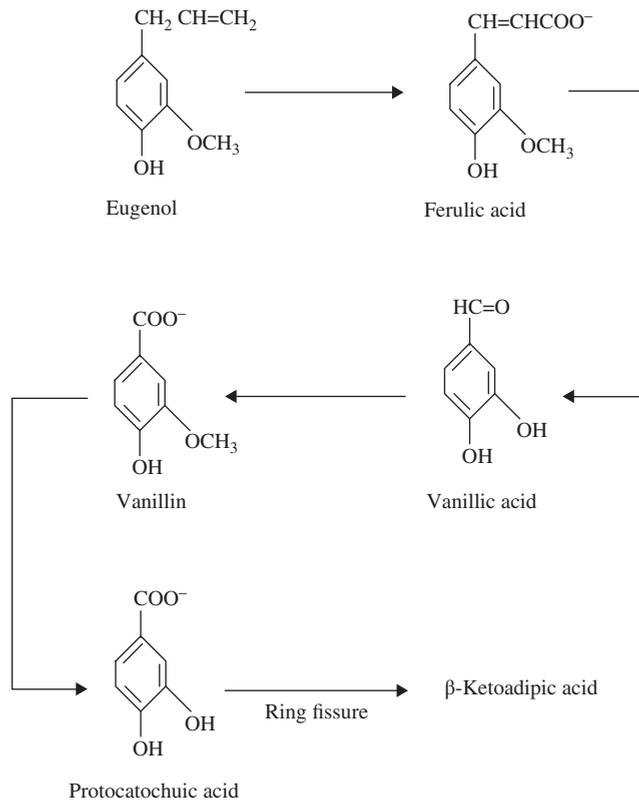


Figure 5.10 Vanillin production by eugenol degradation by *Corynebacterium* species.

from eugenol and isoeugenol by strains of *Serratia*, *Klebsiella*, or *Enterobacter*. Yields of up to 3.8 g/L have been reported. The vanilla flavor is also produced by culturing callus cells derived from *Vanilla fragrans* or *Vanilla phaeantha*. This vanilla production can be enhanced in an immobilized culture system by continuously removing the flavor components from the growth medium. The vanilla flavor recovery was also achieved by desorption from the adsorbent (activated charcoal) using 50% ethanol solution. More recent works on vanillin can be found elsewhere (Walton et al., 2003).

5.3.2.8 Flavor enhancers Microbial cultures can also be used to produce flavor enhancers or potentiators, which are compounds that do not possess any aroma on their own but are capable of enhancing flavors already present. Such compounds include inosine-5'-monophosphate (5'-IMP) and guanosine-5'-monophosphate (5'-GMP) as well as MSG. Among 5'-ribonucleotides, only purine nucleotides have a umami taste. The base component is a purine with a hydroxy group at the 6-position and the phosphoric moiety attached to the 5' position of D-ribose. 5'-Nucleotides can be produced through enzymatic hydrolysis of yeast RNA, chemical hydrolysis of the RNA, and direct fermentation to 5'-IMP or microbial production of inosine and subsequent chemical phosphorylation to 5'-IMP. Since 1975, about 50% of the 5'-nucleotides in Japan were produced by the

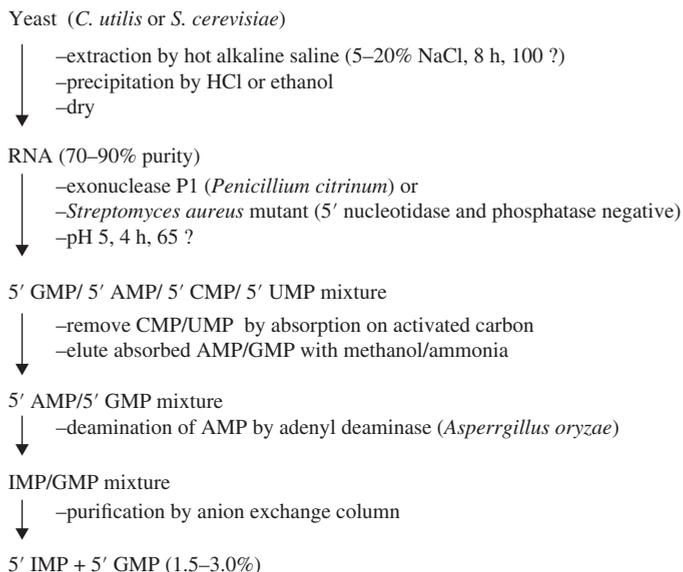


Figure 5.11 Production of 5'-nucleotides by enzymatic hydrolysis of RNA.

enzymatic hydrolysis method (Figure 5.11). In this method, yeast cells having a high RNA content are harvested at early log phase and the RNA is extracted with hot alkaline solution. After separating the cell residue, the RNA is precipitated by adding HCl or ethanol and then dried. In the chemical hydrolysis of RNA by heating at 130 °C for 3–4 h in calcium hydroxide, nucleosides are produced. The chemical phosphorylation of these nucleosides to 5'-IMP and 5'-GMP is then achieved similarly, by deamination of adenosine to inosine. For direct fermentation, 5'-IMP can be produced by various methods – mainly fermentative production of inosine and subsequent chemical phosphorylation to 5'-IMP or direct fermentation to 5'-IMP. In the fermentative production of inosine, adenine auxotrophs (Ade⁻) of several genera, such as *B. subtilis* and *Brevibacterium ammoniagenes*, or biotin auxotrophs (Bio⁻) of *Microbacterium* can accumulate up to 30–35 g of inosine per liter. The mutants possess a block in nucleotide biosynthesis, which has the effect of eliminating end product control. The inosine produced is precipitated from a culture filtrate at pH 11 and crystallized. The chemical phosphorylation of inosine is carried out using trialkyl phosphate with PCL₃, yielding about 90% of 5'-monoester. Mutants of *B. ammoniagenes* that are able to grow on adenine but lacking nucleotidase (which breaks down 5'-IMP) and insensitive to Mn²⁺ could yield 5'-IMP in amounts up to 20–27 g/L. The Ajinomoto Company has developed a process producing for 5'-IMP using a nitrosoguanidine-treated mutant of *B. ammoniagenes*. By adding inosine (50–2000 mg/L) or hypoxanthine (25–1000 mg/L) during fermentation in sugar or starch hydrolysate, a significant increase in the amount of 5-IMP was obtained.

Two processes are currently available for industrial production of 5'-GMP: fermentative production of AICAR (5-amino-4-imidazole carboxamide ribose), which is chemically converted to 5'-GMP, and production of guanosine by direct fermentation, followed by chemical phosphorylation. The purine auxotroph and its mutants, which lack AICARP (5-amino-1-(5'-phosphoribosyl)-imidazole-4-carboamide) formyl-transferase

activity, have been used in industrial production. Under optimal fermentation conditions, this strain produces 16 g/L AICAR from 80 g/L glucose. For maximal AICAR production, the medium must contain a purine source such as dry yeast or yeast RNA. Sporulation has a negative effect on AICAR production but can be suppressed by inhibitors such as butyric acid or by reducing the oxygen supply. The AICARP formed is excreted into the fermentation medium as the dephosphorylated form, AICAR. AICAR is first converted to guanosine in several chemical steps and then phosphorylated. In guanosine production by direct fermentation, guanosine-excreting strains such as *B. subtilis* mutants are chiefly used for commercial production. The high guanosine-excreting mutant is mainly due to several factors: adenine auxotrophy, the absence of GMP reductase, the lack of feedback regulation by GMP, and the increased activity of IMP dehydrogenase and GMP synthetase. The production of guanosine by *B. subtilis* has been improved through a gene cloning approach. The gene for IMP dehydrogenase (responsible in catalyzing the rate-limiting step) from *B. subtilis* was first cloned in *E. coli*, then reintroduced into *B. subtilis*, thereby increasing the number of gene copies. This resulted in an increased production of guanosine.

The amino acid L-glutamate and its salt, MSG, are well-known flavor enhancers, and the industry produces more than 500,000 tons/year. L-Glutamate production has been found to occur in a wide variety of bacteria, streptomyces, yeasts, and fungi. Some *Brevibacterium flavum* and *C. glutamicum* species are major glutamate producers, but most commonly glutamate is produced by the fermentation of glucose and sucrose as carbon and energy sources by *C. glutamicum*. This organism accumulates α -ketoglutaric acid because it is incapable of performing a complete tricarboxylic acid (TCA) cycle. As a consequence, the excess α -ketoglutaric acid is channeled toward the synthesis of glutamic acid. In addition, growth under biotin-limiting conditions results in an increased ability of the organism to excrete glutamic acid into the media. This makes the purification process simpler. If biotin is limiting, phospholipid synthesis is limited, and thus the cell membranes become leaky. Thus, all glutamate producers require biotin, lack L-glutarate dehydrogenase, and show increased activity of glutamate dehydrogenase. Under optimal culture conditions, glutamate-producing bacteria convert about 50–60% of the added carbon source to L-glutamate. In a typical fermentation from glucose with *Brevibacterium divaricatum*, 0.65 mL/L of oleic acid is added. After beginning the growth of culture at pH 7.8 and 38 °C, a fed batch is operated and the fermentation is stopped after 30–35 h with a glutamate yield of 100 g/L.

5.3.3 Enzymatic flavor generation

Although chemical methods are widely employed in the production of commercial flavors, enzymatic processes are also contributors to flavor development using an increasing range of foods: soy sauce, wine, sausages, beer, sauerkraut, and miscellaneous dairy products. Among the multitude of reactions catalyzed by enzymes are those involving modification of appropriate substrates or precursors leading to the creation of intensely flavored enzyme-modified products. These flavor compounds are produced as a result of the catabolism of complex food ingredients comprising proteins, lipids, and carbohydrates by selected enzymes.

Lipolyzed milk fat was one of the very first fermentation flavors to be produced on a large scale. Cultured cream was subjected to a controlled enzymatic hydrolysis using lipase, a type of enzyme that hydrolyzes triglycerides and liberates individual acids. The importance of free fatty acids in the characteristic flavors of various dairy products is an undisputed fact, and the different fatty acids display considerably different flavor characteristics. Lipolyzed milk fat products can be used for the enhancement of butterlike flavors

and flavor development in milk chocolate. An early extension of this technology led to the development of enzyme-modified cheeses discussed in Section 5.1.5.1. Many cheese flavors are already made on a large scale using lipases and proteinases. The products thus obtained either can be applied directly to flavor sauces, dressings, dips, and snacks or can be combined as biobases with other raw materials to create cheese flavors.

Extraction of flavor constituents is achieved by pectinases and cellulases. In plants, the aromatic components are often present bound to carbohydrates in the form of a glucoside. These aromatic compounds (which form the aglycon) vary in nature from phenols, lactones, alcohols, acids, and aldehydes. In the case of pineapple, the aglycone has been found to be the 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF), and it is the major component of pineapple flavor concentrates. Glycosidically bound DMHF does exist in pineapple, and the levels of DMHF are more than doubled when pineapple juice is treated with almond β -glucosidase.

Specific flavor precursors are also formed by the action of enzymes on proteins and carbohydrates. For example, proteases and amylases hydrolyze protein and starch to simple amino acids and sugars, respectively. These metabolites may be used in further reactions involving caramelization and Maillard browning to generate particular flavor notes. The production of certain meat and savory flavors employs this technology.

Major volatile flavor constituents such as the “green notes” of many fruits and vegetables arise from the action of endogenous enzymes on flavor precursors present in the tissue. Thus, the action of lipoxygenase on the polyunsaturated fatty acids linoleic and linolenic acids, followed by the action of hydroperoxide lyase, gives rise to hexanal and 2-hexenal in apples. 2,6-Green components such as the C-9 compounds nonenal and nonadienal and the C-6 compounds hexanal and hexenal play an important role in the overall flavors of cucumber and apple/tomato-type aromas. They arise from the degradation of unsaturated fatty acids via a lipoxygenase-catalyzed formation of hydroperoxides followed by cleavage by a hydroperoxide lyase. For this purpose, cloning and expression of hydroperoxide lyase from tomato and red-bell pepper in *P. pastoris* have been tried (Atwal et al., 2005).

5.3.4 Amino acids

The manufacture of amino acids reflects the increased recognition of the nutritional and taste values of amino acids in many foods and beverages, such as fruit juice, cheese, beer, seafood, soup, and tea. Today, amino acids are used not only as food additives ($\approx 66\%$) but also as feed additives (31%) and components in pharmaceuticals, cosmetics, and other industrial raw materials (3%). In the food industry, amino acids are used alone or in combination to enhance flavors; the production volumes of the most important ones are given in Table 5.5. The well-known flavor enhancer MSG has been mentioned. Sodium aspartate and alanine are added to fruit juices or glycine to sweeteners to round off the taste. L-Cysteine acts as an antioxidant in fruit juices and improves baking quality. L-Tryptophan with L-histidine also acts as an antioxidant to preserve powdered milk from rancidity. L-Phenylalanine and L-aspartate are the building blocks of the dipeptide, low-calorie sweetener, aspartame. L-Methionine, L-lysine, L-threonine, and L-tryptophan are deficient in grain products as food or feed additives. However, 95% of the world production is accounted for by L-glutamic acid (MSG), DL-methionine, and L-lysine.

Since the separation of protein hydrolysates to produce the required amino acid in a relatively pure form on a large scale is an expensive procedure, fermentation processes

Table 5.5 World production and applications of amino acids for the food and feed industry

Amino acid	Application	Annual production, tons	Titer, g/L	Price, US\$/kg*
L-Alanine	Flavor enhancer	200	110*	–
DL-Alanine	Flavor enhancer (beverage)	1,500	–	–
L-Aspartic acid	Flavor enhancer, aspartame sweetener	8,000	–	3–4
L-Cysteine	Antioxidant in bread	1,000	–	–
L-Glutamic acid (MSG)	Flavor enhancer	2,500,000	30	2.5–3
L-Glycine	Sweetener components	6,000	–	4.5
L-Lysine	Dietary and feed additive	1,500,000	120*	3–100
DL-Methionine	Feed additive	250,000	–	56–86
L-Phenylalanine	Aspartame sweetener	8,000	50*	–
L-Threonine	Feed additive	500	70*	–
L-Tryptophan	Dietary supplement	300	60*	–

*Metabolically engineered *E. coli* or native *C. glutamicum* (Becker and Wittmann, 2012).

Source: Author's compiled data, Kumon and Kawakita (1991) and Becker and Wittmann (2012).

have been developed for all amino acids except glycine, L-cysteine, and L-cystine. At present methionine and lysine are still partially produced by chemosynthetic processes and thus occur as racemates. There are four ways of producing L-amino acids: extraction of amino acids from protein hydrolysates (L-cysteine, L-cystine, L-leucine, L-asparagine, and L-tyrosine are still produced in this way), chemical synthesis of racemates with subsequent isolation of the L-form by aminoacylase, microbiological production using wild-type strains or auxotrophic/regulatory mutants, and enzymatic bioconversion. In the direct fermentation method, strains that over-produce amino acids have been developed by mutation and selection for growth on amino acid analogues that interfere with utilization or synthesis of the particular amino acid. Direct fermentation on cheap carbon sources such as glucose, starch hydrolysates, and *n*-paraffins is favorable with respect to costs because there is no need for the expensive chemical or biosynthesis of precursors.

L-Lysine producers are among L-glutamate producing mutants of *Corynebacterium* and *Brevibacterium*, which are homoserine auxotrophs, or among methionine–threonine double auxotrophs. L-Phenylalanine has been produced (21.7 g/L) in a medium containing 13% glucose using a mutant of *Brevibacterium lactofermentum*. This mutant was selected from double auxotrophs for tyrosine and methionine. More recently, metabolically engineered (systems-wide global pathway engineering) of *C. glutamicum* provided the best lysine producer known so far (Becker et al., 2012). A mutant of *C. glutamicum* lacks *homoserine dehydrogenase*, so that the inhibition of end product, threonine on lysine synthesis by *asparto (aspartate) kinase* does not occur. As the auxotrophic mutant does not synthesize threonine or methionine, these amino acids must be added in the growth medium. By auxotrophic mutant, L-lysine is overproduced in the range of 30–35 g/L but using fed-batch fermentation of *C. glutamicum* mutant strains, impressive L-lysine-HCl concentrations of up to 170 g/L have been reported (Eggeling and Sahm, 2009; Brautaset and Ellingsen, 2011). Metabolic engineering of *C. glutamicum* has significantly increased L-lysine production from 35–50 to 80 g/L. Metabolic engineering of *E. coli* has also overproduced up to 50 g/L (Sheng et al., 2012).

More recently L-phenylalanine was produced from *E. coli* using recombinant DNA techniques. The gene coding key enzymes in the L-phenylalanine biosynthesis pathway

was cloned into a vector containing a promoter and an operon of bacteriophage lambda. L-Aspartate production from glucose fermentation has been achieved by conversion of fumaric acid directly to L-aspartic acid using bacterial aspartase or immobilizing whole cells. L-Tryptophan has been produced by immobilized cells or enzymes; but to optimize the process, recombinant DNA technology has also been used. Because of the expense of the starting materials (indole, serine, pyruvate), direct fermentation of L-tryptophan has been carried out. However, the yield was generally low due to the complex regulatory mechanism of tryptophan biosynthesis.

Many recent activities in this metabolic engineering have led to remove bottleneck or metabolic conversion, engineering of central carbon metabolism leading to increased supply of precursors, and transport engineering leading to reduced intracellular pool of the aromatic amino acids using *C. glutamicum* and *E. coli* (Iketda, 2006).

In amino acid production by enzymatic conversions, microbial cells or enzymes are used to convert either an intermediary in the synthetic pathway leading to the amino acid or a related compound not normally an intermediate in the pathway. Such intermediate compounds are called amino acid precursors. L-Threonine hydratase catalyzes the conversion of L-threonine to α -ketobutyrate, an intermediate in the formation of L-isoleucine. This enzyme is subject to feedback inhibition by isoleucine, and thus the addition of L-threonine or α -ketobutyrate does not produce isoleucine. However, if D-threonine is added as a precursor, the enzyme is induced by D-threonine and does not suffer from feedback inhibition by isoleucine.

5.3.5 Economics

Although many microbial processes have been known to produce interesting flavors, the numbers of industrial applications are very limited at this time, mainly because in most cases their yields are low. The microbial flavors are often present only in very low concentrations in the fermentation broths, and thus the costs for downstream processing are high. The development of specific fermentation techniques and recovery methods is consequently an important challenge for researchers in this field. To be competitive, the price per kilogram of microbial flavors must be cut significantly (between \$200/kg and \$2000/kg). The market price of natural aromas in 1991 was 10–100 times higher than that of synthetic aromas. Another obstacle to commercialization is the framework of legal regulations, to which new products derived even from microorganisms are subject before the status “natural” is awarded. The procedure required can be very time-demanding and expensive. Despite these difficulties, microorganisms are already being used to produce a number of flavor compounds on an industrial scale. Many important flavor companies use fermentation techniques for the production of aroma compounds, yet only a few mention the specific products, to protect proprietary secrets.

Recently in Germany a microbial process involving the bioconversion of castor oil by *Yarrowia lipolytica* has been developed to produce 4-decalactone, a peach aroma. A yield up to 6 g/L has been obtained by fermentation. Unilever in the United Kingdom also developed a commercial process to produce lactone, a butter flavor for margarines, from baker's yeast using the starting material, 5-ketododecanoic acid. Butyric acid, a natural cheese aroma in butter and some cheeses, is also produced microbiologically by the American company Hercules Inc. *Clostridium butyricum* converts glucose to butyric acid anaerobically, yielding up to 1.2% in the fermentation broth.

Esterification with ethanol gives rise to ethyl butyrate, an important fruity flavor with a low odor threshold. Ethyl butyrate is one of the most common chemicals used in flavors

and fragrances. It can be used in a variety of flavors: orange (most common), cherry, pineapple, mango, guava, bubblegum, peach, apricot, fig, and plum. In industrial use, it is also one of the cheapest chemicals, which only adds to its popularity. Biotechnologically produced natural ethyl butyrate costs about \$180/kg, while the price of its synthetic counterpart is only \$4/kg (Armstrong et al., 1989). Natural ethyl butyrate for fruit juices costs about \$5000/kg. Methyl ketones are produced in the United Kingdom (Stafford Speciality ingredients) on a commercial scale by bioconversion of coconut oil using *A. niger*. The yield of the conversion is about 40%, and 2-undecanone accounts for 60% of the resulting end product. Autolysed yeast extracts and 5'-nucleotides, which are used as natural flavor enhancers, are classic examples of biotechnological processes. Because of predictions for solid growth in the flavor industry, flavor business worldwide has grown from \$4.5 billion to \$7.5 billion in 2000. The world production of biochemically produced amino acids was 740,000 tons in 1990, of which 370,000 and 90,000 tons/year were MSG and lysine, respectively (Kumon and Kawakita, 1991). However, recent annual market volumes of two major amino acids, L-glutamate and L-lysine alone were estimated to be around 2.5 and 1.5 million tons, respectively, with a 6–8% growth (Becker and Wittmann, 2012). Because of possible safety and health concerns with respect to the so-called MSG syndrome, many companies are marketing MSG-free or MSG replacer products, containing yeast extracts or yeast nucleotides. New innovations including recombinant DNA or metabolic engineering techniques, molecular recognition separation techniques, well-integrated fermentation, and downstream processing are all required for the economic optimization of amino acid production. The global amino acids market is expected to be worth over US\$9.4 billion by 2018 at an estimated compound annual growth rate (CAGR) of 2.4%. Commercially, amino acids can be used as animal feed supplements, as nutritional additives, and as flavoring for human food. It is also used in medical, cosmetics, research, therapeutic, and industrial applications. Amino acids are increasingly being used as a nutritional protein to fortify animal feeds and as food supplements for humans. It is also used for a variety of inventions in the field of medicine since they can be used for making any number of polypeptides. Their current uses in food additives and animal feed will continue to grow since there are no substitutes for amino acids. Some of the companies in this industry are Ajinomoto, RSP amino acids, Biaffin, AnaSpec, ChemPep Inc, IRIS Biotech, PepTech Corporation, and Synthetech. Ajinomoto is one of the leading manufacturers of amino acids. It offers amino acids for research, product development and process development. It has developed and used innovative techniques to manufacture these acids (<http://www.transparencymarketresearch.com/construction-equipment-market.html>).

Summary

Biotechnology has an important future role in the flavor and amino acid industries. Because of the growing demand for natural additives for foods, the commercial importance of biotechnologically produced flavors and other ingredients should certainly grow in the near future. However, demand will be generated from major food industries, which will require low cost-effective ingredients able to withstand modern food processing procedures. In recent years, tremendous progress has been made in metabolic engineering and systems/synthetic metabolic engineering and already some amino acids such as L-lysine were overproduced over 120 g/L. However, there is a new challenge ahead with regard to the high complexity of the underlying secondary metabolism, superimposed by many metabolic and transcriptional controls.

5.4 Vitamins and pigments

5.4.1 Introduction

Vitamins are complex organic compounds that have no energetic value to human nutrition but are required in trace amounts for normal cell function. Humans vary in their daily requirements for vitamins from one individual to another, depending on age, physical activity, climatic conditions, and medical or physiological need. The absence of vitamins, or inadequate quantities of them, may cause very severe physiological disorder and even death. Interest in vitamin production from microbes and other foods derives largely from the recognition that the water-soluble and the fat-soluble vitamins are all essential for human life. The water-soluble groups are vitamin C, vitamin B complexes (B_1 , B_2 , B_5 , B_6 , B_{12}), H (biotin), folic acid, and P (nicotinamide). The function of water-soluble vitamins is to act as cofactors of important enzymes of the intermediate metabolism. The fat-soluble vitamins (vitamins A, D, E, and K) have a coenzyme-like function. Most vitamins are manufactured synthetically to meet the requirements of purity and stability, but only vitamin B_{12} and riboflavin are produced commercially by fermentation. Microbial processes are known for other vitamins but are not used on a commercial scale.

It has often been said that people eat not only with their mouths but also with their eyes. Although color does not usually contribute to the nutritional quality of the food or even to its taste, it is an important aspect of foods by which consumers may judge dietary input. In today's society, where the use of some synthetic pigments is restricted because of potential health hazards, biotechnologically derived natural food pigments may offer some solutions. However, the production of pigments by fermentation is rather limited at the present time.

5.4.2 Production of vitamins

Vitamin C (L-ascorbic acid) is important as a vitamin supplement in beverages and as an antioxidant to prevent the loss of color and aroma of packaged foods or to maintain the quality of the nutrients. World production of synthesized vitamin C is currently estimated at approximately 110,000 tons annually. Main producers have been BASF/Takeda, DSM, Merck, and the China Pharmaceutical Group Ltd. By 2008 only the DSM plant in Scotland remained operational outside the strong price competition from China. The world price of vitamin C rose sharply in 2008 partly as a result of rises in basic food prices but also in anticipation of a stoppage of the two Chinese plants as part of a general shutdown of polluting industry in China. Even today, most L-ascorbic acid is produced by a modification of the *Reichstein-Grussner synthesis*, a lengthy and capital-intensive route. In this process, D-glucose is catalytically hydrogenated to D-sorbitol, fermentatively oxidized to L-sorbose by *Azotobacter suboxydans*, and chemically converted in several steps to L-ascorbic acid. This very effective method can produce about 1 kg of vitamin C from 2 to 4 kg of glucose, but it is not completely a chemical process. A complete fermentation process will eliminate toxic chemical materials and a number of reaction steps in the process of synthesis. In animals, L-ascorbic acid is a part of the glucuronic acid pathway and in plants, its biosynthesis starts with D-glucose oxidation at the C_2 to produce D-glucosone, then epimerization of C_5 , and then finally C_1 oxidation to produce L-ascorbic acid. Some microorganisms are able to synthesize this vitamin from sugar but, in most cases, intermediates of the classical synthesis are produced. In direct synthesis by fermentation using the green alga mutant *Chlorella pyrenoidosa*, high yields of L-ascorbic acid ranging from 1.05 to 1.46 g/L have been obtained after 68–101 h of one-step fermentation in glucose medium. *Candida norvegensis* also produced vitamin C from L-galactono- γ -lactone at concentrations of 1.44 g/L

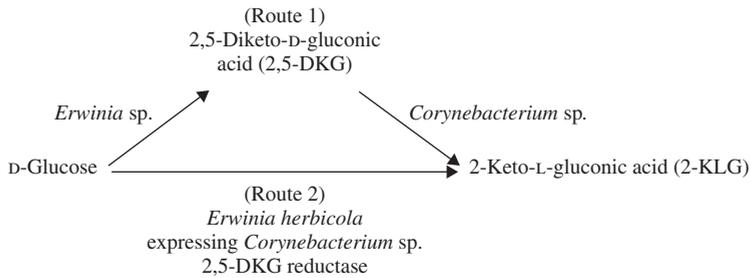


Figure 5.12 Conversion of D-glucose to 2-KLG by way of (1) two-stage fermentation process using mutants of *Erwinia* and *Corynebacterium* species and (2) direct conversion of 2,5-DKG into 2-Keto-L-gluconic acid by cloning and expressing 2,5-DKG reductase of *Corynebacterium* into *Erwinia herbicola*. Source: Adapted from Anderson et al., 1985, *Science* 230:144–149.

after 20 h of fermentation, but this process entails several fermentation and chemical steps to produce L-galactono- γ -lactone from lactose solution. This method has high potential for commercial production but may not be competitive with the Reichstein–Grüssner synthesis at the moment. The last intermediate in the Reichstein–Grüssner synthesis is 2-keto-L-gulonic acid (2-KLG) and it can be easily converted into L-ascorbic acid by a simple acid- or base-catalyzed cyclization. Using this as a starting point, the microbial conversion of 2,5-diketo-D-gluconic acid (2,5-DKG) into 2-Keto-L-gluconic acid has been achieved (Sonoyama et al., 1982). A number of microorganisms such as *Corynebacterium*, *Erwinia*, *Brevibacterium*, *Arthrobacter*, *Micrococcus*, *Acetobacter*, *Gluconobacter*, and *Citrobacter* are able to carry out the microbial conversion of 2,5-DKG into 2-KLG. D-Glucose was converted by dehydrogenases of *Erwinia* species to form 2,5-DKG, which can be reduced by the 2,5-DKG reductase of *Corynebacterium* to produce 2-KLG. By means of a two-stage fermentation process using mutants of *Erwinia* and *Corynebacterium* species, D-glucose was further converted to 2-KLG (Figure 5.12). Although tandem fermentation simplified the route from D-glucose to L-ascorbic acid, the gene cloning technique has been used successfully to combine the relevant traits of two bacteria in a single microorganism. To accomplish this route, the 2,5-DKG reductase gene from *Corynebacterium* was identified, cloned, and expressed into *Erwinia herbicola*, a bacterium that is able to convert D-glucose into 2,5-DKG (Anderson et al., 1985). Thus, the resultant organism is able to convert D-glucose directly into 2-KLG in a single fermentation.

Another approach involving direct microbial production of L-ascorbic acid is the conversion of L-sorbose to 2-KLG. High yields of L-ascorbic acid were produced using a *Gluconobacter oxydans* or mixed-culture fermentation with a *Gluconobacter* and *Bacillus megaterium*.

The Scottish Crop Research Institute (SCRI) created a strain of yeast that can synthesize vitamin C in a single fermentation step from galactose, a technology expected to reduce manufacturing costs considerably (Hancock et al., 2000). SCRI is successfully scaled-up for commercial production by a yeast-based, single-step process. This could prove to be a better alternative to the Chinese technology of vitamin C production. US-based Genentech Inc has also patented methods for producing ascorbic acid using recombinant strain combining one or more enzymes in the metabolic path converting glucose to 2-KLG.

One particularly useful system employs genes encoding membrane-bound L-sorbose dehydrogenases or membrane-bound pyrroloquinoline quinone (PQQ)-bound D-sorbitol dehydrogenases. An example of such a system uses a gene from *Gluconobacter oxydans*

N44-1 encoding L-sorbose dehydrogenase (SNDH*ai*), which converts L-sorbose to L-ascorbic acid (WO 2005/017159). As there is a continuing need in even more optimized fermentation systems for the microbial production of Vitamin C to get higher yields, under suitable culture conditions host cells expressing SNDH*ai* can be used for further optimizing the direct production of Vitamin C (Beuzelin-Ollivier et al., 2013).

Vitamin B₂ (riboflavin), a yellow, water-soluble fluorescent substance, is used in the fortification of flour and breakfast cereals, and in dietetic products. Riboflavin is involved in the electron transfer in oxidation–reduction reactions and in energy metabolism. Although foods such as liver, milk products, and green vegetables contain riboflavin, yeast is a rich source. Commercial production may be achieved by three processes: complete chemical synthesis, complete microbial synthesis by fermentation, and a mixed process that uses microbial synthesis of ribose followed by chemical transformation. Complete microbial synthesis accounts for about 30% of production, while mixed synthesis still, for the most part, dominates world production. The industrial production of D-ribose is based on the use of *Bacillus* mutants lacking in transketolase, a major enzyme of the pentose phosphate pathway.

The fermentative production of riboflavin is normally carried out by *Ascomycetes* fungi such as *Eremothecium ashbyii* and *Ashbya gossypii*. *A. gossypii* is the only organism used in commercial production, and a riboflavin yield of up to 6.4 g/L was obtained after improvements of the medium and the production conditions. Edible oils such as corn or soybean were superior carbon sources, and glycine and purines stimulated riboflavin production. A majority of *Candida* species produce substantial amounts of riboflavin on a simple medium, but this genus has extremely high tolerance for iron. More recently, riboflavin production of up to 21 g/L was reported from *Candida flareri* and its mutant. Among other molds, *A. niger*, *Aspergillus flavus*, *Penicillium chrysogenum*, and *Fusarium* have been reported as flavin producers. Another riboflavin production process has been developed by the Japanese, using *S. cerevisiae* grown on calcium acetate. The productivity of this process was 5.8 g/L with no major impurity problems, compared with molasses-grown cells. Also, genetically engineered *B. subtilis* produced 4.5 g/L of riboflavin.

Vitamin B₁₂ (cobalamin) has been produced by fermentation because the chemical synthesis was too complicated to compete with the biotechnological method. Some industrial processes still depend on *Propionibacterium*, which produces the product intracellularly. Fermentation is carried out in a two-stage process that is strictly anaerobic in the first stage (to accumulate the precursors), and aerobic in the second stage (to transform the precursors to vitamin B₁₂). Since vitamin B₁₂ is produced in different forms such as adenosylcobalamin, hydroxycobalamin, or methylcobalamin, heating must be applied to the culture in the presence of cyanide to produce cyanocobalamin, which subsequently is subjected to adsorption chromatography steps for final purification. The *Pseudomonas denitrificans* process developed in 1952 by Merck, Sharp and Dohme is of greatest economic importance, but few data have been published on this subject. A chlorophyll-producing *Rhodospseudomonas* strain fused with methanol utilizing *Protaminobacter*, a vitamin B₁₂ producer, yielded a high concentration (135 mg/L). Several companies are developing genetically engineered *B. megaterium* to improve productivity by cloning some of the genes of the cobalamin pathway. The first enzyme in the biosynthesis of cobalamin, δ -aminolevulinic acid synthetase, was overexpressed 50 times (Brey et al., 1986). Metabolic engineering of cobalamin (vitamin B₁₂) production in *B. megaterium* has also been successfully reported (Biedendieck et al., 2010). More recently, a breakthrough on the overproduction of vitamin B12 by metabolic engineering has been achieved in *B. megaterium* to produce all of the components of the anaerobic B12 pathway (Moore et al., 2013).

Rhône-Poulenc (France), which has merged into Sanofi-Aventis, used genetically engineered versions of one or both of these species. Two strains, *P. denitrificans* and *Propionibacterium shermanii* are presently the FDA-preferred bacterial fermentation organisms for vitamin B₁₂ production. The total world production of vitamin B₁₂, by four companies (the French Sanofi-Aventis and three Chinese companies) is estimated to about 35 tons in 2008. Most of this production is used as an additive to animal feed.

Although the biosynthetic capacity of microorganisms makes it theoretically feasible to produce all the vitamins by fermentation, the chemosynthetic methods still dominate industrial production. The trend toward natural products is rapidly changing this situation, however. D-Biotin is synthesized in low amounts by microorganisms, but by using strain improvements and gene cloning techniques an *E. coli* strain was induced to excrete about 16 mg/L of biotin from DL-dethiobiotin. Biosynthesis in microorganisms starts from pimelic acid, and four enzymes from pimelic acid to dethiobiotin are involved (Osawa et al., 1989). The direct production of D-biotin from pimelic acid has also been tried using a mutant of *Bacillus sphaericus*. The biotin biosynthesis pathway has been extensively studied in *E. coli* and *B. subtilis* and more recently also the pathways in rhizobia and plants (*Arabidopsis thaliana*) (Muralla et al., 2008). Engineering of biotin-prototrophy in *P. pastoris* for robust production processes has also achieved by integration of four genes involved in the biotin biosynthesis from brewing yeast into the *P. pastoris* genome rendered *P. pastoris* biotin-prototrophic (Gasser et al., 2010). The engineered strain has successfully been used as production host for both intracellular and secreted heterologous proteins in fed-batch processes, employing mineral media without vitamins. Another field of application for these truly prototrophic hosts is the production of biochemicals and small metabolites, where defined mineral media leads to easier purification procedures. Pantothenic acid (vitamin B) is currently being produced by chemical synthesis. However, an efficient bioconversion method for the reduction of ketopantoyl lactone to D-antoyl lactone has been accomplished by *Rhodotorula minuata* and *Candida parapsilosis*. Other attempts to synthesize pantothenic acid depend on the conversion by *Nocardia asteroides* of DL-pantoyl lactone to D-pantoyl lactone. D-Pantoyl lactone and L-alanine are then chemically condensed to produce D-pantothenic acid.

Various biotechnological processes have been developed for industrial scale riboflavin biosynthesis using different microorganisms, including filamentous fungi such as *Ashbya gossypii*, *Candida famata* and *Candida flaveri*, as well as the bacteria *Corynebacterium ammoniagenes* and *Bacillus subtilis* (Stahmann et al., 2000). However, only genetically modified *Bacillus subtilis* is now successfully employed at a commercial scale to produce riboflavin for feed and food fortification purposes. The chemical company BASF has installed a plant in South Korea, which is specialized on riboflavin production using *A. gossypii*. The concentrations of riboflavin in their modified strain are so high, that the mycelium has a reddish/brownish color and accumulates riboflavin crystals in the vacuoles, which will eventually burst the mycelium. Riboflavin is often overproduced, possibly as a protective mechanism, by certain bacteria in the presence of high concentrations of hydrocarbons or aromatic compounds. One such organism is *Micrococcus luteus* (American Type Culture Collection strain number ATCC 49442), which develops a yellow color due to production of riboflavin while growing on pyridine, but not when grown on other substrates, such as succinic acid (Sims and O'Loughlin, 1992).

Thiamine (vitamin B₁) is presently produced by chemical synthesis. Many microorganisms may synthesize thiamine only in low quantities, but *Saccharomyces carlsbergensis* has been reported to produce relatively high amounts (1.036 mg/g dry matter) of this vitamin (Vandamme, 1989). *Pyridoxine* (vitamin B₆) is efficiently produced by chemical synthesis

because of its simple structure. No microbiological method so far has been able to compete with the chemical method. Although the present production of folic acid is also based on chemical synthesis, this vitamin has also been biosynthesized only in small amounts by using various kefir starters in a combination of *Bifidobacterium* spp., *Lactobacillus bulgaricus*, or *Streptococcus thermophilus*, and *Lactobacillus acidophilus*. Niacin (nicotinamide) is manufactured commercially by chemical means, and there is no known microorganism that can produce it. Most of the industrial production of vitamin E (tocopherol) is based on chemical synthesis. Vitamin E may also be extracted from natural sources such as sunflower oil, palm oil, or soybean oil. However, there is no known microbial overproducer of vitamins D and K. There are no microorganisms or plants known to synthesize vitamin A (retinol). However, β -carotene or provitamin A is produced by plants and algae. The green algae *Dunaliella bardawil* is currently being grown to produce β -carotene, and this β -carotene may then be synthesized enzymatically by β -carotene 15,15'-dioxygenase.

5.4.3 Production of pigments

The worldwide market for inorganic, organic and special pigments had a total volume of around 74 million tons in 2006. Asia has the highest rate on a quantity basis followed by Europe and North America. In 2006, a turnover of US\$17.6 billion (13 billion euro) was reached mostly in Europe, followed by North America and Asia. The global demand on pigments was roughly US\$20.5 billion in 2009, around 1.5–2% up from the previous year. It is predicted to increase in a stable growth rate in the coming years. The worldwide sales are said to increase up to US\$24.5 billion in 2015, while it reached US\$27.5 billion in 2018 (<http://www.acmite.com/market-reports/chemicals/world-pigment-market.html>). One class of pigments that has received substantial attention is the carotenoids, particularly β -carotene. There are several types of microbial carotenoid: β -carotene, lycopene, lutein, zeaxanthin, canthaxanthin, astaxanthin, and rhodoxanthin. Only β -carotene and lycopene are currently produced by microorganisms for use in foods, to intensify or modify the color of fats, oils, cheeses, and beverages. They are also used as a feed supplement to enhance the color of egg yolks and chicken flesh. β -Carotene, lutein, and other xanthophylls are abundant in green plants, algae, cyanobacteria, and photosynthetic bacteria. Processes for the large-scale production of β -carotene and lutein from fungi and algae, respectively, were developed in the 1960s but were later abandoned because they could not compete with synthetic manufacturing.

β -Carotene is a precursor of vitamin A, and its production has been studied using various species of algae, fungi (*Neurospora crassa*, *Penicillium sclerotiorum*, *Phycomyces blakesleanus*) and also yeasts (*Rhodotorula*). However, these microorganisms do not produce β -carotene in economical quantities. *Blakeslea trispora* was the basis for the large-scale industrial production of β -carotene. The mating of the two sexual forms resulted in a dramatic increase of β -carotene yield. While certain nutrients such as amino acids, hydrocarbons, and lipids stimulate the formation of β -carotene, certain chemicals that activate the enzymes in the carotenogenic biosynthetic pathways have been found to have a greater effect. These compounds have been found in *Phycomyces* and *Blakeslea* and include β -ionone, limonene, citrus pulp, retinol, and certain aromatic compounds and nitrogenous heterocyclic compounds (iproniazid, isoniazid). Under optimal conditions, involving the addition of antioxidants and carotenogenic substances, the final yield is low (3–3.5 g/L). For this reason, β -carotene is not produced commercially, except in a few countries in eastern Europe.

Golden rice is a variety of *Oryza sativa* rice produced through genetic engineering to biosynthesize β -carotene, a precursor of vitamin A, in the edible parts of rice (Ye et al.,

2000). The objective was to produce a fortified food to be grown and consumed in areas with a shortage of dietary vitamin A, a deficiency which is estimated to kill 670,000 children under 5 each year. In 2005, a new variety called *Golden Rice 2*, which produces up to 23 times more β -carotene than the original golden rice, was announced (Paine et al., 2005). Although golden rice was developed as a humanitarian tool, it has met with significant opposition from environmental and anti-globalization activists. The detailed techniques will be discussed in Section 6.1.

When another microbial source, the green microalga *Dunaliella salina*, is exposed to a particular set of stress conditions (high light intensity, high temperature, nitrogen and phosphorus deficiency, and particularly high salinity), exceptionally high quantities ($\leq 14\%$ dry weight) of β -carotene accumulate in its cells. Maximal β -carotene yield results from the use of a two-stage process in which the switching of the growth to the production phase is achieved by going from low to high salinity. Commercial production is carried out in large open ponds situated in or near salt lakes or solar salt works. This type of nonaxenic culture is possible because the conditions of high salinity (20–30% NaCl, w/v) create a medium that is virtually free of competing microorganisms and predators. A typical biomass yield per liter is 1 g of cells, containing about 10% (w/w) of β -carotene (Nelis and De Leenheer, 1991). Natural β -carotene is currently marketed as a powder or as a suspension in vegetable oil, at a higher price than its synthetic counterpart. By overexpressing a gene encoded for a regulatory enzyme, carotenoid compounds could be increased in plants and algae. The so-called *pds* gene is encoded for the enzyme phytoene desaturase, which involves in pathway from phytoene to β -carotene.

Lycopene is a deep red tomato-like pigment and has been found to be precursor in the biosynthesis of acyclic xanthophylls (as in the case of photosynthetic bacteria) or β -carotene. Hence, to obtain lycopene through fermentation, these biosynthetic reactions should be inhibited. In *B. trispora*, the cyclization of lycopene to β -carotene can be prevented by changing the growth conditions or by adding inhibitors to the medium. A neutral to slightly alkaline pH favors lycopene formation, whereas at more acidic pH values, biosynthesis proceeds further to β -carotene. Several substituted amines and nitrogenous heterocyclics inhibit cyclization of lycopene to β -carotene. The yield for *Blakeslea* is only 0.15–1.0 g/L, while for *Streptomyces* the yield is 0.5 g/L so far (Nelis and De Leenheer, 1991). Recently, Microbia Inc. (USA) developed metabolic engineering capabilities to develop microbial strains that produce commercially significant levels of lycopene over 5 g/L via fermentation. This company has established the feasibility of this platform for producing a wide variety of ingredients, spanning the food, feed, and commodity chemical sectors (www.foodproductdesign.com/news/2010/01/microbia-targets). Also an improved method of production of lycopene by the fermentation of selected strains of *B. trispora* has been reported (EP 1471151 B1, 2009).

Xanthophylls are mixtures of colored carotenoids, which include lutein, canthaxanthin, cryptoxanthine, neoxanthin, violaxanthin, and zeaxanthin. *Basidiomycetes* such as *Dacrymyces deliquescens* and the green algae *Spongiococcus excentricum*, as well as *C. pyrenoidosa*, have been extensively studied for the production of xanthophyll pigments. *D. deliquescens* may produce up to 40 mg/L of xanthophylls, while *C. pyrenoidosa* produced 650 mg/L from glucose, urea, and phosphate under external light (Nelis and De Leenheer, 1991). Heterotrophic microbial production of xanthophyll pigments from microalgae has recently been patented (WO 2012047120A1, 2012). *Lutein* is a carotenoid typical of higher photosynthetic organisms. It is abundant in green algae and plants, but absent from cyanobacteria and phototropic bacteria. Several patents describe special conditions for growing lutein-rich microalgae that belong to *Chlorella*, *Chlorococcum*, *Chlamydomonas*, *Spongiococcum*, and other genera. Like lutein, *zeaxanthin* is also a typical chloroplastidic

pigment. It differs, however, from lutein in that it also occurs in cyanobacteria and in a limited number of nonphotosynthetic bacteria (where it is sometimes present as a glycoside). Although information is scarce, the commercial production of zeaxanthin from a species of *Flavobacterium* appears to be approaching the stage of commercialization. *Brevibacterium* KY-4313, a hydrocarbon-utilizing bacterium, produces canthaxanthin together with smaller amount of echinenone and β -carotene. *Brevibacterium* represents the most thoroughly investigated canthaxanthin-producing microorganism showing potential for biotechnological use. A marine bacterium, *Rhodococcus maris*, and the green microalga, *Dicryococcus cinnabarinus*, have also been shown to produce canthaxanthin.

Astaxanthin is somewhat similar to canthaxanthin in its distribution in the microbial world. *Phaffia rhodozyma*, a red yeast, is unusual in that it ferments sugars and contains astaxanthin, but other red yeasts are strictly aerobic and contain β -carotene and/or monocyclic carotenes. Mutant strains displaying a peculiar morphology and enhanced pigmentation have been obtained by growing the *Phaffia* parent strain in the presence of antimycin A, a powerful inhibitor of the respiratory chain. To facilitate the liberation of the pigment, a preliminary autolysis of the cells in distilled water or a citrate buffer has been suggested. Alternatively, the resistant cell walls could be digested by the enzymes secreted by *Bacillus circulans*. The commercial use of the *Phaffia* pigment may be restricted, however, because its absolute configuration differs from that which normally occurs in salmonids. In April 2009, the US FDA approved astaxanthin as an additive for fish feed only as a component of a stabilized color additive mixture. The color additives astaxanthin, ultramarine blue, canthaxanthin, synthetic iron oxide, dried algae meal, *Tagetes* meal and extract, and corn endosperm oil are approved for specific uses in animal foods. *Haematococcus* algae meal (algaltech.com) and *Phaffia* yeast for use in fish feed to color salmonoids were added in 2000. Since 1997, there have been five novel food applications concerning products that contain astaxanthin extracted from these novel sources. In each case, these applications have been simplified or substantial equivalence applications, because astaxanthin itself is recognized as a food component in the EU diet. The cost of astaxanthin production, high commercial price, and lack of leading fermentation production systems, combined with the shortfalls of chemical synthesis, resulted in alternative fermentation production methods such as metabolic engineering. The metabolic engineering of bacteria (*E. coli*) recently allowed production of astaxanthin at >90% of the total carotenoids, providing the first engineered production system capable of efficient astaxanthin production (Scaife et al., 2009; Lemuth et al., 2011). Recent work has suggested that efficient biosynthesis of astaxanthin can proceed from β -carotene to astaxanthin via zeaxanthin. Carotenoid extraction has been studied extensively; for example, the extraction of canthaxanthin (a precursor to astaxanthin) was studied within an *E. coli* production process and found that extraction efficiency was increased substantially when two solvents, acetone and methanol, were used sequentially rather than as a combined solution (Scaife et al., 2012). *Monascus* pigments, produced by the fungi *Monascus anka* and *Monascus purpureus*, are the best known of the microbial colorants. The pigments have been used traditionally in the orient as a general food colorant (wine, bean curd, rice) and as a medicine. The heat stability and a color range from yellow and red make them useful colorants. *Rhodoxanthin*, a deep red carotenoid identified in the nonphotosynthetic methylotroph *Pseudomonas extorquens*, may serve as an alternative to astaxanthin. *Monascus* sp. belongs to the family *Monascaceae* of the phylum. *Monascus* sp. has been used primarily in Southern China, Japan, and Southeast

Asia for making red rice wine, red soybean cheese and Anka (red rice) (Lin et al., 2008; Dikshit et al., 2013).

5.4.4 Economics

The production of vitamins is an important sector of the food industry. Vitamin output in 2013–2015 is expected to be around 220,000–280,000 tons, of which, export volume by China will make up 75–80%, China's vitamin C production capacity accounted for 80–90% of the global total.

In 2009, the UK market for dietary supplements and vitamins was worth more than £670 million. In 1978, it represented \$670 million for the most important vitamins. The worldwide production of B₂, riboflavin was estimated about 9600 tons/year during 2011 and 2012, mostly produced by China companies and recent years have seen a considerable growth in the commercial and nutritional importance of this vitamin. Vitamin B₁₂ world production exceeded 12,000 kg in 1980. Vitamin C production alone was well over 10,000 tons in 1985. Only vitamin B₁₂ and riboflavin are produced commercially by a biological method. Microbial processes are known for other vitamins but are not used on a commercial scale.

B₁, B₅ (calcium pantothenate), and B₆ still maintained a good momentum in B vitamins, whose export volume reached 5260, 9642, and 4487 tons, respectively.

The global aquaculture industry increases in the proportion of 24% per year and it is expected to be \$49 billion in 2010 (<http://www.articlesfactory.com/articles/business/production-methods-of-natural-astaxanthin.html>). Natural astaxanthin market is mainly affected by the global production capacity constraints and the international market price is above \$3,000 per kilogram. Astaxanthin feed market is well over 185 million dollars with the growth rate of 8% per annum. It shows great market potential. Natural astaxanthin is also a feed additive for treating egg poultry and livestock.

Summary

Few known microorganisms lend themselves to commercial exploitation for pigments. Only β -carotene is produced, on a limited scale and at high cost, from one microbial source. Plant cell culture is another way to produce pigments, but it also is an inherently expensive technique. Only products of substantial price and volume can be justified as targets for commercial development of plant cell culture, and then only when concerns for supply and quantity remove the feasibility of cheaper routes. The primary determinant of economic viability is likely to be the volumetric productivity of the components by the process. The main problems to be solved are low product yields, slow growth of the producing microorganisms, and high medium costs; additional complexity results from the presence of intracellular products and multistep pathways. The ultimate solution to these obstacles may eventually be delivered by the application of genetic engineering and metabolic engineering technologies. Either the biosynthetic pathway for a given pigment could be cloned and expressed in a fast-growing microbe or the pathway could be metabolically engineered in the parent organism to obtain overexpression. Astaxanthin production in *E. coli* has recently achieved up to over 90% of the total carotenoids. A great challenge therefore exists for researchers in this very promising area.

5.5 Mushrooms

5.5.1 Introduction

Edible mushrooms are the fruiting bodies of macroscopic, edible fungi and have formed part of the human diet as a rich source of protein (24–44% in *Agaricus*), vitamin B, and vitamin C for centuries. Wild mushrooms grow on decomposing organic matter and on different substrates under different climate conditions. The increased demand for wild-grown fungi has led to the development of various techniques for culturing mushrooms on certain waste materials under controlled environmental and nutritional conditions. Cultivated edible mushrooms are one of the few examples of a microbial culture used directly as a human food. In Europe and North America, the most widely cultivated species is *Agaricus bisporus*, which is cultivated on composted wheat straw. Each year, millions of tons of these mushrooms are produced for direct consumption and for the food industry.

The United States produced 862 million pounds of mushrooms during 2010–2011, up 9% from the previous season. The total value of the crop was \$1.02 billion in 2011, up 10% from the previous season. The number of commercial mushroom growers was 282. Four other popular species that have specific light requirements to induce fruit formation are the Japanese shiitake mushroom, *Lentinus edoides*; the winter mushroom, *Flammulina velutipes*; the Chinese straw mushroom, *Volvariella volvacea*; and the oyster mushroom, *Pleurotus ostreatus* (as well as other *Pleurotus* species). Large-scale production is found mainly in North America, Europe, Australia, and Southeast Asia. *A. bisporus* is now cultivated in at least 70 countries around the world. Global production in the early 1990s was reported to be more than 1.5 million tons, worth more than USD 2 billion (Chang, 1993). During the 2010–2011 season, the sale of *Agaricus* mushrooms totaled \$966 million (www.agmrc.org/commodities_products/specialty_crops/mushrooms-profile/). The bottom mushroom, *A. bisporus*, accounts for 60% of the world total cultivated mushrooms, followed by the shiitake with about 14%, then the straw and oyster mushrooms, each with about 8%. The remaining 10–15% is provided by cultivated mushrooms, which include the ear fungi (*Auricularia* spp.), the white jelly fungus *Tremella fuciformis*, the winter mushroom *Flammulina velutipes*, and the viscid mushroom *Pholiota nameko*.

Mushrooms have good quality proteins with lysine and tryptophan as well as carbohydrates (4.5–5.0%) in the form of glycogen, chitin, and hemicellulose instead of starch. The fat is very low (0.3%) but is rich in linoleic acid, an essential fatty acid. Furthermore, mushrooms contain good source of vitamin C and vitamin B complex, particularly thiamine, riboflavin, niacin, biotin, and pantothenic acid. Folic acid and vitamin B12, which are absent in most vegetables, are present in the mushrooms. They also supply a range of valuable minerals especially potassium and iron (Mehta et al., 2011). Mushrooms have been used not only as a source of food but medicinal resource as well (Aida et al., 2009). β -Glucan is one of the major structural component of fungal cell walls, that appear to stimulate both the innate and adaptive immunity of the host, followed by a wide range of immunopharmacological activities, particularly antitumor activities, via their cytokine production and signaling cascade (Cheung, 2008). Shiitake extract was found to be effective as an antimicrobial substance and was significantly more antibacterial than ciprofloxacin. Polysaccharides extracts of medicinal mushrooms act as natural antioxidants and possess immunomodulatory properties (Koushki et al., 2011). Though packaging, coating, refrigeration, and dipping in sorbitol and CaCl_2 dipping are the most common methods to extend the shelf life of mushrooms, the alternative to extend mushrooms' shelf life during postharvest storage and commercialization was modified atmosphere packaging (Koushki et al., 2011).

5.5.2 Cultivation

5.5.2.1 Cultivation of the common white mushroom *A. bisporus* is one of the most commonly cultivated mushrooms to enjoy true economic importance in the Western world. The old practices pioneered by French horticulturalists have been gradually refined and modified to the point of developing biotechnological methods for the mass production of this fungus on a large scale. Figure 5.13 shows the stages of the cultivation of *A. bisporus*.

The modern cultivation of mushrooms includes two major process steps: the preparation of suitable composts and the germination of spores and fruitification. Nutrients are provided as composts, usually from mixtures of wheat straw and horse manure. A mixture of horse manure and various supplements (soybean meal, cottonseed meal, etc.) is subjected to hot rotting. Modern mushroom growing is a carefully controlled activity, and all the processes take place in specially built mushroom houses, inside which the environmental conditions can be effectively controlled. It is essential to ferment or compost the straw mixture to obtain a medium that will remain stable and in which available nutrients are reduced at low levels for composting organisms. *A. bisporus* derives most of its nutrients from lignin, cellulose, hemicellulose, and protein, and the bacterial biomass contributes nutrients to this species. The length of time required to prepare the compost varies, depending on the age of the manure, but normally it takes at least 2 weeks to achieve the desired conditions. If the compost is not adequately prepared, mesophilic bacteria may take over the growth of *A. bisporus*.

In the hot rotting process, the temperature inside the heap reaches 60–70 °C. Composting is accelerated by the repeated turning over of the heap layer. After 8–9 days the compost is packed into boxes and subjected to pasteurization at 60 °C, which will kill animal pests as well as harmful microorganisms. The pasteurized compost is cooled to 25 °C,

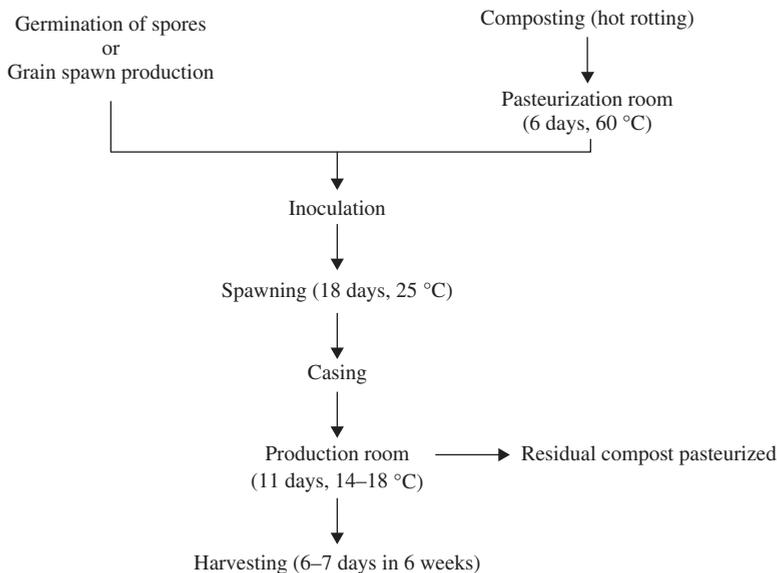


Figure 5.13 Flow diagram of stages in the production of *Agaricus bisporus*.

inoculated with fungal spores at amounts of 0.5–1% inoculum (v/v), and placed in an incubation chamber with a controlled air temperature (17 °C). After a few weeks of lag period, rapid mycelial growth sets in. The compost bed is then covered with a casing of soil or other suitable material, usually a mixture of peat neutralized with chalk or vermiculite (an artificial potting medium). The mycelia colonize this layer, and mushroom fruiting bodies are produced. When the growth reaches the surface, the temperature is lowered to 15–18 °C and incubation is further continued for 11 days with a copious supply of oxygen. The germination of spores is carried out by taking spores or fragments from a mature fruiting body selected for desirable qualities such as color, taste, and vigor. The master culture is prepared by germination of the spores on sterile nutrient medium and is used to prepare the spawn, which is then distributed to growers. Formerly, spawn was produced by inoculating a suitable sterile medium such as horse manure, but nowadays a variety of grain spawn seeded in wheat, rye, or sorghum is available commercially.

The first mushrooms may be harvested after about 3 weeks. Following the first crop, several additional periods of fruiting usually occur at intervals of 2 or 3 weeks. During the whole harvesting period of 6 weeks, yields of 0.5–1 kg of mushrooms per kilogram of compost dry matter (or 15–30 kg/m² cultivation area) are obtained. The residual compost, which has been degraded to about 50%, is pasteurized and used as a fertilizer.

Major problems in the cultivation of mushrooms are the preparation of uniformly good compost, and the prevention of infection of the cultures by harmful organisms such as viruses, bacteria, fungi, and worms, as well as insects. The external environment is also important in culturing mushrooms. Carbon dioxide is stimulatory to vegetative cells but inhibitory to reproductive fruit formation. In addition, microquantities of ethanol, acetone, ethylene, acetaldehyde, and some organic volatiles have been shown to stimulate fruit formation. High relative humidity (>50%) and temperatures greater than 18 °C tend to cause proliferation of *Pseudomonas tolaasii*, responsible for a common bacterial disease called mummy.

5.5.2.2 Cultivation of shiitake mushrooms The shiitake, *Lentinus edodes*, grows naturally in logs of the shii tree, *Pasania cuspidata*, and other deciduous trees such as beech, chestnut, horn beam, and oak. The logs are soaked in water and pounded to make holes, into which are hammered small cylinders of wood chips or sawdust inoculated with a spawn of mycelia. The infected logs are then placed in a carefully selected site in the forest, known as the *laying yard*. The best laying yards are usually in well-ventilated clearings or positioned at the edge of the forest. Cultivation also can take place in a polypropylene bag or space bag, which contains sterilized sawdust and rice bran (9:1 v/v) with a moisture content of about 65%. When mycelia have grown inside the bag, the bag is transferred to the clearinghouse and opened, and the mushrooms are allowed to grow at 80–90% humidity. This method is currently used in Taiwan and Japan. After storage for a year, the logs are thoroughly watered. The formation of fruiting bodies takes place at 12–20 °C. Since shiitake mushrooms contain substantial amounts of 5'-ribonucleotides, especially 5'-guanylate (1–2 mg/g dry mushroom), their extracts have been used as flavor potentiators. The increased content of guanylate is likely due to the hydrolysis of RNA by ribonuclease during cooking at 70 °C.

5.5.2.3 Cultivation of the oyster mushroom The cultivation of the oyster mushroom, *P. ostreatus*, is frequently carried out in both temperate and tropical climates. The requirements are simpler and less costly than those of *A. bisporus*, but only 3% of the world mushroom production has been attributed to *Pleurotus*. The substrates do not have to be composted. Heat-treated straw of wheat and rice, heated sufficiently to eliminate any

contaminants, is commonly used for the commercial production of cultivated mushrooms. Before inoculation with the fungal spores, this substrate is chopped, treated with water, pasteurized at 80 °C, and compressed into balls. The yield of fruiting bodies may reach to 40–50% of the dry substrate used.

5.5.2.4 Cultivation of other mushrooms Rice or paddy straw mushrooms, *V. volvaceae*, have traditionally been grown on paddy straw, but other vegetable wastes (cotton, banana leaves, sawdust, etc.) may be used. The method used to grow this mushroom is similar to that of the common white mushroom. However, the paddy straw mushroom is mainly cultivated on a small scale, using less scientific, traditional methods. The jelly fungus, *Auricularia polytricha*, is highly prized by gourmets in the Far East and is produced in large quantities in China. This crop is grown on logs or in polyethylene bags. Several other fungi have been grown on a small or experimental scale. These are *P. nameko*, *Pleurotus* spp., *Stropharia rugosoannulata*, *Auricularia* spp., *T. fuciformis*, *Tuber* spp., *Tricholoma matsutake*, *F. velutipes*, *Morchella esculenta*, *Fistulina hepatica*, *Armillaria*, and *Dictyophora indusiata*. The major obstacle for large-scale production of many fungi is the difficulty of obtaining economically viable yields.

5.5.3 Culture preservation

Successful mushroom production depends largely on the proper maintenance of stock culture and spawn. The spawn must be of excellent productivity, having good flavor, texture, and color, as well as resistance to pest and disease. Stock cultures of both sporulating and nonsporulating fungi are often maintained in an actively growing stage, commonly by freezing in liquid nitrogen (–196 °C), and transferred at defined time intervals. When sporulation or mycelia production is abundant, fungi are cultured on agar slants or millet on which mycelia had grown. If sporulation is sparse or the culture is nonsporulating and the mycelia are sparse, cultures are grown in broth medium and fragmented in a sterile Waring Blender. The yeastlike fungi *T. fuciformis*, yields colonies in liquid culture, and thus pipetted small cells are able to grow in shake flasks containing a suitable medium. Spawn conservation by cryogenic freezing rather than periodic transfers of cultures eliminates degeneration, mutation, and contamination.

Summary

Over the past two decades, fresh and processed mushroom consumption has increased drastically. Mushrooms are highly valued as a good nutrition source, and more than 30 species are sold commercially. Mushroom cultivation is interesting because of the conversion of what otherwise would be waste material into a nutritious foodstuff. The only successful species are the saprophytic species, which feed on decaying organic matter. Mushroom production in a controlled environment has become an example of highly advanced technology, but further developments will surely lead to an increased efficiency and yields of the products. Recent advances in submerged culture have also led to the isolation of mushroom flavor compounds, which may be used as flavorants.

Mushrooms have good quality of proteins, carbohydrates, minerals as well as good source of vitamin C and vitamin B complex, and folic acid and vitamin B12, which are absent in most vegetables. They have medicinal properties including a wide range of immunopharmacological activities, particularly antitumor activities.

5.6 Cocoa, tea, and coffee fermentation

5.6.1 Introduction

Cocoa, coffee, and tea are plants that grow between approximately the same latitudes and can be regarded as plants of the rain tropics and subtropics. Cocoa is believed to have originated in the Orinoco and Amazon valleys of South America. Coffee may well be a native of Ethiopia and is now well established in Africa, South America, and India. The worldwide production of cocoa bean has increased to 3,607,052 ton in 2004. The production increased by 131.7% in 30 years, representing a compound annual growth rate of 2.8% (www.oacng.com/2013/07/world-production-top-cocoa-beans.html). Malaysia and Indonesia and the Ivory Coast have become the major producers in the world cocoa market. Tea is largely produced in China, India, Sri Lanka, Japan, and Taiwan.

Fermentation is the first stage in the preparation of cocoa beans for the market, and the main purpose is to prepare a stable product with the qualities of flavor and aroma desirable for the manufacture of cocoa and chocolate. The coffee fermentation process is designed to improve the appearance of the finished green beans, resulting in a higher grading of the liquors. Tea fermentation is different: it is an oxidative process that is initiated enzymatically and does not require any microflora involvement. However, leaf microflora such as bacteria and yeasts have an influence on the quality and taste of black tea. It is under optimum fermentation conditions that all three crops will develop a flavor of the desired standard. Thus, it is very important to understand the microbiological, biochemical, and chemical aspects of all three processes.

5.6.2 Cocoa fermentation

The seed of the cocoa tree, *Theobroma cacao*, consists of two cotyledons and a radicle surrounded by a testa or seed coat, which is enveloped by a layer of mucilaginous endocarp termed the *pulp*. The cocoa tree bears fruits called pods, and each pod contains 40–50 seeds covered with a white mucilage. The chemical and biological changes taking place during fermentation result in the breakdown and removal of the pulp and cause the death of the bean, with consequent release of enzymes capable of reducing astringency. The appropriate color for cocoa beans depends on the attainment of the right degree of fermentation. The detailed cocoa fermentation process and microbiology aspects can be reviewed (Nielsen et al., 2013).

5.6.2.1 Harvesting Harvesting practices and processing before fermentation differ not only from country to country but also from farm to farm. This step consists of picking and breaking the ripe pods, removing the beans, and transporting them to the site of the fermentation processes. When the beans are received within 24 h of the removal of the pods, the fermentation procedure may not be affected. On smaller farms, however, the pods may be stored for a few days before fermentation, in which case the process may be affected. A flow diagram for the production of cocoa is shown in Figure 5.14.

5.6.2.2 Fermentation Fermentation is one of the most important steps in cocoa processing because the chocolate flavor of cocoa develops during this stage. Three main methods are currently used in various parts of the world. The first is a simple method called *heap fermentation*. Here the beans are piled up on top of and underneath plantain leaves,

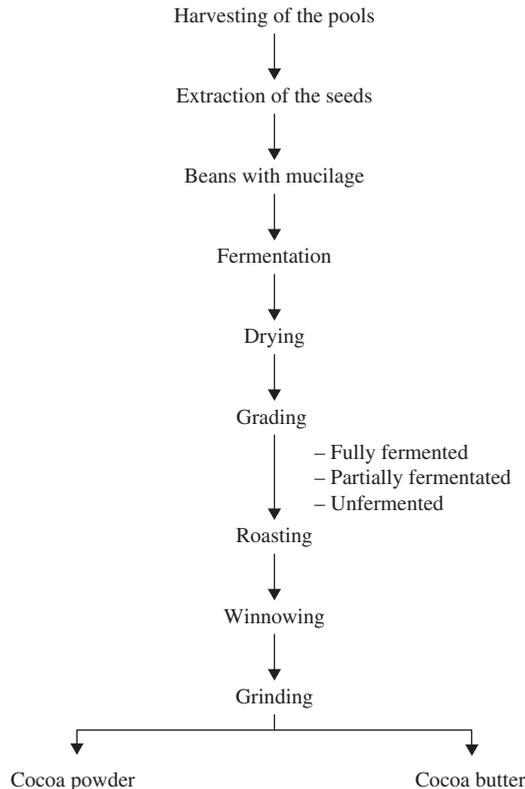


Figure 5.14 Flow diagram for the production of cocoa.

which cover the surface completely. The pile is kept together for 6 days, being turned on the second and fourth day to avoid variation in flavor and quality and to aid aeration. Heap fermentation is limited to the West African countries of Ghana, Nigeria, and the Ivory Coast.

The second method, called *box fermentation*, uses large hardwood boxes holding up to 1.5 tons. These boxes have slatted bottoms or holes in the sides and bottoms to aid drainage and oxygenation. Total fermentation time is 4–9 days. Box fermentation is common in Trinidad, Brazil, and Malaysia.

In the third method, fermentation is carried out in properly equipped buildings called *fermentation houses*. This method is used mostly on organized plantations. The fermentation house is usually a building (24 ft long \times 21 ft wide) with a row of fermentation boxes on each side and a passage down the middle. A wide space between the eaves of the roof and the top of the wall on the lee side of the house permits free movement of air and the escape of gases from the fermented cocoa. During fermentation, the external appearance of the beans changes. Initially they are pinkish with a covering of white mucilage, but gradually the color darkens and the mucilage disappears. When this oxidative color change is uniform (orange-brown), the beans are ready for drying.

The fermentations involved in the processing of cocoa beans are carried out by yeasts and acetic and lactic acid bacteria. The first major microbial activity is an alcoholic fermentation, which may be detected by the aroma of ethanol. Only few microorganisms are to be found in fresh cocoa pulp. But after 24 h of exposure to the air, yeast are found to proliferate rapidly, forming about 90% of the microbial population. Many enzymes participate in the breakdown of cocoa pulp, resulting in the hydrolysis of polysaccharides and the reduction of disaccharides to monosaccharides, which are fermented by yeasts; then ethanol is produced. These reactions are exothermic and will raise the temperature of the fermenting cocoa. Acetic and lactic acid fermentations occur simultaneously. The predominant aroma in cocoa fermentation is acetic acid, which is brought about by the oxidation of ethanol to acetic acid. The organisms involved include *Acetobacter rancens*, *Acetobacter xylinum* and *Acetobacter ascendens*. These reactions raise the temperature to 50 °C. The elevated temperature and the presence of acetic acid kill the fermenting yeasts, darkening the cocoa beans, and killing the embryos in the beans. LAB can be found at the beginning and persist until the end, while their major activity is the metabolism to lactic acid of a wide range of sugars. Other substrates the LAB may attack are malic and citric acids, which produce lactic acid, with the production of acetic acid and carbon dioxide. However, these bacteria have a low optimum growth temperature and thus would not be favored by the elevated temperatures of fermenting cocoa beans. The activity of acetic and LAB is believed to be essential in the development of the chocolate flavor. However, more work is needed to understand the complex biochemistry involved in this process and to identify specific microorganisms and their role. When fermentations are unsuccessful, the reason is often the presence of overripe pods or underripe pods, which may not contain sufficient sugar in the pulp. Beans from diseased pods and wet beans should not be fermented with the healthy ones, to avoid common infections with molds.

5.6.2.3 Drying and roasting Fermented beans must be dried to a final moisture content of about 6–8% to ensure good keeping qualities by preventing mold growth, and to permit storage for considerable periods. Drying can be achieved either by exposure to the sun or by artificial heating. In sun drying, large quantities of beans are laid on raised platforms with sliding roofs, which can be drawn across for protection during rain. This method takes over a week in dry weather and as long as 3 weeks in moist climates. Artificial or mechanical drying has the objective of removing excess moisture from the beans in as short a time as possible. This is done by subjecting the beans to a stream of hot air, and the total drying time is about 48 h. The roasting temperature may range from 70 to 180 °C. This method has several advantages: it has a large capacity; it is less labor intensive; and the enzymatic activities are rapidly stopped. The important reactions during roasting are of course the development of flavor by Maillard reactions between amino acids and sugars, and Strecker degradation. Good flavor depends on the bean variety and on the curing process used. More than 300 different compounds formed during roasting have been identified.

5.6.2.4 Winnowing and grinding Winnowing is a simple process for separating the nib, which is the cellular mass containing 50–56% cocoa fat (butter) or kernel from the inedible shell. This is one of the most important operations in cocoa processing, for failure to remove the shell results in lower quality cocoa and chocolate products. Grinding of the nib or kernel of the cocoa beans is the first step in chocolate liquor production by a pin or a vertical or horizontal ball mill. Cocoa powder is the food prepared by pulverizing the material remaining after part of the cocoa butter has been extracted. Cocoa butter is the fat obtained by subjecting chocolate liquor to hydraulic pressure.

5.6.3 Coffee fermentation

The name coffee is derived from the Turkish pronunciation “kahveh” of the Arabic word “gahweh.” The coffee fruits, known as *cherries*, usually contain two seeds embedded in an outer fleshy coat. Coffee beans are the fermented and roasted seeds from the berries of the coffee tree, *Coffea arabica*. The coffee tree can grow to a height of 10 m, but it is pruned to ease the harvesting of the fruits. The green coffee bean is composed of about 60% carbohydrate, 14% protein, and 13% lipid, with small quantities of the nonvolatile acids, trigonelline, caffeine, and theobromine. The purpose of fermentation is to remove the mucilage, which adheres close to the parchment layer or outer covering of the seed. The best quality beans are blue, whereas yellow or brown coloration is indicative of poor quality. The flavors of coffee develop only during roasting and begin to form when the bean reaches 180–190 °C. Coffee is believed to be native to Ethiopia and other African countries. Today it is grown in about 50 countries including the Ivory Coast and Brazil (one of the largest producers). World coffee production for 2013/2014 is forecast at 146 million bags with 10% growth rate, down 4.4 million bags from the previous year due primarily to Brazil's Arabica trees entering the off-year of the biennial production cycle, and to a lesser extent, Central America and Mexico's continued struggle with coffee leaf rust (<http://www.fas.usda.gov/psdonline/circulars/coffee.pdf>).

5.6.3.1 Green coffee processing Coffee beans can be cured by either a dry or a wet process. The dry process is a nonmicrobial method that produces natural coffee. The over-ripe fruits may be left on the tree until partially dried by the sun, or artificial means of drying may be applied. This is a cheap process, which is used mostly in Brazil and in other countries where water is scarce in the harvesting season, but it is rarely widely used elsewhere. The wet process, a microbial method used for the production of high quality coffee, involves mechanical removal of the outer pulp, leaving the mucilage layer adhering to the bean. The mucilage may be removed by natural fermentation, by enzymes or alkali, or by mechanical force. The composition of the mucilage layer governs the course of fermentation. Polysaccharides are found to form 77.6% of the dry weight. This group of compounds is composed mainly of pectin and pectic acid. The pectin present in coffee has been found to have a degree of esterification of 70%, and chain length varies from 140 to 250 units. Several hydrolytic and oxidative enzymes such as galacturonase, α -galactosidase, peroxidase, and polyphenol oxidase have also been detected. Reducing sugars, sucrose, caffeine, chlorogenic acid, and amino acids are also found in the mucilage.

5.6.3.2 Coffee fermentation The fermentation of coffee is normally carried out in deep tanks open to the atmosphere, and it takes place spontaneously by bacterial and enzymatic action. There is a pronounced increase in the number of gram-negative microorganisms, and degradation of the pectinaceous mucilage is almost complete after 7–8 h. The predominant microflora of the coffee bean consists of bacteria from the genera *Erwinia*, *Escherichia*, and *Paracolobactrum*, filamentous fungi (*Aspergillus*, *Penicillium*, *Cladosporium*, and *Fusarium*) and yeasts (*Saccharomyces maxilianus*, *Saccharomyces bayanus*, *S. cerevisiae* var. *ellipsoides*, *Schizosaccharomyces* spp.). *Erwinia dissolvens* is of particular importance, since it is the only one of the species that produces pectinolytic enzymes. LAB such as *Lactobacillus brevis* occur at later stages of fermentation. The fermentation period may last from 6 to 72 h depending on the rate of mucilage digestion, which in turn depends on the temperature, the thickness of the mucilage layer, and the concentration of pectic enzymes. The duration of fermentation has also been found

to depend on coffee variety, climate and regional factors, degree of anaerobiosis, and microflora affecting degradation of mucilage.

Commercial enzymes such as Pectozyme, Cofepec, and Ultrazym, which are a mixture of pectinase, hemicellulase, and cellulase, are also used. Because of financial constraints, however, these enzymes have not been widely used. The anaerobic process produces the best quality coffee, and the higher ethanol content is produced during the anaerobic conditions. Acetic acid is detected early in the fermentation, and the presence of acetaldehyde may be indicative of spoilage. Propionic and butyric acid levels also increase after 23 h, and they have been associated with the formation of off-flavors.

5.6.3.3 Washing, soaking, and drying After fermentation, residual mucilage is removed by washing (Figure 5.14). The washing process is followed by a period of soaking, which improves coffee quality. During this period, monosaccharide levels as well as activities of α -galactosidase increase correspondingly. Actually, the greater α -galactosidase activity and low oligosaccharide levels in high quality coffee support this hypothesis. These chemical changes may improve the quality of the final beverage. Soaking is followed by drying, which reduces the moisture content of the bean to 10–11%.

Coffee quality and drying temperature appear to be linked to one another and sun drying seems to produce the best results. The optimum drying temperature was shown to be 45 °C; a temperature between 50 and 60 °C produced poor quality drinks, while temperatures of 60–90 °C produced a reasonable beverage. The final step is roasting the beans, which is usually achieved by hot combustion gases in rotating cylinders. It is at this stage that the full coffee flavor and aroma develop. A total of 670 flavor compounds are known to be produced in roasted coffee (Table 5.6). These are extremely important because minute quantities of many flavors must be in balance in a cup of coffee of the highest quality. More updated

Table 5.6 Class of volatile compounds identified in roasted coffee

Sulfur compounds
Thiols
Hydrogen sulfide
Thiophenes (esters, aldehydes, ketones)
Thiazoles (alkyl, alcoxy and acetal derivatives)
Pyrazines
Pyrazine itself
Thiol and furfuryl derivatives
Alkyl derivatives (primarily methyl and dimethyl)
Pyridines
Methyl, ethyl, acetyl and vinyl derivatives
Pyrroles
Alkyl, acyl and furfuryl derivatives
Oxazoles
Furans
Aldehydes, ketones, esters, alcohols, acids, thiols, sulfides and in combination with pyrazines and pyrroles
Aldehydes and ketones
Aliphatic and aromatic species
Phenols

Source: Adapted from Buffo and Cardelli-Freire, 2004. *Flavour Fragr. J.* 19:99–104. Reproduced with permission of Wiley.

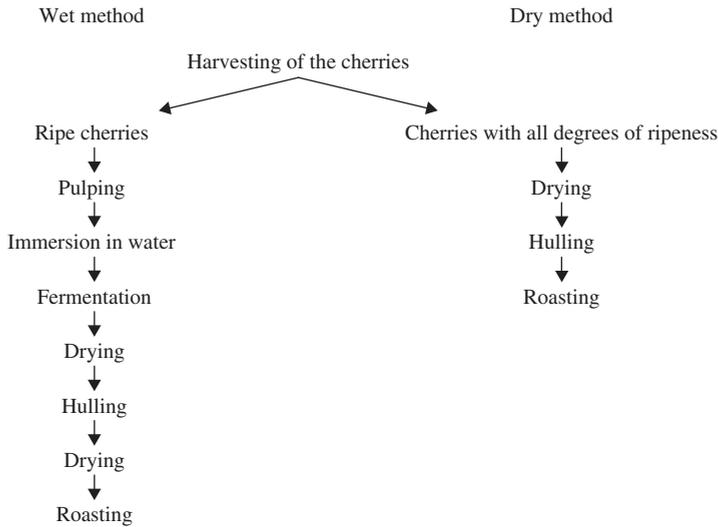


Figure 5.15 A flow diagram for the production of coffee.

volatile composition and antioxidant activity of Arabica coffee can be reviewed (Figure 5.15) (Cheong et al., 2013).

5.6.3.4 Over- and underfermentation Overfermentation of the bean gives poor quality coffee, characterized as having an onionlike flavor. Overfermented beans, commonly termed stinker beans, are produced at the death of the embryo due to the increase of end product fermentation or to anaerobic conditions of river water containing a high biochemical oxygen demand (BOD). If the coffee is underfermented, some mucilage will remain on the bean after washing, and a green fruit flavor will result. Figure 6.14 shows a flow diagram of coffee processing.

5.6.4 Tea fermentation

Tea is the name given to the processed leaves of the tea plant, *Camellia sinensis* or *Thea sinensis*. All teas are products of *C. sinensis* leaves and buds, the only difference is how they are processed: non-fermented tea (e.g., green tea), semi-fermented tea (e.g., oolong tea), fermented tea (e.g., black tea), non-oxidized tea (e.g., white tea) and post-fermented tea (e.g., dark tea). Black tea is processed in a way that allows for fermentation, whereas green tea's processing avoids the fermentation process. As a result, green tea retains maximum amount of antioxidants and the rich nutrients and grassy flavors of these light teas. To avoid the oxidation of green tea, white tea and the rare yellow tea, growers immediately apply heat to and dry the tea leaves soon after picking. Oolong is a traditional Chinese tea (*C. sinensis*) produced through a unique process including withering under the strong sun and oxidation before curling and twisting. White tea is minimally processed and not oxidized, meaning it retains the natural antioxidants, but does not develop as much flavor, color, or caffeine. For post-fermentation of dark tea mainly produced in China, the tea leaves undergo a microbial fermentation process after they

are dried and rolled. This tea is then left to age and ferment for long periods of time until the tea leaves turn a dark black color. With unfermented tea, the leaves are heated after harvesting to inactivate the enzymes of the plant. There is no fermentation in this process, which means that neither color change nor flavor development occurs. The primary objective in green tea manufacture is to prevent catechin oxidation, while imparting an attractive appearance of leaf and developing the characteristic green tea aroma. Tea has been cultivated for a few thousand years, but nowadays tea is grown in Asia, Africa, the Middle East, and South America. The tea plant is a tree that can grow up to 18 m tall, but in a tea plantation, the plants are pruned to form bushes of low height, to facilitate the harvesting of the leaves. Teas are traditionally classified based on how they are processed: nonfermented tea (e.g., green tea), semi-fermented tea (e.g., oolong tea), fermented tea (e.g., black tea), and post-fermented tea (e.g., dark tea) (Alcazar et al., 2007). Prices of black tea remain firm in the global market despite world tea production surging by 13.3% in first 5 months of the current year to 686.7 million kg (<http://articles.economictimes.indiatimes.com/keyword/tea-production/featured/5>). A rising demand from emerging markets such as the United States, Australia, Iran, Iraq, Egypt, Pakistan, and Middle East has pushed up global tea prices. Tea production has gone up in leading producing nations of India, Sri Lanka, and Kenya. Kombucha tea is a popular fermented tea with a symbiotic culture of bacteria and yeasts. It has been claimed that Kombucha tea helps to detoxify the liver, boosts the immune system, improves digestion, and helps with joint pain, due to probiotics and enzymes.

Modern clinical medicine recognizes a variety of health benefits of tea, such as moderating the risk of cancer, reducing cholesterol, preventing cardiovascular diseases, and lowering blood pressure (Wikipedia.org). These could be due to both flavonoids and epigallocatechin gallate (EGCG) which are antioxidants. Tea also contains catechins, a type of antioxidant. In a freshly picked tea leaf, catechins contain up to 30% of the dry weight. Catechins are highest in concentration in white tea and green tea. The FDA in 2005 reports that green tea consumption has no known beneficial effect on breast or prostate cancer. All tea leaves contain fluoride and aluminum. Although low concentrations of fluoride are maintained in many public water supplies for dental health, very high fluoride intake (over 2 mg/day for children, 4 mg adults) increases the risk of osteofluorosis and fractures. There is evidence that over-intake of teas produced using mature leaves (e.g., brick tea) or a combination of mature and young (e.g., through inefficient mechanical harvesting) can cause fluorosis in humans. As with fluoride, the tea plant is a natural bioaccumulator of aluminum, particularly in older leaves, with aluminum concentrations of up to 30,000 ppm aluminum by dry weight. While large doses of aluminum can cause toxicity in humans, but there is no evidence that consuming any quantity of made tea has had this result. The caffeine in tea is a mild diuretic. Tea also contains oxalate, overconsumption of which can cause kidney stones, as well as binding with free calcium and other minerals in the body. However, the bioavailability of oxalate from tea is low, thus negative effect requires a large intake of tea. Tea contains more caffeine than coffee by dry weight. A typical serving, however, contains much less, since tea is normally brewed much weaker. Also contributing to caffeine content are growing conditions, processing techniques, and other variables. A preliminary metagenomic study of pure tea during pile fermentation (Lyu et al., 2013) revealed that major taxonomic bacterial phyla are Proteobacteria (23.56%), Actinobacteria (23.35%), and Firmicutes (11.37%), and one dominant eukaryotic phylum, Ascomycota (30.47%). Metagenomics, that is, direct analysis of the total community DNA, is able to obtain plentiful information content, including, taxonomic, metabolic potential and functional profiles of microbial communities (see Part I).

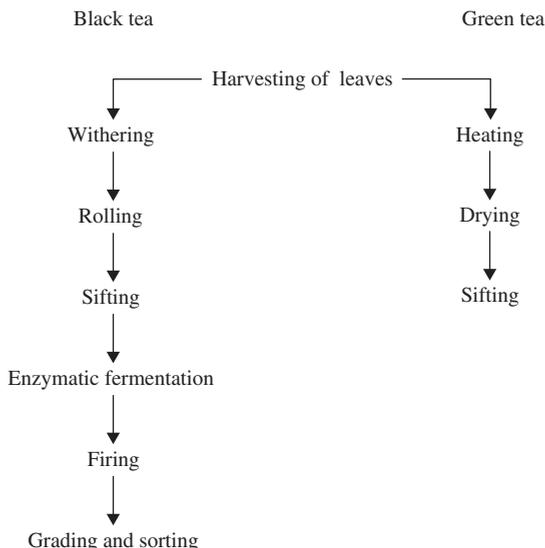


Figure 5.16 A flow diagram for the production of tea.

5.6.4.1 Black tea The black tea process involves four basic steps: withering, rolling, fermenting, and firing as in Figure 5.16. Once plucked from the tea bushes, the leaves are spread thinly on wire netting trays or mats that allow free access of air. The rate of withering may be controlled by changing temperature and humidity levels, whereas in other systems control may be exerted only by varying the depth of the tea leaves. It is important that this step be carried out slowly and evenly for 18–20 h. During this period, the leaf moisture content is reduced from 75–80% to 55–65% without heat buildup in troughs, though exact values depend on the growing region. Also, important chemical changes such as increases of amino acids, caffeine, organic acid, and polyphenolase activity are taking place in this step; hence the name *chemical withering*. Amino acids are formed as precursors of compounds ultimately leading to the production of flavor and nonenzymatic browning.

The tea leaves are then subjected to a rolling process, which applies pressure sporadically, damages the leaf structure, and initiates fermentation. For proper rolling, it is important to have achieved the correct moisture content during withering. The rolling process twists the leaves, bruising them and spreading the juices over the surface. The enzymes present oxidize the polyphenols of the juices and the color gradually changes from green to brown. After rolling, the leaves are sifted to cool them and to allow the separation of the dhools, the very fine particles that must be coaxed away from the rest of the leaf fragments. The dhools are then allowed to ferment by spreading them in thin layers (5–8 cm thick) on the factory floor or on racked trays at a relative humidity of 100% and the lowest feasible temperature for approximately 3 h. Complete oxidation is normally judged by the development of a coppery color and a fermented tea aroma. The fermented surfaces are washed daily to discourage the growth of unwanted bacteria. Soon it is possible to detect loss of astringency due to oxidation of polyphenols, darkening of the leaves due to the type of tanning reaction, and the development of the characteristic taste and smell of tea.

Fermentation is based on the activities of oxidizing enzymes native to the plant, but the microflora of the leaves have an influence on the quality and taste of black tea.

Fermentation is finally arrested by a firing process in an oven with a stream of hot air (82–95 °C), which also dries the product to about 3% moisture content. Proper drying control is crucial to product quality. One important effect of firing is the color change brought about by the conversion of chlorophyll to pheophytins (dark brown) and pheophorbide (black). Roasting temperatures of 82–95 °C result in the loss of volatile flavor components and coagulation of leaf proteins. A high proportion of the tea aroma, containing more than 500 components, is known to be generated during the postharvest and firing process. However, it is probable that only a few dozen components are significant for the tea aroma. The tea is then sorted and graded by using a series of oscillating screens.

5.6.4.2 Oolong tea In the production of oolong tea, the withering process is reduced to induce a limited form of fermentation, and thus this tea retains a considerable amount of the original polyphenolic material. The tea leaves are laid in bamboo baskets for a period of 5–6 h at 28.3–29.4 °C. The fermentation is terminated by drying with an agitator for 10 min at 204 °C. The leaves are then fired for 3 h and kept for another 5–12 h at 100 °C. Long firing times produce high quality tea. Growing seasons and processing technique are two important factors that affect the quality of partially fermented tea. The best quality tea is made in the spring, followed by winter and autumn. Tea produced in summer has the worst quality. Slight changes in the technique of each step may affect the final quality with respect to appearance, color, aroma, and taste. Manufacture is often a cottage industry that uses a series of withering, gentle rolling, and drying steps. The color of oolong tea is intermediate between that of green and black teas.

Summary

The fermenting process as applied to cocoa, coffee, and tea may be regarded as an art rather than a science. The factors controlling the fermentation processes and their effects on the biochemistry of flavor development are poorly understood even with the large volume of literature available on these topics. Fundamental work on the biochemistry and chemistry of flavor development is thus needed to establish criteria for the development of controlled fermentation systems. The use of specific strains of microorganisms and added enzymes to better control fermentation are improvements made to these processes in recent years. Although many health claims of the products have been reported, more works are needed to clarify the benefits.

5.7 Bacteriocins

5.7.1 Introduction

Bacteriocins are proteinaceous toxins produced by bacteria and archaea members to inhibit the growth of similar or closely related bacterial strain(s). Bacteriocins were first discovered by Gratia in 1925 (Garneau et al., 2002) during searching for ways to kill bacteria, which also resulted in the development of antibiotics and the discovery of bacteriophage, all within a span of a few years. Due to the alarming rise in antibiotic resistance and adverse effects provoked by a number of antibiotics, bacteriocins have already been applied in several fields: human health, food industry, animal health, in particular as a substitution of

the traditional antibiotics. Among these LAB bacteriocins that are commercially marketed, nisin groups produced by *L. lactis* subsp. *lactis* and pediocins produced by *Pediococcus* sp. are the most popular because of their antilisterian property. Despite their widespread use in foods, their applications in livestock and aquaculture have been largely limited.

5.7.2 Classification

Bacteriocins produced by bacteria and some archaea members to inhibit the growth of similar or closely related bacterial strain(s) can be divided into several types (Table 5.7). The inhibitory spectrum of bacteriocins can be narrow and confined to closely related species, or it can be relatively broad, inhibiting a range of target organisms (Mantovani et al., 2011). The bacteriocin family is the most abundant and diverse group of bacterial defenses. The application of bacteriocins in livestock to control or/and to maintain intestinal microflora of animals by feeding bacteriocin-producing strains has been largely achieved (Riley, 2009). Novel alternative strategies to reduce or eliminate animal pathogens have also been tested by different research groups. The alternatives include bacteriocins, probiotic microorganisms, and bacteriophages (Joerger, 2003). LAB can act antagonistically against a wide range of foodborne pathogens and spoilage organisms such as *Salmonella* (Gupta and Savalia,

Table 5.7 Bacteriocins of bacteria and archaea

	Bacteriocins	Bacteriocin types/ class	Size, kDa	Examples
Gram-negative bacteria	Colicins	Pore formers Nucleases	20–80	Colicins A, B Colicins E2, E3
	Colicin-like	NA	20–80	S-pyocins Klebicins
	Phage-tail like	NA	>80	R and F pyocins
	Microcins	Posttranslationally modified Unmodified	<10	Microcin C7 Microcin B17 Colicin V
Gram-positive bacteria	Class I	Type A-positively charged and linear	<5	Nisin
		Type B-uncharged or negatively charged globular		Mersacidin
		Type C-synergistic		Lacticin 3147
	Class II	Class IIa-antilisterial Class IIb-synergisti	<10	Pediocin PA1 Carnobacteriocin B2
	Class III	Type IIIa-bacteriolytic enzymes Type IIIb-nonlytic peptides	>10	Lysostaphin Helveticin
Class IV	Cyclic peptides	<10	Enterocin AS-48	
Archaea	Halocins	Microhalocins	<10	Halocin A4, C8, G1
		Protein halocins	>10	Halocin H1, H4
	Sulfolobacin	NA	~20	Sulfolobacin

Source: Adapted from Bakkal et al. (2012). Bacteriocins of aquatic microorganisms and their potential applications in the seafood industry. In *Health and Environment in Aquaculture*, E. Carvalho, Ed., pp. 303–328.

2012). Among many LAB bacteriocins, nisin is the most extensively characterized and used (Mohanasrinivasan et al., 2012).

Bacteriocins of gram-positive bacteria are as abundant and even more diverse as those found in gram-negative bacteria. The gram-positive bacteriocins resemble many of the antimicrobial peptides produced by eukaryotes; they are generally cationic, amphiphilic, membrane-permeabilizing peptides, approximately 2–6 kDa in size (Riley, 2009). Bacteriocins produced by gram-positive and gram-negative bacteria differ by several ecological and evolutionary aspects. In gram-positive bacteria, the biosynthesis of bacteriocins is self-regulated and bacteriocin production is not a lethal event. The spectrum of antimicrobial activity is broader than the peptides from gram-negative species and bacteriocin release is controlled by specific regulatory mechanisms (Mantovani et al., 2011). Additional roles have been proposed for some bacteriocins produced by gram-positive bacteria, such as chemical mediators in quorum sensing and communication molecules in bacterial consortia (Gobbetti et al., 2007). Quorum sensing is one of well-studied systems involved in bacteriocin gene control. Quorum sensing is a cell-density-dependent regulatory system in which autoinducing signal molecule mediates cell-to-cell communication (Wang et al., 2013). By using this system, each bacterial cell senses the number of cells of same species or same strain and adjusts the timing of expression of certain genes. LAB often use quorum sensing for the control of bacteriocin expression in which LAB attack the competitor only when the concentration of the bacteriocin producers is high enough to suppress the growth of competitive strain.

Nisin was first discovered in the late 1920s and early 1930s when it was described as a toxic substance produced by *L. lactis* subsp. *lactis*. Approved by the FDA for food applications, nisin received GRAS status in 1988 and was authorized for food preservation in the European Union by Directive 95/2/EC in 1995 under the code E234. The status as GRAS for use as an antimicrobial agent on cooked meat and poultry products was affirmed in 2001 by the FDA. Nisin is a small peptide of 34 amino acids with a molecular mass 3354 kDa ribosomally synthesized and post-translationally modified peptide contains five lanthionine rings, which can be divided into two parts. The N-terminal half including three rings (A, B, and C) is more hydrophobic than the C-terminus containing two rings (D and E) in Figure 5.17. The rigid ring structures are separated by a flexible hinge region (Mohanasrinivasan et al., 2012), including one Dhb, two Dha one lanthionine, and

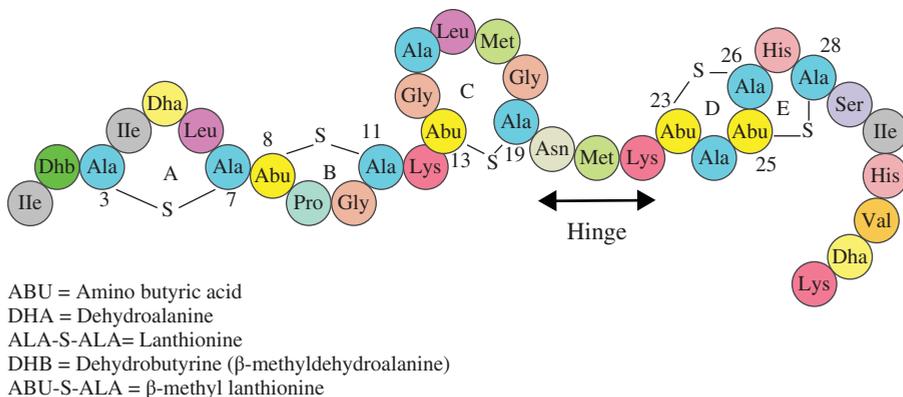


Figure 5.17 Structure of nisin. Source: <http://hu.wikipedia.org/wiki/Nisin>. Reproduced under a Creative Commons License. (See insert for color representation of this figure.)

four methylanthionine residues (form the five lanthionine rings) in its structure. The rigid ring structures are separated by a flexible hinge region. The ring structures give nisin a screw-like conformation that possesses amphipathic characteristics. In water, its solubility and stability increases with decreasing pH, showing maximum solubility 57 mg/mL at pH 2, and maximum stability at pH 3. Nisin belong to the lantibiotic class of bacteriocins, cationic, and hydrophobic peptide. Nisin provides a paradigm for studies of lantibiotic structure, biosynthesis and mode of action of antimicrobial peptides, and is often referred to as the *prototypical* lantibiotic (Mantovani et al., 2011).

Pediocins belong to the Class II of unmodified bacteriocins which subdivided into the groups of the pediocin-like bacteriocins and the two-peptide bacteriocins. This class comprises over 50 members with diverse origins. They are generally small (<5 kDa) and are heat-stable membrane-active and cationic peptides with similar primary structures. Their activity is retained at a wide pH range. They are sensitive to most proteases. The pediocin-like bacteriocins (36–48 residues) are produced by many LAB and share a 40–60% amino acid sequence similarity (Zacharof and Lovitt, 2013). In general, class IIa bacteriocins have a rather narrow spectrum of activity. The peptides of this group are known as *antilisterian* or *Listeria-active* peptides and they are characterized by a -YGNGV-N-terminus (Papagianni and Anastasiadou, 2009).

The positively charged residues in class IIa bacteriocins are located mostly in the hydrophilic N-terminal region in Figure 5.18. It has been shown for pediocin AcH/PA-1 that electrostatic interactions and not the -YGNGV- motif, govern the binding of the pediocin and its fragments to phospholipids vesicles. Lys11 and His12 that are part of the cationic patch in the N-terminal β -sheet-like region of pediocin AcH/PA-1 are of special importance for the electrostatic interactions and subsequent mutagenesis studies, in charged residues of pediocin AcH/PA-1 and in sakacin P. Earlier research confirmed these two amino acids were replaced by neutral residues. The C-terminal region is important in determining the target cell specificity for class IIa bacteriocins (Drider et al., 2006). This has been shown by combining N- and C-terminal regions from different class IIa bacteriocins (hybrid bacteriocins), which displayed target cell specificities similar to the bacteriocins from which the C-terminal was derived.

5.7.3 Mode of action

Different mechanisms of action have been proposed for bacteriocins: alteration of enzymatic activity, inhibition of spore germination and inactivation of anionic carriers through the formation of selective and nonselective pores. LAB bacteriocins can work via different

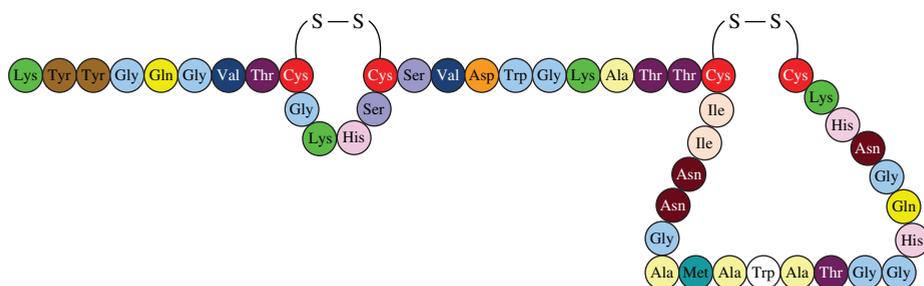


Figure 5.18 Structure of Pediocin PA-1. Source: Desriac et al., 2010. *Marine Drugs* 8:1153–1177. (See insert for color representation of this figure.)

mechanisms to exert an antimicrobial effect, but the cell envelope is generally the target. The initial electrostatic attraction between the target cell membrane and the bacteriocin peptide is thought to be the driving force for subsequent events.

The first step in the mechanism of action of nisin is considered to be the binding of the peptide to the cytoplasmic membrane of target bacteria. Nisin has different antimicrobial activities based on both high-affinity targets and low-affinity membrane interactions. The C-terminal region of nisin containing four out of the six positively charged residues of nisin A (Lys-22, His-27, His-31, Lys-34) was shown to play a dominant role in the membrane-binding step. This part of the molecule inserts into the cell membrane, while nisin's N-terminus binds with high affinity to the Lipid II molecule, a hydrophobic carrier for peptidoglycan monomers, using this compound as a specific receptor to integrate into the bacterial membrane and to form pores that increase membrane permeability; nisin–Lipid II interaction compromises the incorporation of precursor units, blocking the biosynthesis of bacterial cell wall (Mantovani et al., 2011). The final pore structure is believed to have a stoichiometry of eight nisin and four Lipid II molecules. Nisin's pore-forming ability induces the loss of membrane integrity and passive efflux of small intracellular metabolites through the lipid bilayer. Because of the loss of ions (potassium, phosphate), amino acids, and adenosine triphosphate (ATP), the proton-motive force is reduced or dissipated and the cell dies. Nisin can also promote the release of certain enzymes, such as *N*-acetylmuramoyl-L-alanine amidase and *N*-acetylglucosaminidase, which hydrolyze the cell wall by binding to teichoic, teichuronic and lipoteichoic acids. Nisin also inhibits the outgrowth of bacterial spores, by uncoupling the establishment of oxidative metabolism or membrane potential and the shedding of external spore structures.

Pediocins are bactericidal to sensitive gram-positive bacteria. The cytoplasmic membrane of gram-positive bacteria is the target of pediocins. All the class IIa bacteriocins whose modes of action have been studied permeabilize the cytoplasmic membrane through pore formation by insertion of the C-terminal regions into the membrane. Being hydrophobic molecules, they destabilize the cytoplasmic membrane when they come in contact with it. This action includes loss of the permeability barrier and loss of the membrane potential, which, in strains that possess an autolytic system, result in cell lysis, but the specific role of the YGNGV motif of the pediocins has not clarified yet. They kill sensitive bacteria by punching holes in their cell membranes, causing a disruption in their transmembrane potential and destroying the delicate balance of which the organisms maintain between themselves and their environment. Higher concentration of pediocin effectively released higher molecular weighted substances. They frequently adopt conformations where polar and nonpolar residues are segregated properly resulting in a typical amphipathic structure that exhibits more peptide internalization and membrane perturbation. Transmembrane potential (negative inside) in bacteria acts as a potential driving force for insertion and internalization of the antimicrobial peptides promoting AMP interaction (Manuel et al., 2009).

5.7.4 Bioengineering of bacteriocins

In the past two decades, there have been significant advances in functional genomic analysis of LAB and their biochemical characterization of bacteriocins. Considerable efforts have been made to functionally characterize bacteriocin operons and to express them in heterologous systems (Tominaga and Hatakeyama, 2007). The genes responsible for bacteriocin production are frequently associated with mobilizable elements, or in the chromosome in association with transposons or plasmids. The low-molecular-weight

bacteriocins of gram-positive bacteria generally appear to be translated as pre-peptides that are subsequently modified to form the mature biologically active (bactericidal) molecules. Specific auxiliary functions required by bacteriocin-producing cells include mechanisms for extracellular translocation of the bacteriocin and for self-immunity to the bacteriocidal activity of the molecule. As is the case for most bacteriocins, the lantibiotics are initially synthesized with an N-terminal leader peptide. In general, the pre-peptide is modified by the action of other proteins encoded by the bacteriocin gene cluster before export.

5.7.4.1 Biosynthesis of nisin The genes involved in biosynthesis of the model lantibiotic nisin are located on a 70 kb conjugative transposon. Biosynthesis of nisin is encoded by a cluster of 11 genes of which the first gene, *nisA*, encodes the precursor of nisin in Figure 5.19. The first gene of the nisin gene cluster, *nisA*, encodes the 57 amino acid nisin precursor, consisting of an N-terminal leader, sequence followed by the propeptide, from which nisin A is matured. The structural gene is followed by ten other genes, that is, *nisB*, *nisT*, *nisC*, *nisI*, *nisP*, *nisR*, *nisK*, *nisF*, *nisE*, *nisG*, encoding regulatory proteins, proteases, transport proteins, and immunity proteins. The proteins that are encoded by these genes have been found to be homologous to gene products of the gene clusters of other lantibiotics, such as those of subtilin, epidermin and Pep5. Thus, as a result of their gene-encoded nature, lantibiotics have been the focus of bioengineering with a view to elucidating structure function relationships (Field et al., 2010b). The majority of works that lead to enhanced

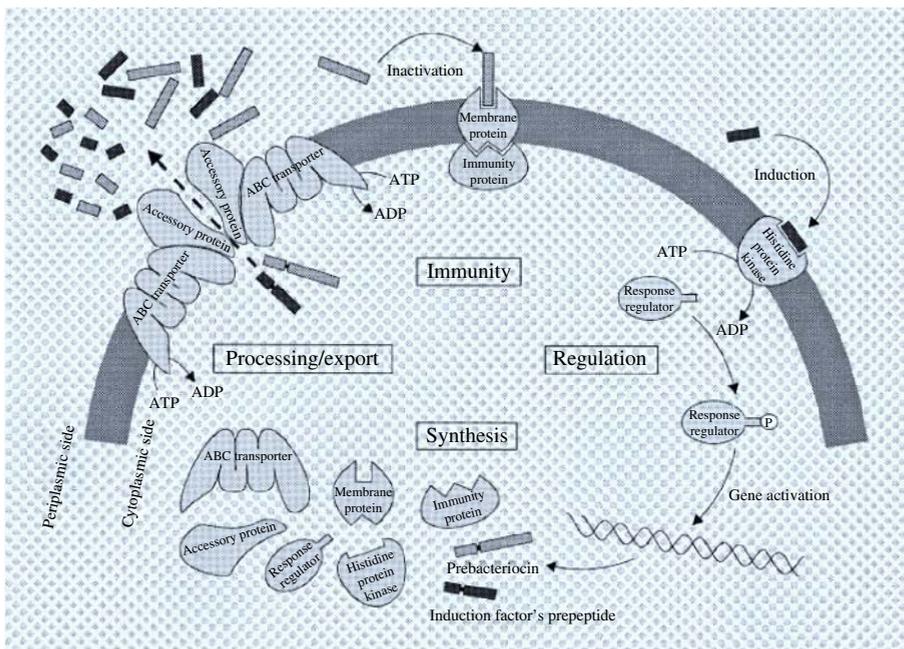


Figure 5.19 Schematic overview of the suggested machinery for production of class IIa bacteriocins: three-component regulatory system, synthesis, processing, excretion and immunity. *Source:* Ennahar et al., 2000. *FEMS Microbiol. Rev.* 24: 85–106. Reproduced with permission of Wiley.

peptides have resulted as a consequence of manipulation of the hinge region (Rouse et al., 2012). The hinge comprises residues 20 (Asn), 21 (Met), and 22 (Lys), which are thought to permit the movement of the N- and C-termini relative to one another during pore formation. The first success in this regard related to the creation of nisin derivatives, N20K and M21K, with enhanced antimicrobial activity against gram-negative bacteria. Subsequent investigations have further highlighted the benefits of manipulating the hinge and finally resulted in the identification of nisin derivatives, such as nisin N20P, M21V, K22T and K22S, which possess enhanced specific activity against gram-positive pathogens. Such activity has been highlighted with the enhanced specific activity of nisin M21V (or nisin V) in foodborne and clinical purpose (Field et al., 2010a).

5.7.4.2 Biosynthesis of pediocin Characteristically, class IIa bacteriocins like other low-molecular-mass bacteriocins are first formed as ribosomally synthesized precursors or pre-peptides, which appear not to be biologically active and contain an N-terminal extension or leader sequence. Subsequent cleavage of the pre-peptide at a specific processing site removes the leader sequence from the antimicrobial molecule concomitantly with its export to the outside of the cell. The leader peptide's removal during transmembrane translocation is accomplished by the same protein that is associated with the bacteriocin transport. The amino acid sequence of a number of class-IIa-bacteriocin leader peptides vary in length from 18 up to 27 residues. One important feature of the majority of these leaders is the presence of two glycine residues in the C-terminus at positions 32 and 31 relative to the processing site, though this is not distinctive of the class IIa. These leaders are believed to serve as signal peptides for the processing and the secretion of class IIa bacteriocins, independently of the GSP, by a dedicated transport system involving two distinct proteins: an ABC-type translocator and an accessory protein. The two conserved glycine residues may serve as a recognition signal for this sec-independent transporter system (Ennahar et al., 2000).

In the case of pediocin PA-1/AcH, the four genes needed for bacteriocin production and secretion are located in one operon (Drider et al., 2006). The four genes are (i) the structural bacteriocin gene, encoding a prebacteriocin; (ii) the immunity gene, encoding an immunity protein that protects the bacteriocin producer from its own bacteriocin; (iii) the gene encoding the ABC transporter for secretion; and (iv) a gene encoding a complementary protein of unknown function (Ennahar et al., 2000). The four genes cluster of pediocin AcH/PA-1 contains common promoter and terminator sequences. PedA encodes a 62 amino acids long pre-pediocin PA-1. Eighteen residue long leader sequences from N-terminal of pre-pediocin are removed during processing and export of pediocin through producer cell membrane. Mature pediocin carries 44 amino acid residues and two intramolecular disulphide bridges at cys9-cys14 and cys24-cys44 positions. PedB immunity gene is located downstream to pedA and encodes a protein of 112 amino acid residues. PedC a 174 amino acid long amphiphilic protein involved along with pedD protein facilitates/accelerates the transmembrane export. PedD gene specifies a polypeptide of 724 amino acid residues. Deletion analysis and site specific mutagenesis of pedD resulted in complete loss of pediocin production, showing its essentiality for secretion in *E. coli*. Its sequence show a very high homology to members of ATP-dependent transport proteins and also to a group of eukaryotic proteins involved in multidrug resistance. Figure 5.19 shows the suggested machinery for production of class IIa bacteriocins (Ennahar et al., 2000).

5.7.5 Applications of bacteriocins

5.7.5.1 Applications in food Although the several methods other than bacteriocins are employed for the preservation of food and beverages, an increasingly health-conscious public may seek to avoid foods that have undergone extensive processing or which contain chemical preservatives. Bacterial fermentation of perishable raw materials has been used for centuries to preserve the nutritive value of food and beverages and to extend shelf life. Bacteriocins produced by many gram-positive and gram-negative species, but those produced by LAB are of particular interest to the food industry, since these bacteria have generally been regarded as safe. The production of bacteriocins by LAB is advantageous for survival of the producing bacteria in a competitive ecological niche; therefore, they could be exploited by the food industry as a tool to control undesirable bacteria in a food-grade and natural manner, which is likely to be more acceptable to consumers. Many LAB produce a high diversity of different bacteriocins and several have been patented for their applications in foods. *Listeria monocytogenes* is a pathogenic bacterium that has been involved in several foodborne outbreaks worldwide and causes special concern with regard to food safety due to its psychrotropic and ubiquitous characteristics. The presence of this pathogen in fermented sausages and in vacuum-packaged meat products is of particular interest for food safety, as these two groups of meat are frequently eaten without reheating. This pathogen has shown to survive at a pH value of as low as 3.6 in foods and in salt concentration of up to 10%, in the presence of surfactants, sanitizers, and after several cycles of freezing and thawing (Hartmann et al., 2011), being a serious risk.

Several possible strategies for the application of bacteriocins in the preservation of foods may be considered: (i) inoculation of the food with LAB as starter or protective cultures that produce the bacteriocin in the product (production *in situ*); (ii) addition of the purified or semi-purified bacteriocin as a food preservative; and (iii) use of a product previously fermented with a bacteriocin-producing strain as an ingredient in food formulation (Jeevaratnam et al., 2005). Bacteriocin production *in situ* by starter cultures has a good chance of finding applications in fermented foods. In nonfermented refrigerated products, such as minimally processed meats or prepackaged vegetable salads, only those strains producing sufficient and potent amounts of bacteriocin but no other metabolic compounds, at levels detrimental to the sensory quality product, can be applied. The direct addition of purified bacteriocins obviously provides a more controllable preservative tool in such products.

There is an enormous amount of information about its application to inhibit a variety of pathogenic and spoilage bacteria in many food products. Nisin is suitable for use in a wide range of foods liquid or solid, canned or packaged, chill or warm ambient storage. On the basis of target microorganisms, its usage falls into three broad categories: (i) to prevent spoilage by gram-positive endospore formers (especially in heat processed food), (ii) to prevent spoilage by LAB and similar organisms like *Brocothrix thermosphacta* and (iii) to kill or inhibit gram-positive pathogens such *L. monocytogenes*, and *Clostridium botulinum*. Usually, it serves as the liquid portion of a product during its processing. It can also be added as a powder; it is essential to ensure uniform dispersal throughout the food matrix in the both ways. The best time to add nisin is at the last practical stage before heat processing (if this is a part of the manufacturing process). In the manufacture of processed cheese, for instance, nisin is usually added to the heated cheese at the same time as the melting salts. Nisin can also be used at high concentrations as a spray or dip for surface decontamination. The level of nisin addition depends on the type of food, severity of heat

process, pH, storage conditions, and the required shelf life. Nisin is often used in acidic foods, but is effective in products across a wide range of pH values 3.5–8.0. It is used in a variety of products including pasteurized, flavored and long-life milks, aged and processed cheeses, and canned vegetables and soups (Jeevaratnam et al., 2005). Nisin has utilized to inhibit undesirable LAB wine and beer (Jeevaratnam et al., 2005). Nisin has also been used in conjunction with other preservative measures to enhance product safety or quality. In canned foods such vegetables, soups, and puddings, nisin has been applied in conjunction with heat to successfully counter heat-resistant spores of flat-sour thermophilic bacteria.

In seafood industry, nisin delayed growth of *L. monocytogenes* in cold-smoked salmon (Bakkal et al., 2012). There has also been encouraging research into nisin-coated packaging. Nisin-coated plastic films reduced the number of *L. monocytogenes* by 3.9 log CFU/cm² at 4 and 10 °C after 56 and 49 days of incubation, respectively (Neetoo et al., 2008). Similarly, nisin-coated plastic films suppressed the growth of other aerobic and anaerobic spoilage microorganisms in a concentration-dependent manner. A combination of nisin and some lactates has been demonstrated to be more active against *L. monocytogenes* due to synergistic action (McEntire et al., 2003). A combinatory treatment of nisin and listeriophage was also found to be effective in controlling *L. monocytogenes*.

Despite a few studies reporting on the applications of pediocins, pediocin PA-1/AcH has been demonstrated to effectively reduce populations of listeria strains in ice cream mix, sausage mix, fresh and ground beef and whole milk (Motlagh et al., 1992). It has been found to be effective against many strains of sublethally stressed gram-positive and gram-negative bacteria. Incorporation of pediocins as preservatives in such foods can help in killing the normally sensitive and resistant but injured cells of spoilage and pathogenic bacteria and ensure longer product shelf life and greater consumer safety (Jeevaratnam et al., 2005). Pediocin PA-1/AcH has a specific application to control *L. monocytogenes* in the production of certain fermented foods, especially in controlled fermentation where specific strains of starter cultures are used. Many refrigerated vacuum-packaged processed food products from meat, dairy, fish, and vegetable groups contain normally psychotropic gram-positive bacteria strains such *Leuconostoc*, *Lactobacillus*, *Carnobacterium*, *Brochothrix*, and *Clostridium*. By incorporating pediocin PA-1/AcH during the formulation of the raw product, spoilage problems in the final product could be reduced. Pediocins are also commercially available but are marketed as fermentates of LAB having GRAS status.

5.7.5.2 Applications in livestock health and aquaculture The application of bacteriocins in livestock has been largely achieved by feeding bacteriocin-producing strains, but very little evidence exists in administering of bacteriocins alone to livestock. Because of lack of evidence, the use of bacteriocins in livestock is largely based on those studies that reported feeding or applying bacteriocin-producing bacteria (BPB). The application of BPB for improvements in productivity has not been limited to cattle, but also to increase the growth rate of swine. In poultry, the use of BPB has been mainly targeted for the control salmonella.

The utilization of BPB as a preharvest food safety strategy is considered one of the most viable interventions for reducing the gastrointestinal colonization of livestock by foodborne pathogens. The BPB can be easily administered to animals by mixing dried or wet cultures with feed or drinking water, and depending on the ability of the particular probiotic strain to colonize the gastrointestinal tract. The feeding can have a direct effect on reducing the existing populations of foodborne pathogens such as salmonella and *E. coli*, and long-term colonization with BPB would prevent further reintroduction of the pathogenic bacteria. The most widely known nisin, lacticin, enterocin, pediocin, and plantaricin have

been extensively studied for their application in foods, but only a few of them have been used in livestock. Nisin has successfully been used to control respiratory tract infection by *Staphylococcus aureus* in animal model (De Kwaadsteniet et al., 2009).

One of the major disease in dairy cattle is 'bovine mastitis' induced by *S. aureus* that is the one of the most pathogen agent implicated in clinical and subclinical mastitis infections. Animal trials with intramammary formulations containing bacteriocins, such as germicidal preparation, were used for cow's teats (Wu et al., 2007). Nisin Z have shown a significant increase in cure rates of infections caused by *Staphylococcus agalactiae*, *S. aureus* and other mastitis pathogens (90.1%, 50%, and 65.2%). Moreover, after 48 h of treatment, no bacteriocin residue was detected in milk (Wu et al., 2007).

Nisin has shown an inhibitory effect against common rumen anaerobes. *In vitro*, this bacteriocin-affected ruminal fermentation in the similar way to monensin, the most common ionophore used as feed additive in cattle rations. Moreover, the introduction of nisin into an artificial rumen system changed the fermentation parameters, such as an increase in hemicellulose degradation and acetate and propionate production, which contributed to the improvement of microbial balance in this environment (Santoso et al., 2006).

Aquacultures are facing the same problems with animal farming; animals are continuously exposed to a wide range of microorganisms, some of which are pathogenic. Many efforts were undertaken to prevent and control this dilemma by husbandry techniques, the use of vaccines (Corripio-Miyar et al., 2007) and antibiotics (Smith, 2007), that can create several negative problems. Laborious, costly, and highly stressful to the animals (vaccines) and especially the selection for antibiotic-resistant bacteria and active residues of the drugs remain long after use. An alternative approach to disease prevention in aquaculture is the use of BPB as probiotics. In aquaculture, aquatic animal and microorganisms share the same ecosystem in the aquatic environment and the interaction between the microbiota, including probiotics, and the host is not limited to the intestinal tract (Zhou and Wang, 2012). Probiotic exclude competitively pathogenic bacteria through the production of inhibitory compounds, improve water quality, enhance the immune response of host species, and enhance the nutrition of host species through the production of supplemental digestive enzymes. Most probiotics used in aquaculture belong to the lactic acid bacteria, of the genus *Bacillus*, to the photosynthetic bacteria or to the yeast, although other genera or species have also been mentioned. The effects of probiotics, photosynthetic bacteria (*Rhodobacter sphaeroides*), and *Bacillus* sp. (*B. coagulans*), on growth performance and digestive enzyme activity of the shrimp, *Penaeus vannamei*, were related with supplementation concentrations of probiotics and thus, the use of a 10 g/kg (wet weight) supplement of probiotics in shrimp diet was recommended to stimulate productive performance. Nutrient and water enrichment with commercial BPB, designated Alchem Poseidon™ (a mixture of *B. subtilis*, *L. acidophilus*, *C. butyricum* and *S. cerevisiae*) significantly improved lysozyme activity, lowered levels of mucosal proteins, and also improved survival after bacterial immersion challenge with *Vibrio anguillarum* (Taoka et al., 2006). Commonly employed BPB developed for terrestrial animals contained the facultative or obligate gram-positive anaerobes found in the GI tract, specifically of the genera *Bifidobacterium*, *Lactobacillus*, and *Streptococcus*. Bacteriocin-producing strains should further be developed to be more effective for aquaculture than the regular probiotic strains in the future.

5.7.5.3 Applications in pharmaceutical and medicine The use of antibiotics for disease control, prophylactic agents, and growth promotion has contributed significantly to the emergence of resistant bacteria pathogenic to humans, animals and plants (Mcmanus et al., 2002). The extensive use of antibiotic and the alarming nature of this antibiotic resistance problem have motivated to find alternatives. Numerous antibacterial agents such as

bacteriophages, probiotic bacteria, antimicrobial peptides, and bacteriocins are now being considered alternatives and chemical or genetic engineering methods are exploited to obtain the desired activities of these varied antimicrobial leads (Lien and Lowman, 2003). As the narrow spectrum of bacteriocins produced by LAB represent an important limitation for the application of these bacteriocins as clinical drugs or as food preservatives (Acuña et al., 2012), some examples of bacteriocins and their pharmaceutical applications are (i) the use of microcins derived from enterobacteria to control gram-negative bacteria (Duquesne et al., 2007). Similarly to pediocin-like bacteriocins, microcins belonging to class IIa such as microcin V are linear polypeptides, and the removal of the leader peptide is the unique posttranslational modification that they undergo before being secreted by the cells. In order to obtain a peptide with a broader antimicrobial spectrum, Acuña et al. (2012) fused by asymmetrical polymerase chain reaction (PCR) the required portions of genes encoding enterocin CRL35 and microcin V, namely, *munA* and *cvaC*. The hybrid bacteriocin purified from *E. coli* extracts, named Ent35eMccV, showed inhibitory activity against enterohemorrhagic *E. coli*, *L. monocytogenes* and other pathogenic gram-positive and gram-negative bacteria (Acuña et al., 2012). Bacteriocins have interest in medicine because they are made by nonpathogenic bacteria that normally colonize the human body. Loss of these harmless bacteria following antibiotic use may allow opportunistic pathogenic bacteria to invade the human body. In this field, several lantibiotics are used in pharmaceutical applications and some have been used in dental caries treatment (mutacin-producing strain) used to control vaginal microbiota with significantly reducing the adherence of the urogenital pathogen *S. aureus* (Zarate and Nader-Macias 2006).

The resistance of spontaneous mutants to bacteriocins have also been reported, that may be related to changes in membrane and cell wall, such as alterations in the electrical potential, fluidity, membrane lipid composition, and load or cell wall thickness or even a combination of all factors. These changes may occur following cell exposure to low concentrations of bacteriocins or as part of an adaptive response to some other stress. The resistance of *L. monocytogenes* to nisin is related to variation in fatty acid composition of cell membranes, reducing the concentration of phospholipids, hindering the formation of pores. The mechanism of resistance to subclass IIa bacteriocins appears to be linked to reduced expression of mannose permease of the phosphotransferase system (Vadyvaloo et al., 2002).

5.7.6 Commercial production of bacteriocins

The only commercially produced bacteriocins are the group of nisin produced by *L. lactis* and pediocin PA-1 by *Pediococcus acidilactici*. Nisin is the most commercially important member of a large class of bacteriocins produced by bacteria that can kill or inhibit the growth of other bacteria. This phase of the Nisin Market Study analyzes the characteristics of the current market for nisin and competing bacteriocins in four main sections highlighting: (i) the general market characteristics for antimicrobial preservatives; (ii) current producers and sellers of commercial grade nisin; (iii) current users of nisin and competing bacteriocins; and (iv) implications for the market opportunities for nisin production in the United States. The global leader in the antimicrobial preservatives industry is Danisco A/S, a Danish company, with Royal DSM (Netherlands), and Kerry Bio-Sciences (Ireland). Danisco's Nisaplin™ is generally considered to be the most commercially available form of nisin for food preservative uses. Danisco's strategic focus for their nisin product line is the US meat and deli food sector in order to take advantage of the FDA approval status of nisin as a natural ingredient. Other players in the global nisin market include Rhodia, S.A.

(France) along with numerous producers and providers of various antimicrobial products based in China. Some of these Chinese sources are in joint ventures or alliances with European-based corporate entities. Bacteriocin preservatives are part of the \$22 billion global food additives market that has grown at an average annual rate of 2.4% per annum between 2001 and 2004. This market is expected to continue growing at 2–3% per annum through 2007 to \$24 billion. The Danish holding company recorded profits of USD 176 million on sales of USD 2.9 billion for the year ending April 30, 2004 (Jones et al., 2005). The Genencor division of Danisco has manufacturing locations in the United States, Finland, Belgium, China, and Argentina (<http://www.genencor.com>). More than half of Genencor's \$410 million yearly sales are outside the United States. Key competitors to Genencor have been identified as Diversa, Novo Nordisk, and DSM (Royal DSM NV) (www.hoovers.com). Several attempts have also been tried to express and secrete pediocin PA-1 in other *L. lactis* hosts, resulting in the enhanced production of pediocin PA-1 and to coproduce the lantibiotic nisin A and pediocin PA-1 and develop novel expression system for large-scale production and purification of recombinant class IIa bacteriocins and its application to Piscicolin 126. More recently Jiménez et al. (2013) expressed the bacteriocin sakacin A (SakA) and two SakA-derived chimeras in LAB and the yeast *P. pastoris* and *K. lactis*.

Summary

Bacteriocins produced by LAB may further be a good solution to the problem of resurgence of resistant strains to antibiotics. It is now evident that the bacteriocin-like products of gram-positive bacteria, especially those with a relatively broad antibacterial spectrum, will continue to be an active area of applied research. The potential for either the discovery or genetic engineering of novel peptides with commercially desirable antibacterial activities offers an irresistible lure. The utilizations of bacteriocins or BPB in livestock and aquaculture are a field with enormous possibilities for both research and commercialization. As more countries develop antibiotic-limiting policies, the need for alternative antimicrobial will probably be the main driving force to continuously identifying novel bacteriocins and testing existing ones. Because of the relative specificity of bacteriocins as compared with antibiotics, it can be anticipated that the identification of broader spectrum bacteriocins will be an active research endeavor. Novel LAB bacteriocins and their bioengineering will be useful in applications, but more details of their actions mechanisms and biosynthetic mechanisms must be determined for further application in food and livestock health. On the other hand, other techniques such as screening must further be undertaken to discover novel bacteriocins. The use of more than one LAB bacteriocin as a combination biopreservatives or antimicrobial could be advantageous over a single bacteriocin especially in medical applications.

5.8 Functional foods and nutraceuticals

Although there is no universally accepted term for functional foods and nutraceuticals, a *functional food* is similar in appearance to a conventional food that is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions. A *nutraceutical* is a product isolated or purified from foods that is generally sold in medicinal forms (capsule or tablet), that suppose to have a physiological benefit or provide protection against chronic

disease. Functional food is a natural or processed food that contains known biologically active compounds which when in defined quantitative and qualitative amounts provides a clinically proven and documented health benefit, and thus, an important source in the prevention, management, and treatment of chronic diseases of the modern age (Martirosyan, 2011). The recently surged market on functional foods and nutraceuticals is due to their increasing popularity with health-conscious consumers and the ability of marketers to create new interest in existing products. During the 1980s, the Japanese Ministry of Health and Welfare introduced a category of foods which had health-promoting effects. This was designed to reduce the escalating cost of health care in Japan. The Japanese termed this food category as Food for Specific Health Use (FOSHU). Today more than 100 food items are approved as FOSHU, making Japan the world leader in the development of functional foods. About 11 very broad classes of ingredients are considered to be health enhancing in Japan: (i) dietary fiber; (ii) oligosaccharides; (iii) sugar alcohols; (iv) amino acids, peptides and proteins; (v) glycosides; (vi) alcohols; (vii) vitamins; (viii) LAB; (ix) minerals; (x) polyunsaturated fatty acids, and (xi) phytochemicals and antioxidants. Most of the functional foods that have been developed are beverages and probiotic/prebiotic containing yogurt products. Some examples include Japan's best-selling soft drink "FibeMini" which contains dietary fiber supplement, minerals, and vitamins. Another example is "omega-3 milk beverages containing flax oils" that are now available to Canadian consumers. Table 5.8 lists some examples of functional foods and nutraceuticals on the markets.

The markets for functional food are very large and growing steadily worldwide, intensely competitive. From a report entitled "Consumer Trends: Functional Foods" released by Agri-Food Trade Service in 2009, the annual growth rate for functional foods are about 8–14%, but the exact size is difficult to measure, and the global market size is estimated from US\$7 billion to US\$167 billion (Lau et al., 2013). Plant-derived functional foods and nutraceuticals can be found in other references (Valls et al., 2013).

5.8.1 Probiotics and prebiotics

After Elie Metchnikoff first introduced the concept of probiotics, in his book entitled "Prolongation of Life" in 1907, many types of bacteria have since surfaced possessing probiotic properties and the most documented groups comprise of LAB and bifidobacteria. Many of these are associated with various habitats such as fermented dairy products, and plant materials, but other habitats such as soil, silage, human oral cavity, the intestinal tract, and vagina. Probiotics are currently defined as "live microorganisms that, when administered in adequate amounts, confer a beneficial effect on the hosts. This definition has evolved much since the birth of the probiotic concept, with earlier definition stressing on the need of the microorganisms to be alive, and later, it should be consumed enough. These probiotic bacteria must be present in numbers high enough to have a physiological effect on the consumer. These numbers should be above 10^9 (1 billion) viable organisms per serving of the product. Survival of the probiotic cultures during distribution, retailing and in the consumer's home is required to maintain efficacy of those food products (probiotic yogurts) or nutraceuticals (capsules of probiotic bacteria). These were very much contributed by the initial roles of probiotics in regulating gut well-being, including the alleviation of lactose intolerance, improvement of diarrhea, and inhibition toward pathogenic bacteria in the gut; thus probiotics need to be alive and delivered via the oral route to reach the gastrointestinal tract. For the past two decades, unconventional new roles have been investigated, that includes the modulation of blood lipid cholesterol levels, topical applications for dermal diseases, alleviation of postmenopausal symptoms and stress management, etc.

Table 5.8 Examples of functional food components

Functional components	Source	Potential benefits
<i>Carotenoids</i>		
α -Carotene/ β -carotene	Carrots, fruits, vegetables	Neutralize free radicals, which may cause damage to cells
Lutein	Green vegetables	Reduce the risk of macular degeneration
Lycopene	Tomato products (ketchup, sauces)	Reduce the risk of prostate cancer
<i>Dietary Fiber</i>		
Insoluble fiber	Wheat bran	Reduce risk of breast or colon cancer
β -Glucan	Oats, barley	Reduce risk of cardiovascular disease; protect against heart disease and some cancers; lower LDL and total cholesterol
Soluble fiber	Psyllium	Reduce risk of cardiovascular disease; protect against heart disease and some cancers; lower LDL and total cholesterol
<i>Fatty Acids</i>		
Long chain omega-3 fatty acids-DHA/EPA	Salmon and other fish oils	Reduce risk of cardiovascular disease. Improve mental, visual functions
CLA	Cheese, meat products	Improve body composition. Decrease risk of certain cancers
<i>Phenolics</i>		
Anthocyanidins	Fruits	Neutralize free radicals; reduce risk of cancer
Catechins	Tea	Neutralize free radicals; reduce risk of cancer
Flavonones	Citrus	Neutralize free radicals; reduce risk of cancer
Flavones	Fruits/vegetables	Neutralize free radicals; reduce risk of cancer
Lignans	Flax, rye, vegetables	Prevention of cancer, renal failure
Tannins (proanthocyanidines)	Cranberries, cranberry products, cocoa, chocolate	Improve urinary tract health; reduce risk of cardiovascular disease
<i>Plant Sterols</i>		
Stanol ester	Corn, soy, wheat, wood oils	Lower blood cholesterol levels by inhibiting cholesterol absorption
<i>Prebiotics/Probiotics</i>		
FOS	Jerusalem artichokes, shallots, onion powder	Improve quality of intestinal microflora; gastrointestinal health
<i>Lactobacillus</i>	Yogurt, other dairy	Improve quality of intestinal microflora; gastrointestinal health
<i>Soy Phytoestrogens</i>		
Isoflavones: daidzein and genistein	Soybeans and soy-based foods	Menopause symptoms, such as hot flashes Protect against heart disease and some cancers; lower LDL and total cholesterol

Abbreviations: CLA, conjugated linoleic acid; FOS, fructo-oligosaccharides; LDL, low-density lipid.

Source: Reprinted from the International Food Information Council Foundation, 2009; and author's compiled data.

Both *in vitro* evidences and *in vivo* clinical data have supported some of these new health claims, while recent molecular advancement has provided strong indications to support and justify the hypotheses. However, probiotics validity and health claims have continuously been rejected on the basis of “biomarker deficiency” by European Food Safety Authority (EFSA) and probiotic word has been banned from Europe since 2012. Despite the difficulty, bile salt hydrolase (BSH)-active probiotic, *Lactobacillus reuteri* (Cardioiva™, Montreal, Canada) has been approved for cholesterol reduction and marketed by the health authority in North America since 2012.

5.8.1.1 Characteristics of lactic acid bacteria (LAB) and bifidobacteria as probiotics

LAB strains that are the major representatives of probiotics are used in dairy food such as fermented milk and pharmaceutical market. LAB are associated with various habitats, particularly those rich in nutrients such as various food substrates and plant materials, and have other habitats such as soil, water, manure, sewage, and silage. Some LAB strains inhabit the human oral cavity, the intestinal tract, and vagina and may beneficially influence these human ecosystems. LAB produce lactic or acetic acid and some carbon dioxide from carbohydrates during fermentation. General characteristics are gram-positive, non-motile, non-spore forming, coccus and rod shaped with less than 50% of G + C content. The major LAB genera include *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*.

L. acidophilus is a gram-positive, catalase-negative, rod-shaped bacterium, no gas from glucose or gluconate, thiamine not required with adolase activity. It is facultative with best growth obtained in the absence of excess oxygen and with energy obtained through homofermentative metabolism of a variety of carbohydrates to mainly D-lactic acid. The optimum growth is between 35 and 38 °C, but grows at 45 °C with no growth at below 15 °C. This became an advantage when this organism is added to nonfermented milk, in that little or no acid development will occur during refrigerated storage of the product. *L. acidophilus*, being a normal inhabitant of the small intestine, is resistant to bile. Growth occurs at initial pH values of 5–7 with optimum of 5.5–6.0. As acidity varies from 0.3% to 1.9% lactic acid, some of the characteristics enable it to provide potential health and nutritional benefits. Therefore, *L. acidophilus* is the most commonly suggested organism for dietary use.

Lactobacillus casei possesses many of the same characteristics as *L. acidophilus* that has potential for health and nutritional benefits. Although its optimum growth temperature is 37 °C, unlike *L. acidophilus*, it grows at 15 °C with no growth at 45 °C. No gas is released from glucose, but gas is released from gluconate and ribose. Four subspecies are currently recognized. It also ferments a number of carbohydrates homofermentatively to L(+)-lactic acid. It is a normal inhabitant of the small intestine and is resistant to bile. The genetic basis for ecological flexibility in *L. casei* is not fully understood; however, comparative genomic analyses have suggested extensive gene loss and gene acquisitions during evolution of *Lactobacilli*, presumably via bacteriophage or conjugation-mediated horizontal gene transfers (HGTs), and these may have facilitated their adaptation to diverse ecological niches (Makarova and Koonin, 2007).

Bifidobacteria are obligate anaerobes in the *Actinomycetales* branch of the high-G + C (>60%) gram-positive bacteria with distinct fructose-6-phosphate “shunt” metabolic pathway (Biavati and Mattarelli, 2006). *Bifidobacterium* spp. has similar characteristics to LAB. This is gram-positive, nonspore forming, nonmotile bacilli, catalase-negative, irregularly shaped rods with club-shaped or spatulate extremities and many of which form branched cells. They are sensitive to oxygen and have more strict growth requirements. As the obligate anaerobes, they are considered normal inhabitants of the small intestine and are bile

resistant. The major fermentation products are acetic acid and lactic acid in the molar ratio (3:2). This becomes an important factor to provide antagonisms toward undesirable microorganisms in the intestinal tract, in that acetic acid is more toxic to microorganisms than lactic acid. Since bifidobacteria are sensitive to oxygen, difficulty may be encountered in maintaining high levels of viability in products during storage unless anaerobic conditions are used. This makes them technologically more difficult to maintain the viability and thus they are commonly used less than lactobacilli (Ouweland et al., 2002).

5.8.1.2 Probiotic selection criteria Harzallah and Belhadj (2013) reviewed the general characteristics required for starter culture bacteria to survive and grow in the intestinal tract. One of the first barriers to survival is the gastric acidity encountered in the stomach. While *L. acidophilus* and *L. casei* are resistant to the acidic conditions of artificial gastric juice at pH 3.0 at 37 °C, *L. delbrueckii* ssp. *bulgaricus* is not. Strains of *Bifidobacteria* vary in their ability to survive transit through the stomach. The LAB also should be able to survive the acid conditions of the stomach and the bile in the upper digestive tract to reach the small intestine. Initially, the candidate strains were selected solely upon their ability to displace and destroy pathogens *in vitro* (Reid and Bruce, 2006). At present, several additional factors appear to be important when choosing practical probiotic strains for human consumption. In order for a microorganism to be classified as probiotic, it must fulfill the following criteria (Table 5.9); (i) human origin, (ii) nonpathogenic properties, (iii) resistance to technological processes, (iv) stability in acid and bile, (v) adhesion to target epithelial tissue, (vi) ability to persist within the GI tract, (vii) production of antimicrobial substances, (viii) ability to modulate the immune system, and (ix) ability to influence metabolic activities. Additional criteria for probiotic selection should be taken into consideration for commercialization purposes. First and foremost, a good probiotic strain should display good technological properties, among which they should be cheaply and easily culturable on a large scale (Ouweland and Vesterlund, 2003). Strains that are viable at higher temperatures as well as in oxygen-poor conditions are favored. Second, storage and packaging materials must adequately protect and preserve the therapeutic activity

Table 5.9 Properties and benefits of good probiotic strains

Property	Benefit
Resistance to pancreatic enzymes, acid, and bile	Survival of passage through the intestinal tract
Adhesion to the intestinal mucosa	Immune modulation; pathogen exclusion; enhance healing of damaged mucosa; prolonged transient colonization
Human origin	Species-dependent health effects and maintained viability
Production of antimicrobial substrates	Antagonism against pathogenic microorganisms
Documented health effects	Proposed health effects are “true”; clinically validated and documented health effects of minimum effective dosage in products
Health	The assessment and proof of a “GRAS” strain, with a previous “history of safe use” and safety in food; nonpathogenic even in immunocompromised hosts
Good technology properties	Strain stability; production at large scale; oxygen tolerance

Source: Adapted from Baek and Lee (2009). Reproduced with permission of Wiley.

of probiotic foods (da Cruz et al., 2007). Although some recent studies show potential benefits in consuming nonviable probiotics, live bacterial strains provide better efficacy as probiotics. Also, beneficial effects to the host are observed when probiotics are consumed in adequate quantities (a minimum of 10^9 viable cells per day). Therefore, aspects such as shelf life and storage conditions must be determined and optimized to ensure the viability of cells at the time of consumption. Third, because multistrain probiotic concoctions may provide enhanced health benefits, many probiotic products often contain more than a single microbial strain. In these cases, strain selection must consider the species compatibility issues. Finally, based on the intended form of the final probiotic product (capsules, tablets, powders, or liquids), special requirements with respect to species selection may be necessary to consider. For example, due to the heating step of the encapsulation process thermostable strains are better choices for probiotics destined to be sold as capsules (Boyle et al., 2006). By far, the most commonly selected probiotic strains are from the *Lactobacillus* and *Bifidobacterium* genera. These strains generally fulfill the criteria as excellent probiotics and are frequently derived from the GI tract of healthy humans or from other non-human strain used in the fermentation of dairy products. Many other strains are also very good probiotics that are regularly found in many commercially available probiotic products. Some less common probiotic selections include yeast species such as *S. cerevisiae* ssp. *bouardii* (Ouwehand and Vesterlund, 2003). Not surprisingly, most of these lactic strains are some of the most abundant bacterial strains residing as part of the natural gut microflora, oral cavity, urogenital tract, and skin. Administering endogenously found microbial strains fulfills the main criteria of a good probiotic. Moreover, the end result is to maintain and/or restore the natural microflora of the GI tract.

5.8.1.3 Beneficial effects of probiotics A number of health benefits for products containing probiotic organisms have been reported. Most of the beneficial effects are associated with gut health, such as balancing intestinal microflora, prevention of infectious diarrhea, improvement in lactose intolerance, improved calcium absorption, synthesis of vitamins and predigestion of protein, fat, carbohydrates, and improved bioavailability of nutrients, antimicrobial activity against gastrointestinal infections, enhancement of bowel motility/relief from constipation, adherence and colonization resistance and maintenance of mucosal integrity. Other new benefits that have surfaced for the past two decades include reduction in serum cholesterol, reduction of mutagens, which may induce tumors, enhancement of immune system response, post menopausal disorders, and skin health. The different beneficial effects of specific probiotic strains can be translated into different health claims. In general, comprehensive and harmonized guidelines are needed on the assessment of the characteristics and efficacy of probiotics and probiotic containing foods. An international expert group of ILSI has evaluated the published evidence of the functionality of different probiotics in four areas of (human) application in Table 5.10: (i) *metabolism*, (ii) *chronic intestinal inflammatory and functional disorders*, (iii) *infections*, and (iv) *allergy*. On the basis of the existing evidence, concrete examples of demonstration of benefits and gaps are listed, and guidelines and recommendations should help design the next generation of probiotic studies (Rijkers et al., 2010).

Decades of evidence have supported the fact that health benefits imparted by probiotic bacteria are strain specific. Strains of the same species have been recognized to exhibit health benefits at varying degrees and to some extent, same strains within the same species may not convey such health roles at all.

Despite many health claims of probiotics, approved health claims by health authorities are currently only two on (i) lactose intolerance and lactose digestion and (ii) cholesterol

Table 5.10 Other potential applications of probiotics*Metabolism*

Metabolism of dietary compounds in the gut lumen
 Lactose digestion
 Lipid metabolism
 Oxalate metabolism
 Composition and metabolic markers of the gut microbiota
 Xenobiotics, phytochemicals
 Indigestible dietary components
 Metabolic activity of gastrointestinal mucosa and liver

IBD and IBS

Inflammatory bowel diseases:
 Crohn's disease
 Ulcerative colitis
 Pouchitis
 Irritable bowel syndrome

Allergic Diseases

Eczema, atopic eczema
 Allergic rhinitis
 Asthma

Reduction of Risk Factors of Infection

Infectious diarrhea (acute and antibiotic associated)
 Traveler's diarrhea
 Necrotizing enterocolitis (infants)
Helicobacter pylori
 Respiratory tract infections (adults and children)
 Ear, nose and throat infections
 Infectious complications in surgically ill patients

Source: Rijkers et al. (2010). *J. Nutr.* 140: 671S–676S.

reduction. Probiotics research is still in the early stages, and far more studies need to be conducted to determine the health benefits and safety of probiotics.

Lactose intolerance Lactose is relevant as a nutrient in all mammalian neonates and all (with rare exceptions) have the ability to digest lactose for a variable time after birth. Inheritance of lactase persistent (LP: adults retain ability to digest lactose) is dominant and lactase nonpersistent (LNP: adults lose ability to digest lactose) is recessive (Lee and Szilagy, 2012). Lactose intolerance is symptoms due to ingestion of lactose. Lactose intolerance (LI) also known as lactose malabsorption occurs when small intestine does not produce enough lactase, an enzyme needed to digest lactose, a sugar in milk and other dairy products. The unabsorbed lactose is metabolized by colonic bacteria to produce gas [hydrogen (H₂) and methane (CH₄)] and short-chain fatty acids (SCFAs). Two major types of lactose intolerance are encountered in the population worldwide. Primary or adult-type maldigestion (or hypolactasia) is an autosomal recessive condition resulting from the physiological decline of lactase activity in the intestinal cells. Secondary-type lactose maldigestion is thought to be caused by a loss of small intestinal mucosa (results in severe diarrhea, bowel resection, etc.). Symptoms related to lactose intolerance appear 30 min to 2 h after

consumption of food products containing lactose. Lactose intolerance leads to symptoms including abdominal pain, bloating, flatulence, and diarrhea, but the cause may be multifactorial. There are geographic patterns of lactase status in population distributions, which may influence different disease risks. Some of these LPs (and even LNPs) may mistakenly ascribe the symptoms of undiagnosed irritable bowel syndrome (IBS) or other intestinal disorders to LI.

About 65% of the world's population are LNP, while the rest are LP. Highest rates of LI are found in the Asian, native Americans, and African American (60–100%), while the lowest rates are found in people of northern European origin including northern Americans. In humans, decline in intestinal lactase is largely irreversible and cannot be reconstituted by regular lactose consumption. Genetic polymorphisms determining LP phenotype are complex and have interesting geographic distributions. Interplay between lactose ingestion and digestion status has several effects on medical problems. The unusual geographic population LP/LNP distributions may affect disease risks (Lee and Szilagyi, 2012).

The two most common tests that form lactose intolerance are measurements of (i) blood glucose concentrations, and (ii) breath hydrogen test following ingestion of a lactose load, though (iii) genetic detection of C/T polymorphism at –13910 upstream of LPH (lactase-phlorizin hydrolase) gene is also used. However, breath hydrogen test has been advocated as the best diagnostic tool for the assessment of LI. During the test, subjects are sampled for hydrogen levels of breath samples at base line and every 30 minutes after the administration of 50 g of oral lactose, for a total period of 180 min. A breath sample with >20 ppm above baseline is considered positive for LI. Most individuals with presumed LI can tolerate up to 15 g of lactose (about one cup of milk). Since the avoidance of milk and milk-containing products can result in a dietary calcium intake that is below the recommended levels of 1000 mg/day for men and women and 1300 mg for adolescents, osteoporosis and associated fractures secondary to inadequate dietary calcium is the perceived major potential health problem associated with real or assumed lactose intolerance. For this reason, different course of action needs to be considered instead of a complete exclusion of dairy products by LI patients. Two possible interventions in the case of LI are the supplement of commercially available lactase (tablets) or the addition of probiotics. Another approach in management of lactose intolerance is to increase the lactose load steadily in one's diet, giving the colon time to adapt. Introduction of lactose to diet causes temporary and transient symptoms in individual (Paolo et al., 2012). Since lactase from intestinal brush border is not an inducible enzyme, the reduction in symptoms may be explained by colonic adaptation (Lee and Szilagyi, 2012).

Microbial β -galactosidase present in yogurt survives gastric passage and supports cleavage of lactose. Moreover, the high viscosity of yogurt compared to milk increases the time for microbial or human β -galactosidase to hydrolyse lactose. One of the health benefits of probiotics is probably generally accepted relief of the symptoms of lactose intolerance. However, reduced levels of lactose in fermented products due to partial hydrolysis of lactose during fermentation are only partly responsible for this greater tolerance for yogurt. Overproducing β -galactosidase mutants are studied to improve probiotic efficacy to treat symptoms of lactose malabsorption (Ibrahim and O'Sullivan, 2000). Nonfermented milk containing cells of *L. acidophilus* also can be beneficial for lactose maldigestors (Kim and Gilliland, 1983). Probiotic supplementation also modifies the amount and metabolic activities of the colonic microbiota and alleviates symptoms in lactose-intolerant subjects (He et al., 2008). The changes in the colonic microbiota might be among the factors modified by the supplementation, which lead to the alleviation of

lactose intolerance. Prebiotics – which are food ingredients that encourage “growth or activity” of microorganisms already in intestines – may ultimately be more effective.

Treatment to reduce lactose exposure consists of a lactose restricted diet or the use of lactase supplements. Other strategies include probiotics, colonic adaptation, and antibiotics. Unfortunately, there is insufficient evidence that these therapies are effective for LI.

Cholesterol-lowering effects Even though cholesterol is a major basic block for body tissues, high level of blood cholesterol is one of the causes of hypercholesterolemia and it was shown that 45% of heart attacks in Western Europe and 35% of heart attacks in Central and Eastern Europe are related to this hypercholesterolemia (Aloğlu and Öner, 2006; Kumar et al., 2012).

Many studies have proposed a hypocholesterolemic effect when fermented products and probiotics are consumed, especially with selected strains of lactic acid bacteria. Fermented milk containing *L. acidophilus* LI was found to lower serum cholesterol. This would translate to 6–10% reduction in risk for coronary heart disease (Anderson and Gilliland, 1999). Xiao et al. (2003) also showed total serum lowering with a yogurt containing *Bifidobacterium longum* BL1. The mechanisms of action proposed for the reduction of cholesterol are not clearly known, but includes the (i) inhibition of exogenous cholesterol absorption in the small intestine by assimilation by the bacteria, as well as suppressing bile acid resorption by bacterial hydrolase activity, (ii) cholesterol binding to the cell surface, (iii) cholesterol precipitation by bile acids, (iv) cholesterol incorporation into cellular membrane, (v) cholesterol conversion to coprostanolin and (vi) deconjugation of conjugated bile acids by bile salt hydrolase BSH, but (vi) seems to be the promising mechanism at the moment.

As probiotic bacteria are reported to deconjugate bile salts by BSH, deconjugated bile acid does not absorb lipid as readily as its conjugated counterpart, leading to a reduction in cholesterol level. *L. acidophilus* is also reported to take up cholesterol during growth and this makes it unavailable for absorption into the blood stream, via several ways, namely, absorption of cholesterol to the cellular surface and incorporation of cholesterol into the phospholipid tails, upper phospholipids, and polar heads of the cellular membrane phospholipid bilayer (Lye, et al., 2010). The detailed genetic organization of BSHs from *Bifidobacterium* strains (Kim et al., 2004; Kim and Lee, 2008), bile salt biotransformations by human intestinal bacteria (Ridlon et al., 2006), and the presence of the bsh gene have also been reported. Most of the *in vivo* trials conducted thus far have focused heavily on verifying the hypocholesterolemic effects of probiotics, rather than the mechanisms involved. Ooi et al. (2010) studied some possible mechanisms using a synbiotic product containing *L. acidophilus* CHO-220 and inulin.

Although various studies in humans (Taylor and Williams, 1998; de Roos and Katan, 2000) showed inconsistent results on the role of fermented milk products as hypocholesterolaemic agents, more reliable effects were documented in few clinical trials (Anderson and Gilliland, 1999; Jones et al., 2012, 2013). The cholesterol-lowering effect of probiotics was found to be highly strain-specific as different strains exhibited different levels of cholesterol-lowering activity (Mishra and Prasad, 2005). Therefore, it is important to identify probiotics strains that exhibit excellent cholesterol-lowering ability. Cholesterol-busting probiotics, which could decrease cholesterol level up to 12% using *L. reuteri* NCIMB30242 in humans, was developed by Micropharma (Montreal, Canada) and have recently been approved for sale. This is the first probiotic in the world to become a recognized biomarker of disease.

Gastrointestinal health Common gastrointestinal diseases and disorders such as diarrhea (including travelers' and antibiotic-associated diarrhea), constipation, as well as inflammatory bowel disease (IBD) have all shown indications of improvement when treated with probiotics. Millions of people, especially children in developing countries, die every year due to diarrhea, an intestinal disturbance often caused by an imbalance of the gut's flora with rotavirus infection and *Clostridium difficile* overgrowth. Antibiotic-associated diarrhea is a major clinical problem occurring in up to 25% of patients, with diarrhea owing to *C. difficile*. The administration of antibiotics allows the proliferation of *C. difficile*, leading to colonization of intestinal pathogens and excretion of exotoxins. *C. difficile* produces toxins A and B that cause mucosal damage and inflammation of the colon, thus accelerating the pathogenesis of colonic lesions. Probiotics produce proteases that directly degrade *C. difficile* toxins and increase the immune response to the toxins (McFarland, 2009). *Saccharomyces boulardii* reduced toxin-induced intestinal secretion and permeability, produced toxin-degrading proteases, and inhibited binding of toxins to the brush border membrane (Chen et al., 2012).

IBD such as Crohn's disease, ulcerative colitis, and pouchitis are immune-mediated diseases with chronic recurrent inflammatory conditions in gut of unknown multifactorial aetiology. Probiotics alleviate IBD via inhibition of pathogenic bacterial growth via secretion of bactericidal compounds, decreasing luminal pH by formation of and colonization resistance (Quigley, 2012). Probiotics also influence gut immunoregulatory activities via modulation of immunocytes apoptosis, reduction of phagocytic activity, downregulation of T-cell responsiveness, induction of antimicrobial substances secretion from specialized intestinal cells such as immunoglobulin A, and alteration of cytokines profile by upregulation of anti-inflammatory cytokines such as IL-10 and downregulation of proinflammatory cytokines.

Substantial experimental evidence exists to suggest that probiotics may be beneficial in the prevention and treatment of colon cancer, but there have been few conclusive human trials (Geier et al., 2006; Chong, 2013).

Helicobacter pylori is considered one of the major risk factors underlying the development of gastric and duodenal ulcers. Current antibiotic-based treatment for *H. pylori* infection is neither sufficient nor satisfactory, with the most successful treatments reaching 75–90% eradication rates. The use of probiotics is a potentially promising tool to prevent *H. pylori*. The growth and attachment of pathogenic *H. pylori* that causes peptic ulcers could be inhibited by probiotic strains (Chenoll et al., 2011).

Oral health The oral microbiota is highly diverse and includes fungi, protozoa, viruses, and bacteria, among which *Archaea* and *Bacteria* are present. Representatives of the *Archaea* are restricted to a few taxa in the genus *Methanobrevibacter*, while there are over 600 species of *Bacteria*, from at least 12 phyla (Wade, 2013). The balance of all these microorganisms can easily be disturbed and a prevalence of pathogenic organisms can lead to various oral health problems including dental caries, periodontitis, and halitosis. Although the evidence for periodontitis is less than that of dental caries, the use of probiotics to manage the oral microflora appears to be very effective adjunct way to control oral conditions (Bowen, 2013). Effect of long-term consumption of a probiotic bacterium *Lactobacillus rhamnosus* GG in milk was effective on dental caries and carries risk in children (Nase et al., 2001). Administration of probiotics reduced counts of oral *Candida* in the elderly and may be a new strategy for controlling oral yeast infections (Hatakka et al., 2007). Other preliminarily findings exhibiting effects of probiotics on oral health include imbalanced oral ecosystem and periodontal disease (Kang et al., 2006). Probiotics was effective on halitosis that definitely inhibited the production of volatile

sulfur compounds. In addition, a reduction of gingivitis and gum bleeding was observed by Krasse et al. (2006) with the application of *L. reuteri*. The oral health applications of probiotics or replacement therapy with *Streptococcus mutans* strains of attenuated virulence and increased competitiveness were more than 700 bacterial taxa in the oral cavity (Devine and Marsh, 2009). However, data on the oral probiotics are still scarce and insufficient, further studies are required to identify the resident oral probiotics, clarify the mechanism of their colonization, and the eventual effect on the oral environment.

Urogenital and vaginal health Infections that involve urogenital microbial flora imbalance such as yeast vaginitis, bacterial vaginosis, and urinary tract infection (UTI) can be recurrent. Current available antimicrobial treatments can often lead to diarrhea, depression, headaches, renal failure, and super infections. Moreover, antimicrobial resistance tends to decrease the effectiveness of this therapy over time. Lactobacilli have been shown to inhibit the growth of *C. albicans* and/or its adherence on the vaginal epithelium in small sample sizes (Falagas et al., 2006). Reid (2005) showed a reduction in and better treatment of urogenital infections using a combination of two different *Lactobacilli* strains. Presently, the only strains clinically shown to have an effect are *L. rhamnosus* GR-1 and *L. reuteri* (Commane et al., 2005), when used intravaginally once weekly or twice daily orally, have reduced recurrences of UTI and restored a normal lactobacilli-dominated vaginal flora (Reid and Bruce, 2006). Recent study on the role of probiotics in a woman's urogenital health confirm that supplemental probiotics containing these two strains of *Lactobacillus* promote the colonization of beneficial microbiota and may help to support overall vaginal health (Vujic et al., 2013). This study carried out with 544 women receiving either a probiotic containing a combination of *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14 or a placebo daily for 6 weeks showed balanced vaginal microbiota (61.5%) of those in the probiotic group compared to 26.9% in the women taking the placebo.

Immunomodulation and skin health During the past decades, probiotics have used extensively for improving gut health but they can also improve skin health such as atopic eczema, atopic dermatitis, healing of burn and scars, skin-rejuvenating properties, and improving skin innate immunity (Lew and Liong, 2013). Cell wall, certain metabolites, and dead bacteria can evoke immune response on skin and improve its anatomical barrier function of innate immunity. They restore the normal microflora of intestine and prevent the inflammation of gut and related disease conditions (Hemarajata and Versalovic, 2012). Different strains of probiotic bacteria such as *L. delbrueckii* subsp. *bulgaricus* along with *L. casei*, *L. acidophilus*, and *Bifidobacterium animales* vary in their mechanism of action for immunomodulation in terms of increasing the production of immunoglobulins; stimulation of cell mediated immunity; increasing the phagocytic activity; and increase in cytokine production, etc. (Deng et al., 2013; Dongarra et al., 2013). Figure 5.20 shows the general mechanism of immunomodulation by beneficial microbes. Usually *Streptococcus* and *Leukonostoc* strains are the strong inducers of Th1 cytokine. Sticking to *Bifidobacterium* and *Propionibacterium* genera promised to have the best anti-inflammatory potential by inducing IL-10 production. The probiotic bacteria act at the local as well as systemic level. At local level these bacteria are presented to the M cells or epithelial cells or interdigitating dendritic cells in the intestinal wall.

The effect of probiotics on the immune system is well documented. The number of people afflicted with atopic diseases such as eczema (dermatitis), allergic rhinitis or asthma is continuously increasing in western societies. These diseases are caused by a hereditary predisposition to developing certain hypersensitivities upon exposure to specific antigens and might be in part attributed to reduced microbial exposure in early

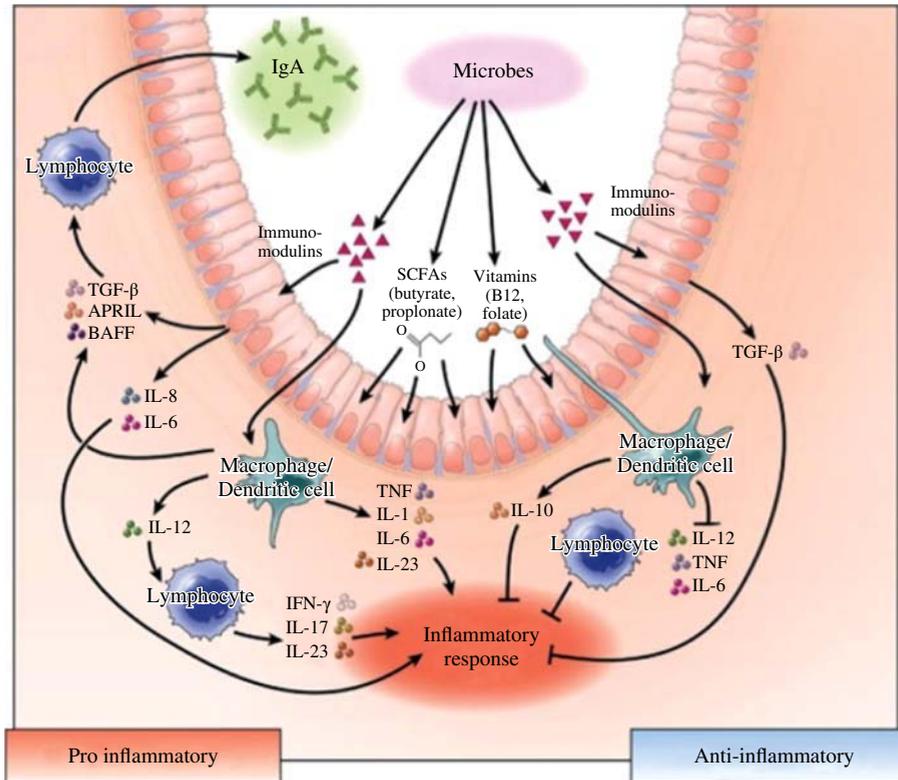


Figure 5.20 Mechanisms of immunomodulation by beneficial microbes. Probiotics can modulate the immune system in the intestine through the luminal conversion process. The bacteria produced secreted soluble factors and metabolites, such as short-chain fatty acids (SCFAs) and vitamins using substrates from the diet. These bioactive compounds can affect the function of intestinal epithelium and mucosal immune cells, resulting in production of cytokine and related factors such as a proliferation-inducing ligand (APRIL) and B-cell activating factor (BAFF). *Source:* Adapted from Preidis and Versalovic, 2009. *Gastroenterology* 136: 2015–2031. Reproduced with permission of Elsevier. (See insert for color representation of this figure.)

life. Reduced symptoms of the atopic eczema and dermatitis syndrome in food-allergic infants have also been observed when treated with probiotics. The intestinal microflora can reduce the allergic response by enhancing antigen exclusion and inducing IgA response (Abrahamsson et al., 2007). Probiotics increase the mucosal barrier of patients with atopic dermatitis and food allergy when infants are treated with LGG fortified formula. The administration of lactobacilli during pregnancy prevents atopic eczema in children aged from 2 to 7 years. However, a mixture of various bacterial strains does not affect the development of atopic eczema, independent of whether they contain lactobacilli or not (Doege et al., 2012). Probiotics have also been described to decrease the expression of cytokines involved in inflammatory pathways. Cell wall components of gram-positive bacteria such as lipoteichoic acid and peptidoglycan are potent immune modulators, in enhancing the production of skin beta defensins. In addition, some strains of LAB could also produce dermal compounds such as hyaluronic acid (HA) (Hor et al., 2014) that

function to stimulate the propagation and migration of human keratinocytes, dermis regeneration, and wound healing.

LAB strains have also been documented to produce sphingomyelinase (SMase), which generates a family of ceramides and phosphorylcholine for the development of extracellular lipid bilayers in the stratum corneum (Lew et al., 2013).

Prebiotics and synbiotics Prebiotics are nondigestible (by the host) food ingredients that have a beneficial effect through their selective metabolism in the intestinal tract (Gibson et al., 2004). A wide range of prebiotics have been isolated from plant materials, including β -glucans from oats, inulin from chicory root, oligosaccharides from beans, onions, and leek are a few examples. They are recognized for their ability to increase levels of health-promoting bacteria in the intestinal tract of humans or animals. Human milk oligosaccharides (HMO) are believed to have a range of biological activities beyond providing nutrition to the infant that may act as prebiotics (Barile and Rastall, 2013). As shown in Table 5.11, with the exception of soybean oligosaccharides and raffinose (which are produced by direct extraction) and lactulose (which is produced by alkali isomerization reaction), they are either synthesized from simple sugars, such as sucrose or lactose, by enzymatic transglycosylation reactions, or formed by controlled enzymatic hydrolysis of polysaccharides, such as starch or xylan (Baek and Lee, 2009). These processes usually produce a range of oligosaccharides differing in their degree of polymerization and sometimes in the position of the glycosidic linkages. Residual substrates and monosaccharides are usually present after oligosaccharide formation, but such sugars can be removed by membrane or chromatographic procedures to form higher-grade products that contain

Table 5.11 Nondigestible oligosaccharides with bifidogenic functions commercially available

Compound	Molecular structure*	Production method
Cyclodextrins	(Gu) _n	Starch by CGTase and α -amylase
Fructooligosaccharides	(Fr) _n -Gu	Transfructosylation from sucrose by β -fructofuranosidase or inulin hydrolysate
Galacto-oligosaccharides	(Ga) _n -Gu	Transgalactosylation from lactose by β -galactosidase
Gentio-oligosaccharides	(Gu) _n	Transgalactosylation of starch by β -glucosidase
Glycosylsucrose	(Gu) _n -Fr	Transglucosylation of sucrose and lactose by cyclomaltodextrin gluca-notransferase
Malto/isomaltooligosaccharides	(Gu) _n	Transgalactosylation of starch by pullanase, isoamylase, α -amylases/transglucosidase
Isomaltulose (or palatinose)	(Gu-Fr) _n	Transglucosidase of sucrose
Lactosucrose	Ga-Gu-Fr	Transglycosylation of lactose and sucrose by β -fructofuranosidase
Lactulose	Ga-Fr	Isomerisation of lactose by alkali
Raffinose	Ga-Gu-Fr	Extraction of plant materials by water or alcohol
Soybean oligosaccharides	(Ga) _n -Gu-Fr	Extraction of soy whey
Xylooligosaccharides	(Xy) _n	Xylan hydrolysis by xylanase or acids

*Ga, galactose; Gu, glucose; Fr, fructose; Xy, xylose; CGTase, cyclodextrin glycosyltransferase.

Source: Adapted from Mussatto et al. (2006) and Baek and Lee (2009).

pure oligosaccharides. Worldwide, there are 13 classes of food-grade oligosaccharides currently produced commercially. Both the volume and diversity of oligosaccharide products are increasing very rapidly as their functional properties become further understood. Detailed production methods for various oligosaccharides have been reviewed by Baek and Lee (2009). The global demand for prebiotic ingredients was dominated by inulin, accounting for over 40% of the overall market in 2011, but mannan oligosaccharides (MOS) and galactooligosaccharides (GOS) are expected to be the fastest-expanding of (<http://www.transparencymarketresearch.com/prebiotics-market.html>). With respect to application areas for prebiotic ingredients, food and beverages, dietary supplements, and animal feed are the major markets where prebiotic sales have seen tremendous potential. Prebiotic demand for food and beverage applications is expected to reach US\$3.7 billion in 2018. The infant formulae market is expected to grow at 11.3%. Prebiotic demand for animal feed applications is expected to cross 70,000 tons by 2018. Lactulose (β -D-galactosyl-D-fructofuranose) and lactitol (β -D-galactosyl-D-glucitol) are synthetic derivatives by alkali isomerization or reduction respectively. Lactulose is also formed in small amounts during sterilization of milk. Neither lactulose, nor lactitol are digested in the small intestine. Their energy content is 2 kcal/g (about 50% of digestible sugars) and they are metabolized by colonic bacteria into SCFAs (acetate, propionate, butyrate). Lactulose and lactitol do not cause tooth decay and their sweetness makes them suitable for application in a large variety of products, including chewing gum, confectionery and ice cream. Lactose can also be used as substrate for the synthesis of oligosaccharides, such as trans-galactosyl-oligosaccharide (TOS) or galactosyl-oligosaccharides. Galactosyl-oligosaccharides are resistant to intestinal hydrolysis and have gained popularity, because of their sweet taste, low energy value (\sim 2 kcal/g), prebiotic properties, and enhancement of mineral absorption. Galactosyl-oligosaccharide are increasingly added to other foods (e.g., chewing gum, dairy products, and baked goods). Industrial production processes can extract oligosaccharides from natural sources, by hydrolyzing polysaccharides, or by enzymatic and chemical synthesis from disaccharide substrates (Baek and Lee, 2009). Slavin (2013) also discussed the mechanisms of fiber and prebiotics well. The combination of probiotics and prebiotics (called *synbiotics*) increase effectiveness of probiotic preparations for therapeutic use. Exact effects are not fully elucidated and can vary by geographic host distribution of resident flora, as well as by diet, aging, and disease states (McFarland, 2006). There are several mechanisms by which affected bacteria exert benefits. These include interference with pathogens, production of antibacterial agents, modification of mucosal permeability, alteration of the innate immune reaction, and metabolism of carbohydrates to SCFA. These result in anti-inflammatory (Broekaert and Walker, 2006), antineoplastic, and other disease-modifying effects. Molecular ecological methods, which reveal the entire range of bacterial diversity and more accurately detect population changes, are now considered more appropriate for evaluating synbiotic effects.

5.8.2 Health claim regulation

Functional foods are regulated as either foods or dietary supplements, depending on how they are labeled. They are usually FDA (USA) preapproved, or structure/function claims, depending on the most recent regulations promulgated by FDA as a result of the Dietary Supplement Health and Education Act (DSHEA). The FDA regulates medical foods somewhat loosely within their own regulatory category (Hasler, 2005). Nutraceuticals are treated differently in different jurisdictions. *Health claims* describing the relationship of diet to a disease can be approved by FDA, but a substance/disease relationship must be based on a sound body of scientific evidence. *Structure/Function claims* are statements of

health-promoting or nutritional benefit allowed on dietary supplement labels. They must describe the support or maintenance of the normal functioning of the body. “Cranberry supports the health of the urinary tract,” is an example of a model structure/function claim.

Canada products are regulated by Health Canada. The EFSA currently requires no less than gold standard clinical trials as proof of efficacy in order to carry a product-specific health claim. The Health Claim Regulation and its practical implementation still have a way to go toward unifying all of the European Union as far as health claims are concerned (Coppens et al., 2006).

Summary

Functional foods, with nutraceuticals represent an emerging trend in food science and the food industry. Modern food biotechnology including enzyme technology, fermentation, and nanotechnology is producing functional foods and nutraceuticals that offer a whole host of increased health benefits, including prevention against illness and chronic and degenerative conditions. This technology also gives insight into quality assurance and food safety and an assessment of where the field currently stands on legal, social, and regulatory aspects of food biotechnology. To address the problem of low oral bioavailability of nutraceuticals, nanotechnology provides a promising approach to the delivery of nutraceuticals. The current challenges are in relation to food-grade encapsulation materials and the toxicity associated with nanoscale formulations.

Among many products, probiotics have been extended to use outside of human nutrition in fermented functional foods resulting from the microbial production of bioactive metabolites such as certain vitamins, bioactive peptides, organic acids, and fatty acids. Probiotic foods *in situ* in the gut through the introduction of a probiotic microorganism may offer the host some added protection against diseases. Bioactive probiotics and derived components can be produced either directly through the interaction of ingested live microorganisms with the host or indirectly as a result of ingestion of microbial metabolites produced during the fermentation process. Although still far from fully understood, several probiotic mechanisms of action have been proposed, including competitive exclusion, competition for nutrients and/or stimulation of an immune response. To battle the increase of health care costs, a preventative approach to medicine with the development of probiotic and prebiotic or symbiotic products is being advanced. Widespread antibiotic resistance has become a cause for concern and new promising antimicrobial treatment options must be explored. Increases in the prevalence of many diseases are fuelling the need for safer, and more effective therapies such as bacteriocins, but more research must be carried out to design more effective bacteriocins.

Bibliography

- Abrahamsson, T. R., T. Jakobsson, M. F. Böttcher, M. Fredrikson, M. C. Jenmalm, B. Björkstén, and G. Oldaeus. 2007. Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial. *J Allergy Clin. Immunol.* 119: 1174–1180.
- Acuña, L., G. Picariello, F. Sesma, R. D. Morero, and A. Bellomio. 2012. A new hybrid bacteriocin, Ent35eMccV, displays antimicrobial activity against pathogenic gram-positive and gram-negative bacteria. *Fed. Eur. Biochem. Soc. Open BIO.* 2: 12–19.

- Adams, M. W. W., F. B. Perler, and R. M. Kelly. 1995. Extremozymes: Expanding the limits of biocatalysis. *Biotechnology* 13: 662–668.
- Adams, R. M., S. Yeast, S. E. Mainzer, K. Moon, A. L. Palombella, D. A. Estell, S. D. Power, and B. F. Schmidt. 1994. Characterization of two cold-sensitive mutants of the β -galactosidase from *Lactobacillus delbrueckii* subsp. *bulgaricus*. *J. Biol. Chem.* 269: 5666–5672.
- Aloğlu H., Öner Z. 2006. Assimilation of cholesterol in broth, cream and butter by probiotic bacteria. *Eur. J. Lipid Sci. Technol.* 108: 709–713.
- Anderson, J. W. and S. E. Gilliland. 1999. Effect of fermented milk (yoghurt containing *Lactobacillus acidophilus* Li on serum cholesterol in hypercholesterolemic humans. *J. Am. Coll. Nutr.* 18: 43–50.
- Anderson, S. C., C. B. Marks, R. Lazarus, J. Miller, K. Stafford, J. Seymour, D. Light, W. Rastette, and D. Estell. 1985. Production of 2-keto-L-gulonate, an intermediate in L-ascorbate synthesis, by a genetically modified *Erwinia herbicola*. *Science* 230: 144–149.
- Anderson, D. M. 1987. Enzymatic production of L-amino acids. In *Biotech 1: Microbial Genetic Engineering and Enzyme Technology*, C. P. Hollenberg and H. Sahn, Eds., pp. 41–59, Gustav Fischer, New York.
- Armstrong, D. W., B. Gillies, and H. Yamazaki. 1989. Natural flavors produced by biotechnological processing. In *Flavor Chemistry: Trends and Developments*. R. Teranashi, R. G. Buttery, and F. Shahidi, Eds., pp. 105–120, American Chemical Society, Washington, D.C.
- Aida, F. M. N. A., M. Shuhaimi, M. Yazid, and A. G. Maaruf. 2009. Mushroom as a potential source of prebiotics: A review. *Trends Food Sci. Technol.* 20: 567–575.
- Alcazar A., Ballesteros O., Jurado J. M., Pablos F., Martin M. J., Vilches J. L., et al. 2007. Differentiation of green, white, black, Oolong, and Pu-erh teas according to their free amino acids content. *J. Agric. Food Chem.* 55: 5960–5965.
- Atwal, A., Bisakowski, B., Richard, S., Robert, N. and Lee, B. 2005. Cloning and expression of hydroperoxide lyase genes from tomato (*Lycopersicon esculentum*) and red bell pepper (*Capsicum annum*) in *Pichia pastoris*. *Process Biochem.* 40: 95–102.
- Baek Y. J. and B. H. Lee. 2009. Probiotics and prebiotics as bioactive components in dairy products. In *Bioactive Components in Milk and Dairy Products*, Y. W. Park, Ed., p. 449, Wiley-Blackwell Publication, New York.
- Bakkal, S., S. M. Robinson and M. A. Riley. 2012. Bacteriocins of aquatic microorganisms and their potential applications in the seafood industry. In *Health and Environment in Aquaculture*, E. Carvalho (Ed.), 303–328.
- Balciunas, E. M., F. A. Castillo Martinez, S. D. Todorov, B. D. Gombossy de Melo Franco, A. Converti, and R. Pinheiro de Souza Oliveira. 2013. Novel biotechnological applications of bacteriocins: A review. *Food Control* 32: 134–142.
- Barile, D. and R. A. Rastall. 2013. Human milk and related oligosaccharides as prebiotics. *Curr. Opin. Biotechnol.* 24: 214–219.
- Becker, J., O. Zeider, S. Häfner, H. Schröder, and C. Wittmann. 2011. From zero to hero-design-based systems metabolic engineering of *Corynebacterium glutamicum* for L-lysine production. *Metab. Eng.* 13: 159–168.
- Becker, J. and C. Wittmann. 2012. Systems and synthetic metabolic engineering for amino acid production – The heartbeat of industrial strain development. *Curr. Opin. Biotechnol.* 23: 718–726.

- Benkerroum, N. 2013. Traditional fermented foods of North African countries: Technology and food safety challenges with regard to microbiological risks. *Compreh. Rev. Food Sci. Food Safety* 12: 54–89.
- Benmechernene, Z., N. I. Fernandez, M. Kihal, K. Böhme, P. Calo-Mata, and J. Barros-Velazquez. 2013. Recent patents on bacteriocins: Food and biomedical applications. *Recent Pat. DNA Gene Seq.* 7: 66–73.
- Berger, J. L., B. H. Lee, and C. Lacroix. 1995. Oligosaccharides synthesis by free and immobilized β -galactosidase from *Thermus aquaticus* YT-1. *Biotechnol. Lett.* 17: 1077–1080.
- Berger, R. G., J. A. M. de Bont, G. Eggink, M. M. R. da Fonseca, M. Gehrke, J. B. Gros, F. V. Keulen, U. Krings, C. Larroche, and D. J. M. J. V. Der Werf. 1999. Biotransformations in the flavour industry. In *Handbook of Flavour and Fragrance Chemistry*, K. Swift, Ed., pp. 139–169, Kluwer Academic Publishers, London, UK.
- Beuzelin-Ollivier, M. G. et al. 2013. Fermentative vitamin C production. US patent no. 8318462.
- Biavati, B. and P. Mattarelli. 2001. The family *Bifidobacteriaceae*. In *The Prokaryotes*, M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer, and E. Stackebrandt, Eds., pp. 1–70, Springer, New York.
- Bierendieck, R. M. Malten, H. Barg, B. Bunk, J. H. Martens, E. Deery, H. Leech, M. J. Warren, and D. Jahn. 2010. Metabolic engineering of cobalamin (vitamin B12) production in *Bacillus megaterium*. *Microbiol. Biotechnol.* 3: 24–37.
- Björkstén, B. and G. Oldaeus. 2007. Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial. *J. Allergy Clin. Immunol.* 119: 1174–1180.
- Bowen, D. M. 2013. Probiotics and oral health. *J. Dent. Hyg.* 87: 5–9.
- Boyle, R. J., R. M. Robins-Browne, and M. L. Tang. 2006. Probiotic use in clinical practice: What are the risks? *Am. J. Clin. Nutr.* 83: 1256–1264.
- Bomgardner, M. M. 2012. The sweet smell of microbes. *Chem. Eng. News* 90: 25–29.
- Bornscheuer, U. T., G. W. Huisman, R. J. Kazlauskas, S. Lutz, J. C. Moore, and K. Robins. 2012. Engineering the third wave of biocatalysis. *Nature* 485 (7397): 185–194.
- Brautaset, T. and T. E. Ellingsen. 2011. Lysine: Industrial uses and production. *Industrial Biotechnology and Commodity Products, Volume 3. Comprehensive Biotechnology*, 2nd ed., pp. 541–554 Elsevier Science & Technology, New York.
- Brey, R. N., C. D. B. Banner, and J. B. Wolf. 1986. Cloning of multiple genes involved with vitamin B₁₂ biosynthesis in *Bacillus megaterium*. *J. Bacteriol.* 167: 623–630.
- Broekaert, I. J. and W. A. Walker. 2006. Probiotics and chronic disease. *J. Clin. Gastroenterol.* 40: 270–274.
- Buffo, R. A. and C. Cardelli-Freire. 2004. Coffee flavour: An overview. *Flavour Fragr. J.* 19: 99–104.
- Capuzzo, A., M. E. Maffei and A. Occhipinti. 2013. Supercritical fluid extraction of plant flavors and fragrances. *Molecules* 18: 7194–7238.
- Cavaco-Paulo, A., and G. Gubitz, Eds. 2003. *Textile Processing with Enzymes*. pp. 240. Woodhead Publishing Ltd Cambridge, UK.
- Chang, S. T. 1993. Mushroom biology: The impact on mushroom production and mushroom products. In *Mushroom Biology and Mushroom Products*, S.-W. Chiu, J. Buswell, and S.-T. Chang, Eds., pp. 3–20, Chinese University Press, Hong Kong. ISBN 962-201-610-3.

- Cheetham, P. J. 1991. The flavour and fragrance industry. In *Biotechnology, the Science and the Business*, V. Moses and R. E. Cape, Eds., pp. 481–506, Harwood Academic Publishers, London.
- Chen, Y. P., P. J. Hsiao, W. S. Hong, T. Y. Dai, and M. J. Chen. 2012. *Lactobacillus kefirifaciens* M1 isolated from milk kefir grains ameliorates experimental colitis in vitro and in vivo. *J. Dairy Sci.* 95: 63–74.
- Chen, L., C. Chen, F. Ge, D. Liu, S. Zhao, and D. Chen. 2013. A preliminary metagenomic study of puer tea during pile fermentation. *J. Sci. Food Agric.* 93: 2870. doi: 10.1002/jsfa.6149.
- Chenoll, E., B. Casinos, E. Bataller, P. Astals, J. Echevarría, J. R. Iglesias, P. Balbarie, D. Ramón, and S. Genovés. 2011. Novel probiotic *Bifidobacterium bifidum* CECT 7366 strain active against the pathogenic bacterium *Helicobacter pylori*. *Appl. Environ. Microbiol.* 77: 1335–1343.
- Cheong, M. W., K. H. Tong, J. J. M. Ong, S. Q. Liu, P. Curran, and B. Yu. 2013. Volatile composition and antioxidant capacity of Arabica coffee. *Food Res. Intern.* 51: 388–396.
- Cheung, P. C. K. 2008. *Mushrooms as Functional Foods*, pp. 23–24, John Wiley & Sons, Inc., Hoboken, NJ.
- Chong, E. S. L. 2013. A potential role of probiotics in colorectal cancer prevention: review of possible mechanisms of action. *World J. Microbiol. Biotechnol.* 30(2):351–374. doi:10.1007/s11274-013-1499-6.
- Christen, P. and A. Lopez-Munguía. 1994. Enzymes and food flavor – A review. *Food Biotechnol.* 8: 167–190.
- Chu, Y. F. 2012. *Coffee: Emerging Health Effects and Disease Prevention*, pp. 352, Wiley-Blackwell, UK.
- Commane, D., R. Hughes, C. Shortt, and I. Rowland. 2005. The potential mechanisms involved in the anti-carcinogenic action of probiotics. *Mutation Res.* 591: 276–89.
- Coppens, P., M. Fernandes da Silva, and S. Pettman. 2006. European regulations on nutraceuticals, dietary supplements and functional foods: A framework based on safety. *Toxicology* 222: 59–74.
- Corripio-Myar, Y., C. Mazorra de Quero, J. W. Treasurer, L. Ford, P. D. Smith, and C. J. Secombes. 2007. Vaccination experiments in the gadoid haddock, *Melanogrammus aeglefinus* L., against the bacterial pathogen *Vibrio anguillarum*. *Vet. Immunol. Immunopathol.* 118: 147–153.
- da Cruz, A. G., J. D. F. Faria, and A. G. F. Van Dender. 2007. Packaging system and probiotic dairy foods. *Food Res. Intern.* 40: 951–956.
- De Kwaadsteniet, M., K. T. Ten Doeschate, and L. M. T. Dicks (2009) Nisin F in the treatment of respiratory tract infections caused by *Staphylococcus aureus*. *Lett. Appl. Microbiol.* 48, 65-70.
- De Roos, N. M. and M. B. Katan. 2000. Effects of probiotic bacteria on diarrhea, lipid metabolism, and carcinogenesis: A review of papers published between 1988 and 1998. *Am. J. Clin. Nutr.* 71: 405–411.
- Deng, J., Y. Li, J. Zhang, and Q. Yang. 2013. Co-administration of *Bacillus subtilis* RJGP16 and *Lactobacillus salivarius* B1 strongly enhances the intestinal mucosal immunity of piglets. *Res. Veterinary Sci.* 94: 62–68.
- Desriac, F., D. Defer, N. Bourgougnon, B. Brillet, P. Le Chevalier, and Y. Fleury. 2010. Bacteriocin as weapons in the marine animal-associated bacteria warfare: inventory and potential applications as an aquaculture probiotic. *Mar. Drugs* 8: 1153–1177.

- Devine, D. A. and P. D. Marsh. 2009. Prospects for the development of probiotics and prebiotics for oral applications. *J. Oral Microbiol.* 1: 1–11.
- Diez-Gonzalez, F. 2007. Use of bacteriocins in livestock. In *Research and Applications in Bacteriocins*, M.A. Riley and O. Gillor, Eds., pp. 218, Horizon Biosciences, USA.
- Dikshit R. and T. Padmavathi . 2013. Exploring *Monascus sanguineus* as a potential natural source for pigment production. *Int. Res. J. Biol. Sci.* 2: 59–67.
- Doerge, K., D. Grajecki, B. C. Zyriax, E. Detinkina, C. Zu Eulenburg, and K. J. Buhling. 2012. Impact of maternal supplementation with probiotics during pregnancy on atopic eczema in childhood – A meta-analysis. *Brit. J. Nutr.* 107: 1–6.
- Dongarra, M. L., V. Rizzello, L. Muccio, W. Fries, A. Cascio, I. Bonaccorsi, and G. Ferlazzo. 2013. Mucosal immunology and probiotics. *Curr. Allergy Asthma Rep.* 13: 19–26.
- Dridger, D., G. Fimland, Y. Hechard, L. M. McMullen, and H. Prevost. 2006. The continuing story of class IIa bacteriocins. *Microbiol. Mol. Biol. Rev.* 70: 564–582.
- Duquesne, S., D. Destoumieux-Garzon, J. Peduzzi, and S. Rebuffat. 2007. Review: Microcins, gene-encoded antibacterial peptides from enterobacteria. *Nat. Prod. Rep.* 24: 708–734.
- Eggeling, L. and H. Sahm. 2009. Amino acid production. In *Encyclopedia of Microbiology*, Volume 1, 3rd ed., pp 150–158, Elsevier, Oxford, UK.
- Ennahar, S., D. Assobhel, and C. Hasselmann. 2008. Inhibition of *Listeria monocytogenes* in a smear-surface soft cheese by *Lactobacillus plantarum* WHE92 a pediocin ACh producer. *J. Food Prot.* 61: 86–191.
- Ennahar, S., T. Sashihara, K. Sonomoto, and A. Ishizaki. 2000. Class IIa bacteriocins: Biosynthesis, structure and activity. *FEMS Microbiol. Rev.* 24: 85–106.
- Falagas, M. E., G. I. Betsi, and S. Athanasiou. 2006. Probiotics for prevention of recurrent vulvovaginal candidiasis: A review. *J. Antimicrob. Chemother.* 58: 266–272.
- Field, D., L. Quigley, P. M. O'Connor, M. C. Rea, K. Daly, P.D. Cotter, C. Hill, and R. P. Ross. 2010a. Studies with bioengineered Nisin peptides highlight the broad-spectrum potency of Nisin V. *Microbiol. Biotechnol.* 3: 473–486.
- Field, D., C. Hill, P. D. Cotter, and R. P. Ross. 2010b. The dawning of a ‘Golden era’ in lantibiotic bioengineering. *Mol. Microbiol.* 78: 1077–1087.
- Florent, J. 1986. Vitamins. In *Biotechnology*, Volume 4, H. J. Rehm and G. Reed, Eds., pp. 115–158, VCH Publishers, New York.
- Frost, G. M. and D. A. Moss. 1987. Production of enzymes by fermentation. In *Biotechnology*, Volume 7a, H. S. Rehm and G. Reed, Eds., pp. 65–211, VCH Publishers, New York.
- Galvez, A., R. L. Lopez, H. Abriouel, E. Valdivia, and N. B. Omar. 2008. Application of bacteriocins in the control of foodborne pathogenic and spoilage bacteria. *Crit. Rev. Biotechnol.* 28: 125–152.
- Garlapati, V. K. and R. Banerjee. 2013. Solvent-free synthesis of flavour esters through immobilized lipase mediated transesterification. *Enzyme Res.* 2013: 367410, <http://dx.doi.org/10.1155/2013/367410>.
- Gasser, B., M. Dragosits, and D. Mattanovich. 2010. Engineering of biotin-prototrophy in *Pichia pastoris* for robust production processes. *Met. Eng.* 12: 573–580.
- Gatfield, I. L. 1988. Production of flavor and aroma compounds by biotechnology. *Food Technol.* 42(10):110–122.
- Garneau, S., N. I. Martin, and J. C. Vederas. 2002. Two-peptide bacteriocins produced by lactic acid bacteria. *Biochimie* 84: 577–592.

- Geier, M. S., R. N. Butler, and G. S. Howarth. 2006. Probiotics, prebiotics and synbiotics: A role in chemoprevention for colorectal cancer? *Cancer Biol. Ther.* 5: 1265–1269.
- Gibson, G. R., H. M. Probert, J. Van Loo, R. A. Rastall, and M. B. Roberfroid. 2004. Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. *Nutr. Res. Rev.* 17: 259–275.
- Gobbetti, M., M. de Angelis, R. Di Cagno, F. Minervini, and A. Limitone. 2007. Cell–cell communication in food related bacteria. *Intern. J. Food Microbiol.* 120: 34–45.
- Goh, E. B., E. E. K. Baido, J. D. Keasling, and H. R. Beller. 2012. Engineering of bacterial methyl ketone synthesis for biofuels. *Appl. Environ. Microbiol.* 78: 70–80.
- Gratia, J. P. 2000. André Gratia: a forerunner in microbial and viral genetics. *Genetics* 156: 471–476.
- Gupta, S., and C. V. Savalia. 2012. Application of biotechnology to improve livestock products. *Vet. World* 5: 634–638.
- Hancock, R. D., J. R. Galpin, and R. Viola. 2000. Biosynthesis of L-ascorbic acid (vitamin C) by *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* 186: 245–250.
- Hartley, B. S., N. Hanlon, R. J. Jackson, and M. Rangarajan. 2000. Glucose isomerase: insights into protein engineering for increased thermostability. *Biochem. Biophys. Acta*, 1543: 294–335.
- Hartmann, A. H., T. Wilke, and R. Erdmann. 2011. Efficacy of bacteriocin-containing cell-free culture supernatants from lactic acid bacteria to control *Listeria monocytogenes* in food. *Int. J. Food Microbiol.* 146: 192–199.
- Harzallah, D. and H. Belhadj. 2013. Lactic acid bacteria as probiotics: Characteristics, selection criteria and role in immunomodulation of human GI mucosal barrier. In *Lactic Acid Bacteria – R & D for Food, Health and Livestock Purposes*, J. Marcelino Kongo, Ed., in tech, Texas, USA.
- Hasler, C. M., Ed. 2005. *Regulation of Functional Foods and Nutraceuticals: A Global Perspective*, pp. 432, Wiley-Blackwell, UK.
- Hatakka, K., A. J. Ahola, H. Yli-Knuutila, M. Richardson, T. Poussa, J. H. Meurman, and R. Korpela. 2007. Probiotics reduce the prevalence of oral candida in the elderly – A randomized controlled trial. *J. Dental Res.* 86: 125–30.
- He, T., M. G. Priebe, Y. Zhong, C. Huang, H. J. Harmsen, G. C. Raangs, J. M. Antoine, G. W. Welling, and R. J. Vonk. 2008. Effects of yogurt and bifidobacteria supplementation on the colonic microbiota in lactose-intolerant subjects. *J. Appl. Microbiol.* 104: 595–604.
- Hebeda, R. E. 1991. Sweeteners, nutritive. In *Encyclopedia of Food Science and Technology*, Y. H. Hui, Ed., pp. 2487–2504, John Wiley & Sons, Inc., New York.
- Henrick, K., D. M. Blow, H. L. Carrell, and J. P. Glusker. 1987. Comparison of backbone structure of glucose isomerase from *Streptomyces* and *Arthrobacter*. *Protein Eng.* 1: 467–469.
- Hemarajata, P. and J. Versalovic. 2012. Effects of probiotics on gut microbiota: Mechanisms of intestinal immunomodulation and neuromodulation. *Therap. Adv. Gastroenterol.* 6: 39–51.
- Hirao, T., T. Nakano, T. Azuma, M. Sugimoto, and T. Nakanishi. 1989. L-Lysine production in continuous culture of a L-lysine hyperproducing mutant of *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* 32: 269–273.

- Homler, B. E., L. D. Stegink, and L. J. Filer, Jr., Eds. 1984. Aspartame, Physiology and Biochemistry, Marcel Dekker, New York.
- Hor, K. C., L. C. Lew, and M. T. Liong. 2014. Effects of ultrasonication on the production of hyaluronic acid by lactobacilli. *Acta Aliment.* 43: 324–332.
- Ibrahim, S. A. and D. J. O’Sullivan. 2000. Use of chemical mutagenesis for the isolation of food grade beta-galactosidase overproducing mutants of bifidobacteria, lactobacilli and *Streptococcus thermophilus*. *J. Dairy Sci.* 83: 923–930.
- Ikeda, M. 2006. Towards bacterial strains overproducing L-tryptophan and other aromatics by metabolic engineering. *Appl. Microbiol. Biotechnol.* 69: 615–626.
- Janssens, L., H. L. De Pooter, N. M. Schamp, and E. J. Vandamme. 1992. Production of flavours by microorganisms. *Process Biochem.* 27: 195–215.
- Jeevaratnam, K., M. Jamuna, and A. S. Bawa, 2005. Biological preservation of foods – Bacteriocins of lactic acid bacteria. *Ind. J. Biotechnol.* 4: 446–454.
- Jiménez, J. J., J. Borrero, D. B. Diep, L. Gútiez, I. F. Nes, C. Herranz, L. M. Cintas, and P. E. Hernández. 2013. Cloning, production, and functional expression of the bacteriocin sakacin A (SakA) and two SakA-derived chimeras in lactic acid bacteria (LAB) and the yeasts *Pichia pastoris* and *Kluyveromyces lactis*. *J. Ind. Microbiol. Biotechnol.* 40: 977–993.
- Joerger, R. D. 2003. Alternatives to antibiotics: bacteriocins, antimicrobial peptides and bacteriophages. *Poultry Sci.* 82: 640–647.
- Jones, K. L. and S. E. Jones. 1986. Fermentation involved in the production of cocoa, coffee, and tea. *Prog. Ind. Microbiol.* 19: 411–456.
- Jones, E., V. Salin, and G. W. Williams. 2005. Nisin and the market for commercial bacteriocins. TAMRC Consumer and Product Research Report No. CP-01-05. July.
- Jones, M. L., C. J. Martoni, and S. Prakash. 2012. Cholesterol lowering and inhibition of sterol absorption by *Lactobacillus reuteri* NCIMB 30242: A randomized controlled trial. *Eur. J. Clin. Nutr.* 66: 1234–1241.
- Jones, M. L., C. Tomaro-Duchesneau, C. J. Martoni, and S. Prakash. 2013. Cholesterol lowering with bile salt hydrolase-active probiotic bacteria, mechanism of action, clinical evidence, and future direction for heart health applications. *Expert Opin. Biol. Ther.* 13: 631–642.
- Kang, M. S., B. G. Kim, J. Chung, H. C. Lee, and J. S. Oh. 2006. Inhibitory effect of *Weissella cibaria* isolates on the production of volatile sulphur compounds. *J. Clin. Periodontol.* 33: 226–232.
- Khattak, F. M., T. N. Pasha, Z. Hayat, and A. Mahmud. 2006. Enzymes in poultry nutrition. *J. Anim. Plant Sci.* 16:1–7.
- Kim, H. S., and S. E. Gilliland. 1983. *Lactobacillus acidophilus* as a dietary adjunct for milk to aid lactose digestion in humans. *J. Dairy Sci.* 66: 959–966.
- Kim, G. B., C. M. Miyamoto, E. A. Meighen, and B. H. Lee. 2004. Cloning and characterization of the bile salt hydrolase genes (bsh) from *Bifidobacterium bifidum* strains. *Appl. Environ. Microbiol.* 70: 5603–5612.
- Kim, G. B. and B. H. Lee. 2008. Genetic analysis of a bile salt hydrolase (bsh) in *Bifidobacterium animalis* subsp. *lactis* KL612. *J. Appl. Microbiol.* 105: 778–789.
- Kirk, O., T. V. Borchert, and C. C. Fuglsang. 2002. Industrial enzyme applications. *Curr. Opin. Biotechnol.* 13: 345–351.

- Klusken, L. D., J. Zeilstra, A. C. Geerling, W. M. de Vos, and J. van der Oost. 2010. Molecular characterization of the glucose isomerase from the thermophilic bacterium *Fervidobacterium gondwanense*. *Environ. Technol.* 31: 1083–1090.
- Koekman, C. Lee A. F. Mayer, A. Meury, N. J. Mouncey, D. Schipper, M. Shinjoh, C. Toepfer, A. W. H. Vollebregt. 2013. Fermentative vitamin C production. US Patent 2013143280.
- Koushki, M. R., A. S. Khoshgozara, M. Mohammadi, Z. Hadian, B. N. Pourfallah, P. Sharayei, and A. M. Mortazavian. 2011. Physicochemical properties of mushrooms as affected by modified atmosphere packaging and CaCl₂ dipping. *Afr. J. Agric. Res.* 6: 5414–5421.
- Krasse, P., B. C. Carlsson, C. Dahl, A. Paulsson, A. Nilsson, and G. Sinkiewicz. 2006. Decreased gum bleeding and reduced gingivitis by the probiotic *Lactobacillus reuteri*. *Swedish Dental J.* 30: 55–60.
- Kumar, M., R. Kumar, R. Hemalatha, V. Verma, A. Kumar, C. Chakraborty, B. Singh, F. Marotta, S. Jain, and H. Yadav. 2012. Cholesterol-lowering probiotics as potential biotherapeutics for metabolic diseases. *Exp. Diabetes Res.* 2012: 1–14, Article ID 902917, <http://dx.doi.org/10.1155/2012/902917>.
- Kumon, S. and T. Kawakita. 1991. Amino acids. In *Biotechnology and Food Ingredients*, I. Goldberg and R. Williams, Eds., pp. 125–152, Van Nostrand Reinhold, New York.
- Laskin, A., Ed. 1985. *Enzymes and Immobilized Cells in Biotechnology*. Benjamin Cummings Publishers, New York.
- Lau, T. C., M. W. Chan, H. P. Tan, and C. L. Kwek. 2013. Functional food: A growing trend among the health conscious. *Asian Social Sci.* 9: 198–208.
- Lee, B. H. 1991a. Bioconversion of starch wastes. In *Bioconversion of Waste Materials to Industrial Products*, A. M. Martin, Ed., pp. 265–311, Elsevier Applied Science, New York.
- Lee, B. H. 1991b. Genetic engineering: Enzyme cloning (16), In *Encyclopedia of Food Science and Technology*, Y. H. Hui, Ed., pp. 1300–1313, John Wiley & Sons, Inc., New York.
- Lee, B. H. and A. Szilagyi. 2012. Lactose: Uses in Industry and Biomedical Importance, Chapter 46. pp. 806–820, Royal Society of Chemistry, UK.
- Lemuth, K., K. Steuer, and C. Albermann. 2011. Engineering of a plasmid-free *Escherichia coli* strain for improved in vivo biosynthesis of astaxanthin. *Microbial Cell Fact.* 10: 29.
- Lew, L. C. and M. T. Liong. 2013. Bioactives from probiotics for dermal health: Functions and benefits. *J. Appl. Microbiol.* 114: 1241–1253.
- Lew, L. C. and M. T. Liong. 2013. Bioactives from probiotics for dermal health: functions and benefits. *J. Appl. Microbiol.* 114:1241–1253.
- Li, S., X. Yang, S. Yang, M. Zhu, and X. Wang. 2012. Technology prospecting on enzymes: Application, marketing and engineering. *Comput. Struct. Biotechnol. J.* 2: 1–11, e201209017, <http://dx.doi.org/10.5936/csbj.201209017>.
- Liang, J. F., Y. T. Li, and V. C. Yang. 2000. Biomedical application of immobilized enzymes. *J. Pharm. Sci.* 89: 979–990.
- Lien, S. and H. B. Lowman. 2003. Therapeutic peptides. *Trends Biotechnol.* 21: 556–562.
- Lin Y. L., Wang T. H., Lee M. H., and Su N. W. 2008. Biologically active components and nutraceuticals in the *Monascus* fermented rice: A review, *Appl. Microbiol. Biotechnol.* 77: 9659–97.

- Lu, Y., G. V. Levin, and T. W. Donner. 2008. Tagatose, a new antidiabetic and obesity control drug. *Diabetes Obes. Metab.* 10: 109–134.
- Lye, H.-S., G. Rusul Rahmat-Ali, and M.-T. Liong. 2010. Mechanisms of Cholesterol removal by lactobacilli under conditions that mimic the human gastrointestinal tract. *Intern. Dairy J.* 20: 169–175.
- Lyu, C., C. Chen, F. Ge, D. Liu, S. Zhao, and D. Chen. 2013. A preliminary metagenomic study of puer tea during pile fermentation. *J. Sci. Food Agr.* 93: 3165–3174.
- MacAllister, R. V. 1980. Manufacture of high fructose corn syrup using immobilized glucose isomerase. In *Immobilized Enzymes for Food Processing*, CRC Press, Boca Raton, FL.
- McNeil, B., D. Archer, I. Giavasis, and L. Harvey, Eds. 2013. Microbial production of food ingredients, enzymes and nutraceuticals. p. 640, Woodhead Publishing Ltd, UK.
- Makarova, K. S. and E. V. Koonin. 2007. Evolutionary genomics of lactic acid bacteria. *J. Bacteriol.* 189: 1199–1208.
- Mantovani, H. C., A. M. O. Cruz, and A. D. Paiva. 2011. Bacteriocin activity and resistance in livestock pathogens. In *Science against Microbial Pathogens: Communicating Current Research and Technological Advances*, A. Méndez-Vilas, Ed., pp. 853–863. FORMATEX, Spain.
- Manuel, N., M. Rafael, A. Miguel, and R. B. Castanho. 2009. Antimicrobial peptides: Linking partition, activity and high membrane-bound concentrations. *Nature Rev. Microbiol.* 7: 245–250.
- Martirosyan, D. M. Ed. 2011. *Functional Foods and Chronic Diseases: Science and Practice*. Food Science Publisher, Texas, USA.
- McEntire, J. C., T. J. Montville, and M. L. Chikindas. 2003. Synergy between nisin and select lactate against *L. monocytogenes* is due to the metal cations. *Intern. J. Food Microbiol.* 66: 1631–1636.
- McFarland L. V. 2006. Meta-analysis of probiotics for the prevention of antibiotic associated diarrhea and the treatment of *Clostridium difficile* disease. *Am. J Gastroenterol.* 101:812–822.
- McFarland, L. V. 2009. Evidence-based review of probiotics for antibiotic-associated diarrhea and *Clostridium difficile* infections. *Anaerobe* 15: 274–280.
- Mcmanus, P. S., V. O. Stockwell, G. W. Sundin, and A. L. Jones. 2002. Antibiotic use in plant agriculture. *Ann. Rev. Phytopathol.* 40 : 443–465.
- Mehta, B. K., S. K. Jain, G. P. Sharma, A. Doshi, and H. K. Jain. 2011. Cultivation of button mushroom and its processing: A techno-economic feasibility. *Intern. J. Adv. Biotechnol. Res.*, 2: 201–207.
- Meister, A., G. M. Brown, and J. M. Williamson. 2006. Biosynthesis of riboflavin, folic acid, thiamine, and pantothenic acid. *Advances in Enzymology and Related Areas of Molecular Biology*, Volume 53. John Wiley & Sons, Inc., New York.
- Mercenier, A., P. H. Pouwels, and B. M. Chassy. 1994. Genetic engineering of lactobacilli, leuconostocs, and *Streptococcus thermophilus*. In *Genetics and Biotechnology of Lactic Acid Bacteria*, M. J. Gasson and W. M. de Vos, Eds., pp. 252–293, Blackie Academic and Professional, London.
- Minifie, B. W. 1999. *Chocolate, Cocoa, and Confectionery: Science and Technology*. AVI Publishing Co., Westport, CT, p. 545.
- Mishra, V. and D. N. Prasad. 2005. Application of in vitro methods for selection of *Lactobacillus casei* strains as potential probiotics. *Intern. J. Food Microbiol.* 103: 109–115.

- Mohanasrinivasan, V., D. C. Subathra, V. Suganthi and E. Selvarajan. 2012. Nisin Based Lantibiotic Engineering: A Novel Approach on Biopreservation. p. 80, LAP LAMBERT Academic Publishing, Germany.
- Moore, S. J., A. D. Lawrence, R. Biedendieck, E. Deery, M. J. Howard, S. Frank, M. J. Howard, S. E. J. Rigby, and M. J. Warren. 2013. Elucidation of the anaerobic pathway for the corrin component of cobalamin (vitamin B₁₂). *Proc. Natl. Acad. Sci. USA* 110(37): 14906–14911.
- Motlagh, A. M., S. Holla, M. C. Johnson, B. Ray, and R. A. Field. 1992. Inhibition of *Listeria* spp. in sterile food systems by pediocin AcH, a bacteriocin produced by *Pediococcus acidilactici* H. *J. Food Prot.* 55: 337–343.
- Mukherjee, M. and A. Sarkar. 2011. Sugar content in artificial sweeteners. *Adv. Appl. Sci. Res.* 2: 407–409.
- Muralla, R., E. Chen, C. Sweeney, J. Gray, A. Dickerman, B. Nikolau, and D. Meinke. 2008. A bifunctional locus (BIO3-BIO1) required for biotin biosynthesis in *Arabidopsis*. *Plant Physiol.* 146: 60–73.
- Mussatto, S. I., Dragone, G., and Roberto, I. C., 2006. Brewers' spent grain: Generation, characteristics and potential applications. *J. Cereal Sci.* 43: 1–14.
- Nase, L., Hatakka, K., Savilahti E., Saxelin M., Ponka A., Poussa T., Meurman J. H. 2001. Effect of long term consumption of a probiotic bacterium *Lactobacillus rhamnosus* GG, in milk on dental caries and caries risk in children. *Caries Res.* 35: 412–420.
- Neetoo H., Ye M., Chen H., Joerger R. D., Hicks D. T., and Hoover D. G. 2008. Use of nisin-coated plastic films to control *Listeria monocytogenes* on vacuum-packaged cold-smoked salmon. *Intern. J. Food Microbiol.* 122: 8–15.
- Nelis, H. J. and A. P. De Leenheer. 1991. Microbial sources of carotenoid pigments used in foods and feeds. *J. Appl. Bacteriol.* 70: 181–191.
- Nielsen, D. S., M. Crafsack, L. Jespersen and M. Jakobsen. 2013. The microbiology of cocoa fermentation. *Nutr. Health* 7: 39–60.
- Ooi, L. G., R. Bhat, A. Rosma, K. H. Yuen, and M. T. Liong. 2010. A synbiotic containing *Lactobacillus acidophilus* Cho-220 and insulin improves irregularity of red blood cells. *J. Dairy Sci.* 93: 4535–4544.
- Ortiz, M. E., J. Bleckwedel, R. R. Raya, and F. Mozzi. 2013. Biotechnological and in situ food production of polyols by lactic acid bacteria. *Appl. Microbiol. Biotechnol.* 97: 4713–4726.
- Osawa, I., D. Speck, T. Kisou, K. Hayakawa, M. Zinsius, R. Gloeckler, Y. Lemoine, and K. Kamogawa. 1989. Cloning and expression in *Escherichia coli* and *Bacillus subtilis* of the biotin synthetase gene from *Bacillus sphaericus*. In *Biotechnology of Vitamins, Pigments and Growth Factors*, E. J. Vandamme, Ed., p. 254, Elsevier Applied Science, London.
- Ouwehand, A. C., S. Salminen, and E. Isolauri. 2002. Probiotics: An overview of beneficial effects. *Antonie van Leeuwenhoek* 82: 279–289.
- Ouwehand, A. and S. Vesterlund. 2003. Health aspects of probiotics. *IDrugs* 6: 573–580.
- Paine, J. A., C. A. Shipton, S. Chaggar, R. M. Howells, M. J. Kennedy, G. Vernon, S. Y. Wright, E. Hinchliffe, J. L. Adams, A. L. Silverstone, and R. Drake. 2005. Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nature Biotechnol.* 23: 482–487.

- Paolo, U. S., M. Scarpa, F. Oppia, and F. Cabras. 2012. Lactose malabsorption and intolerance: What should be the best clinical management? *World J. Gastrointest. Pharmacol. Ther.* 3: 29–33.
- Papagianni, M. and S. Anastasiadou. 2009. Pediocins: The bacteriocins of *Pediococci*. Sources, production, properties and applications. *Microbial Cell Fact.* 8: 1–16.
- Pedersen, D. C. 1991. Alternative sweeteners. In *Biotechnology and Food Ingredients*, I. Goldberg and R. Williams, Eds. pp. 393–413, Van Nostrand Reinhold, New York.
- Peña-Montes, C., M. E. Mondragón-Tintor, J. A. Castro-Rodríguez, I. Bustos-Jaimes, A. Navarro-Ocaña, and A. Farrés. 2013. Immobilization and biochemical properties of the enantioselective recombinant NStcI esterase of *Aspergillus nidulans*. *Enzyme Res.* 2013, 11 pages, Article ID 928913, <http://dx.doi.org/10.1155/2013/928913>.
- Perlman, D. 1979. Microbial process for riboflavin production. In *Microbial Technology*, Volume 1, H. J. Peppler and D. Perlman, Eds., Academic Press, New York.
- Preidis G. and Versalovic J. 2009. Targeting the human microbiome with antibiotics, probiotics, and prebiotics: gastroenterology enters the metagenomics era. *Gastroenterology* 136: 2015–2031.
- Quigley, E. M. 2012. Probiotics in the management of functional bowel disorders: Promise fulfilled? *Gastroenterol. Clin. North Am.* 41: 805–819.
- Rabinovich, M. L. 2013. Biocatalysis and enzyme technology. *Biotech. J.* 8: 764–767. doi: 10.1002/biot.201200401.
- Rafiqul, I. S.M. and A. M. M. Sakinah. 2013. Processes for the production of xylitol – A review. *Food Rev. Intern.* 29: 127–156.
- Rao, D. R., S. Reddy, R. Pulusani, and P. E. Cornwell. 1984. Biosynthesis and utilization of folic acid and vitamin B₁₂ by lactic cultures in skim milk. *J. Dairy Sci.* 67: 1169–1174.
- Rao, L., Y. Xue, Y. Zheng, J. R. Lu, and Y. Ma. 2013. A novel alkaliphilic *Bacillus* esterase belongs to the 13th bacterial lipolytic enzyme family. *PLoS One* 8(4): e60645. doi:10.1371/journal.pone.0060645.
- Rashmi, D. and T. Padmavathi. 2013. Exploring *Monascus sanguineus* as a potential natural source for pigment production. *Intern. Res. J. Biol. Sci.* 2: 59–67.
- Reetz, M. T. 2013. Biocatalysis in organic chemistry and biotechnology: Past, present, and future. *J. Am. Chem. Soc.* J135: 12480–12496.
- Reid, G. 2005. The importance of guidelines in the development and application of probiotics. *Curr. Pharm. Des.* 11: 11–16.
- Reid, G. and A. W. Bruce. 2006. Probiotics to prevent urinary tract infections: The rationale and evidence. *World J. Urol.* 24: 28–32.
- Ridlon, J. M., D. J. Kang, and P. B. Hylemon. 2006. Bile salt biotransformations by human intestinal bacteria. *J. Lipid Res.* 47: 241–259.
- Rijkers, G. T., S. Bengmark, P. Enck, D. Haller, U. Herz, M. Kalliomaki, S. Kudo, I. Lenoir-Wijnkoop, A. Mercenier, E. Myllyluoma, S. Rabot, J. Rafter, H. Szajewska, B. Watzl, J. Wells, D. Wolvers, and J. M. Antoine. 2010. Guidance for substantiating the evidence for beneficial effects of probiotics: Current status and recommendations for future research. *J. Nutr.* 140: 6715–6765.
- Riley, M. A. 2009. Bacteriocins, biology, ecology, and evolution. In *Encyclopedia of Microbiology*, M. Schaechter, Ed., pp. 32–44, Elsevier.

- Romero, D. A. 1992. Bacteria as potential sources of flavor metabolics. *Food Technol.* 46(11):122–126.
- Rose, A. H. 1982. Fermented foods. In *Economic Microbiology*, Volume 7, Academic Press, London.
- Rouse, S., D. Field, K. M. Daly, P. M. O'Connor, and P. D. Cotter, et al. 2012. Bioengineered nisin derivatives with enhanced activity in complex matrices. *Microb. Biotechnol.* 5: 501–508.
- Salaspuro, M. 2009. Acetaldehyde as a common denominator and cumulative carcinogen in digestive tract cancers. *Scandinavian J. Gastroenterol.* 44: 912–925.
- Santoso, B., B. Mwenya, C. Sar, and J. Takahashi. 2006. Ruminant fermentation and nitrogen metabolism in sheep fed a silage-based diet supplemented with *Yucca schidigera* or *Y. schidigera* and nisin. *Animal Feed Sci. Technol.* 129: 187–195.
- Sarrouh, B., T. M. Santos, A. Miyoshi, R. Dias, and V. Azevedo. 2012. Up-to-date insight on industrial enzymes applications and global market. *J. Bioprocess Biotech.* 8: 4–14.
- Scaife, M. A., A. M. Burja, and P. C. Wright. 2009. Characterization of cyanobacterial β -carotene ketolase and hydroxylase genes in *Escherichia coli*, and their application for astaxanthin biosynthesis. *Biotechnol. Bioeng.* 103: 944–955.
- Scaife, M. A., C. A. Ma, T. Ninlayarn, P. C. Wright, and R. E. Armenta. 2012. Comparative analysis of β -carotene hydroxylase genes for astaxanthin biosynthesis. *J. Nat. Prod.* 75: 1117–1124.
- Seitz, E. W. 1991. Flavor building blocks. In *Biotechnology and Food Ingredients*, I. Goldberg and R. Williams, Eds., pp. 375–392, Van Nostrand Reinhold, New York.
- Shankar, P., S. Ahuja, and K. Sriram. 2013. Non-nutritive sweeteners: Review and update. *Nutrition*, doi: 10.1016/j.nut.2013.03.024.
- Sheng, Y., H. Huang, and D. Wang. 2012. Recombinant microorganism and method for producing L-lysine. US patent 8268597.
- Shi, L. E., G. Q. Ying, Z. X. Tang, J. S. Chen, W. Y. Xiong, and H. Wang. 2009. Continuous enzymatic production of 5'-nucleotides using free nuclease P1 in ultrafiltration membrane reactor. *J. Membr. Sci.* 345: 217–222.
- Sims, G. K. and E. J. O'Loughlin. 1992. Riboflavin production during growth of *Micrococcus luteus* on pyridine. *Appl. Environ. Microbiol.* 58: 3423–3425.
- Slavin, J. 2013. Fiber and prebiotics: Mechanisms and health benefits. *Nutrients* 5: 1417–1435.
- Smith, P. 2007. Antimicrobial use in shrimp farming in Ecuador and emerging multi-resistance during the cholera epidemic of 1991: A re-examination of the data. *Aquaculture* 27: 1–7.
- Sonoyama, T., H. Tani, K. Matsuda, B. Kageyama, M. Tanimoto, K. Kobayashi, S. Yagi, H. Kyotanim, and K. Mitsushima. 1982. Production of 2-keto-L-gulonic acid from D-glucose by two-stage fermentation. *Appl. Environ. Microbiol.* 43: 1064–1069.
- Stahmann, K. P., J. L. Revuelta, and H. Seulberger. 2000. Three biotechnical processes using *Ashbya gossypii*, *Candida famata*, or *Bacillus subtilis* compete with chemical riboflavin production. *Appl. Microbiol. Biotechnol.* 53: 509–516.
- Swati, S., A. P. Vartak, and R. Vince. 2012. The butter flavorant, diacetyl, exacerbates β -amyloid cytotoxicity. *Chem. Res. Toxicol.* 25(10): 2083–2091.
- Taoka, Y., H. Maeda, J. Y. Jo, M. J. Jeon, S. C. Bai, W. J. Lee, K. Yuge, and S. Koshio. 2006. Growth, stress tolerance and non-specific immune response of Japanese

- flounder *Paralichthys olivaceus* probiotics in a closed recirculating system. *Fish Sci.* 72: 310–321.
- Taylor, G. R. and C. M. Williams. 1998. Effects of probiotics and prebiotics on blood lipids. *Brit. J. Nutr.* 80: S225–230.
- Tominaga, T., and Y. Hatakeyama. 2007. Development of innovative pediocin PA–1 by DNA shuffling among class IIa bacteriocins. *Appl. Environ. Microbiol.* 73: 5292–5299.
- Qin, X. 2011. What made Canada become a country with the highest incidence of inflammatory bowel disease: Could sucralose be the culprit? *Can. J. Gastroenterol.* 25: 511–516.
- Qin, X. 2012. Etiology of inflammatory bowel disease: A unified hypothesis. *World J. Gastroenterol.* 18: 1708–1722.
- Vadyvaloo, V., J. W. Hastings, M. J. Van Der Merwe, and M. Rautenbach. 2002. Membranes of class IIa bacteriocin-resistant *L. monocytogenes* cells contain increased levels of desaturated and snort-acyl-chain phosphatidylglycerols. *Appl. Environ. Microbiol.* 68: 5223–5230.
- Valls, J., N. Pasamontes, A. Pantaleón, S. Vinaixa, M. Vaqué, A. Soler, S. Millán and X. Gómez. 2013. Prospects of functional foods/nutraceuticals and markets. In *Natural Products*, K. G. Ramawat and J. M. Merillon, Eds., pp. 2491–2525, Springer-Verlag, Wien.
- Vandamme E. J., Ed. 1989. *Biotechnology of Vitamins, Pigments and Growth Factors*, p. 189, Elsevier Applied Science, New York.
- Verrips, C. T., A. M. Ledebøer, L. Edens, R. Klok, and J. Maat. 1990. DNA sequences encoding the various allergic forms of mature thaumatin and cloning vehicles, etc. US Patent 4891316.
- Vujic, G., K. A. Jajac, S. V. Despot, and V. V. Kuzmic. 2013. Efficacy of orally applied probiotic capsules for bacterial vaginosis and other vaginal infections: a double-blind, randomized, placebo-controlled study. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 168: 75–79.
- Wade, W. 2013. Detection and culture of novel oral bacteria, Chapter 2. In *Oral Microbial Ecology: Current Research and New Perspectives*, N. S. Jakubovics and R. J. Palmer Jr., Eds. Caister Academic Press, Norfolk.
- Waldek, S., M. R. Patel, M. Banikazemi, R. Lemay, and P. Lee. 2009. Life expectancy and cause of death in males and females with Fabry disease: Findings from the Fabry Registry. *Genet. Med.* 11: 790–796.
- Walton, N. J., M. J. Mayer, and A. Narbad. 2003. Vanillin. *Phytochemistry* 63: 505–515.
- Wang, W. L., J. Liu, Y. B. Huo, and J. Q. Ling. 2013. Bacteriocin immunity proteins play a role in quorum-sensing system regulated antimicrobial sensitivity of *Streptococcus mutans* UA159. *Arch. Oral Biol.* 58: 384–390.
- Wanarska, M. and J. Kur. 2012. A method for the production of D-tagatose using a recombinant *Pichia pastoris* strain secreting β -D-galactosidase from *Arthrobacter chlorophenolicus* and a recombinant L-arabinose isomerase from *Arthrobacter* sp. 22c. *Microbial Cell Fact.* 11: 113.
- Wells, J. A. and D. A. Estell. 1988. Subtilisin: an enzyme designed to be engineered. *Trends Biochem. Sci.* 13: 291–292.
- Whitaker, J. R., A. G. J. Voragen, and D. S. W. Wong. 2002. *Handbook of Food Enzymology*, CRC Press, pp. 1128, USA.
- Woodley, J. M. 2013. Protein engineering of enzymes for process applications. *Curr. Opin. Chem. Biol.* 17: 310–316.

- Wu, J., S. Hu., and L. Cao. 2007. Therapeutic effect of nisin Z on subclinical mastitis in lactating cows. *Antimicrob. Agents Chemother.* 51: 3131–3135.
- Xiao, J. Z., S. Kondo, N. Takahashi, K. Miyaji, K. Oshida, A. Hiramatsu, K. Iwatsuki, S. Kokubo, and A. Hosono. 2003. Effects of milk products fermented by *Bifidobacterium longum* on blood lipids in rats and healthy adult male volunteers. *J. Dairy Sci.* 86: 2452–2461.
- Yamaguishi, C. T. Sanada, C. T. Gouvea, P. M. Ashok, P. Woiciechowski, A. L. Parada, J. L. Soccol, C. R. 2009. Biotechnological process for producing black bean slurry without stachyose. *Food Res. Intern.* 42: 425–429.
- Ye, X., S. Al-Babili, A. Klöti, J. Zhang, P. Lucca, P. Beyer, and I. Potrykus. 2000. Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* 287(5451): 303–305.
- Ye, R., H. Pyo, and D. G. Hayes. 2010. Lipase-catalyzed synthesis of saccharide-fatty acid esters using suspensions of saccharide crystals in solvent-free media. *J. Am. Oil Chem. Soc.* 87: 281–293.
- Zacharof, M. P., and R. W. Lovitt. 2013. Low molecular weight liquid media development for *Lactobacilli* producing bacteriocins. *J. Chem. Technol. Biotechnol.* 88: 72–80.
- Zarate G. and M. E. Nader-Macias. 2006. Viability and biological properties of probiotic vaginal lactobacilli after lyophilization and refrigerated storage into gelatin capsules. *Process Biochem.* 41: 1779–1785.
- Zhang, E., L. Zhang, and G. Y. Shi. 2013. Cloning and expression of D-arabinose isomerase from *Geobacillus pallidus* and its application in D-tagatose conversion. *Food Sci.* 34: 121–126.
- Zhou, X. and Y. Wang. 2012. Probiotics in aquaculture – Benefits to the health, technological applications and safety, Chapter 8. In *Health and Environment in Aquaculture*, E. Carvalho, Ed., pp. 215–226, In tech.

Part II

Questions and Answers

1. What is different between fungi, yeast, and mold?

Answer: A fungus is a eukaryotic organism that is a member of the kingdom Fungi. The fungi are heterotrophic organisms possessing a chitinous cell wall. The majority of species grow as multicellular filaments called hyphae forming a mycelium; some fungal species also grow as single cells. Sexual and asexual reproduction of the fungi is commonly via spores, often produced on specialized structures or in fruiting bodies. Some species have lost the ability to form reproductive structures, and propagate solely by vegetative growth. Yeasts, molds, and mushrooms are examples of fungi.

2. What do bread and beer have in common?

Answer: Yeasts break glucose down and produce alcohol and carbon dioxide as their by-products.

3. What is the importance of yeast in the food process?

Answer: Yeast is a living organism used in bread (to make it rise), beer, wine, and spirits (to make ethanol). In bread dough the yeast will consume the natural sugars in the flour as soon as water is added. As it consumes the sugars ferments producing pockets of CO₂ and alcohol. When baking, the heating process gets rid of the ethanol to leave the pockets, making the bread light and fluffy.

4. In yeast fermentation of different foods and beverages, only one species is commonly used. Name the species and discuss the mating. What are the characteristics of bottom and top yeasts?

Answer: *S. cerevisiae* is used in brewing beer, when it is sometimes called a top-fermenting or top-cropping yeast. It is so called because during the fermentation process its hydrophobic surface causes the flocs to adhere to CO₂ and rise to the top of the fermentation vessel. Top-fermenting yeasts are fermented at higher temperatures than the lager yeast *Saccharomyces pastorianus*, and the resulting beers have a different flavor than the same beverage fermented with lager yeast. Lager yeast normally ferments at a temperature of approximately 5 °C (41 °F), where *S. cerevisiae* becomes dormant.

5. Name the four active forms of yeasts and discuss each one of them.

Answer:

1. Compressed yeast (active *S. cerevisiae* yeasts containing about 70% water and 27% (w/v) dry matter, marketed as cake or cake-crumbled products).
 2. Cream yeast (yeasts containing about 85% water is delivered directly in chilled steel containers for bakeries, that is favored by many bakeries).
 3. Active dry yeast (similar to compressed yeasts, but the pressed cake is extruded through a perforated plate to produce strands (about 3 mm diameter), further break down into smaller strands (0.3–1 mm), dried over on a continuous belt drier (6 h at 25–45 °C), and packaged in small pouches under nitrogen. Unground, granular yeast are also sold air-packed in cartons or drum for large bakeries).
 4. Instant active dry yeast comparable to those of compressed yeasts, but use special strains of the yeast, *S. cerevisiae* which can retain maximum activity through an air-lift fluidized-bed drier. Yeasts are dry very fast in finer particles, but are highly unstable when exposing to air, thus require packaging under nitrogen or vacuum.
6. What is inactive yeast? What are yeast derivatives?

Answer: Dried forms of brewer's yeast as a byproducts of beer manufacture, and primary grown yeasts (*S. cerevisiae*, *Kluyveromyces*, *C. utilis*) in other carbohydrates (sulfite liquor wastes, or other food wastes); yeast derivatives are yeast autolysates or yeast extracts produced by the autolysis process (40–55 °C), which triggers autolytic enzymes (such as proteases, carbohydrases, and nucleases, etc.) to achieve maximum solubilization of yeast cell contents. Name of yeast derivatives are: (i) Flavor enhancer nucleotides, 5'-IMP 5'-GMP, (ii) invertase, which hydrolyzes sucrose to glucose and fructose for chocolate enrobing, (iii) lactase (beta-galactosidase) from *Kluyveromyces marxianus* (or *K. lactis*) grown in whey lactose, (iv) astaxanthin red pigment by red yeast, *P. rhodoryma*, that are used in fish feed, and (v) glycan, the crude cell fraction of yeast, that can be used in emulsifier, stabilizer, thickener, or texturizer to develop low fat, low calorie salad dressings and other food formulations, as well as oligosaccharides (bifidogenic factor), and immunogenic ingredients.

7. What are the ingredients in beer?

Answer: Beer is traditionally produced from water, malted barley, and hops. Today some brewers use rice or corn to replace a portion of the malted barley. Yeast is used in the fermentation of beer, but it is usually removed before packaging.

8. What is malt in beer processes? Why is malt used in beer?

Answer: Malting is a controlled process of seed germination. During malting the seed is soaked in water to raise the moisture content and promote germination for 4–5 days, after which it is dried (kilned). Barley is easy to malt and produces the best beer; Malt is the source of "extract" in brewing. Barley contains about 65% starch. Enzymes that develop during the malting process convert this starch into fermentable sugars during brewing. The yeast, in turn, converts the sugars to alcohol and carbon dioxide during fermentation. Compounds in the malt also contribute to beer color, body, flavor, and foam.

9. Outline the brewing process.

Answer: See Figure 3.3.

10. What are hops? Which compound of hops makes bitterness in beer?

Answer: Hops are dried blossoms of the female flowers of the hop plant, *H. lupulus*. They are used primarily as a flavoring and stability agent in beer, to which they impart a bitter and tangy flavor. Hop resins are composed of two main acids: α - and β -acids, but only α -acids (humulones) are responsible for the bitter flavor in the beer.

11. What is light beer? How to produce light beer?

Answer: Light beer is lower calorie beer, which contains reduced dextrin content. Many breweries add enzymes (α -amylase and glucoamylase) to aid in breaking down unfermentable dextrans that would normally be left in the finished beer. Adjuncts such as rice and corn in their beers are another way. Many light brewers also dilute the beer with water to hit a desired gravity.

12. Outline the wine-making process.

Answer: See Figure 3.4.

13. What is malolactic fermentation during wine aging?

Answer: In the aging process of red wines, a secondary fermentation, called malolactic fermentation occurs. During this process lactic starter, *O. oeni* converts tart-tasting malic acid, naturally present in grape must, is converted to softer-tasting lactic acid, lowering the total acidity and raising the pH, that create a rounder, fuller mouth feel wine.

14. What is ice wine?

Answer: Ice wine is a type of dessert wine produced from grapes that have been frozen while still on the vine. The sugars and other dissolved solids do not freeze, but the water does, allowing a more concentrated grape must to be pressed from the frozen grapes, resulting in a smaller amount of more concentrated, very sweet wine. With ice wines, the freezing happens before the fermentation, not afterwards.

15. What are the health benefits and harmful effects of beer and wine?

Answer: Both beer and wine contain significance amounts of polyphenols, antioxidant phytochemicals that occur naturally in plants and these have been shown to reduce “bad” LDL cholesterol, reduce the risk of heart disease and certain cancers and prevent free radicals from causing cell damage. Drinking alcohol decreases the risk for some diseases, but increases others. At a moderate level, the benefits simply outweigh the harms. However, the presence of ethyl carbamate, a suspected carcinogen, is subjected to regulation in many countries. The compound is produced from the degradation of the amino acid arginine, which is present in both grape must and beer that released through the autolysis of dead yeast cells. While the use of urea as a source of yeast assimilable nitrogen was the most common cause of ethyl carbamate in wine, *O. oeni* has been known to produce both carbamyl phosphate and citrulline, which can be precursors to ethyl carbamate formation.

16. Corn crop is the main feedstock used for producing ethanol fuel in the United States. What are the controversies surrounding ethanol fuel production from corn? Which raw materials are future target?

Answer: Controversies are around corn ethanol's energy balance and carbon intensity considering the full life cycle of ethanol production, as well as its social and environmental impacts, as bioethanol is competing with food crop. Cellulose biomass, switch grass, and seaweed are possible raw materials.

17. What is the bottleneck for cellulosic bioethanol commercialization?

Answer: Start-up costs for pilot scale lignocellulosic ethanol plants are high. Most pretreatment processes are not effective when applied to feedstocks with high lignin content, such as forest biomass. Enzymes (cellulases and hemicellulases) used in the production of cellulosic ethanol are expensive. It requires 40–100 times more of the enzyme to be present in its production. There is also relatively high capital costs associated with the long incubation times for the vessel that perform enzymatic hydrolysis. Altogether, enzymes comprise a significant portion of 20–40% for cellulosic ethanol production.

18. What are the differences between leavened and unleavened bread?

Answer: Leavened bread has “leavening” added to the dough. Leavening can be yeast, baking soda, or powder. Unleavened bread is made without leavening agents; such bread does not rise and is usually flat.

19. Why do you think soy flour helps bread to rise?

Answer: Soy flour improves the binding of the proteins in the dough and allows for more efficient and effective CO₂ capture.

20. How is sourdough bread made?

Answer: Sourdough is a bread product made by the long fermentation of dough using naturally occurring lactobacilli (*L. sanfrancisco*, etc.) and yeasts (*Candida humilis* or *Saccharomyces exiguous*). In comparison with breads made quickly with cultivated yeast, it usually has a mildly sour taste because of the lactic acid produced by the lactobacilli.

21. What is staling bread? Which mechanism makes this? How can this be prevented?

Answer: Staling is a chemical and physical process in bread and other foods that reduces their palatability. One important mechanism is the migration of moisture from the starch granules into the interstitial spaces, degelatinizing the starch. The starch amylose and amylopectin molecules realign themselves causing recrystallization. This results in stale bread's leathery, hard texture. To prevent staling, glycerol monostearate, hydrocolloids (sodium alginate, xanthan gum, etc.), and enzymes (fungal amylases and proteases) can be added.

22. What are the benefits of probiotic yeasts?

Answer: Some commercial probiotic supplements use the yeast *Saccharomyces boulardii* to maintain and restore the natural flora in the gastrointestinal tract. *S. boulardii* has been shown to reduce the symptoms of acute diarrhea, reduce the chance of infection by *Clostridium difficile*, reduce bowel movements in diarrhea-predominant irritable bowel syndrome (IBS) patients, and reduce the incidence of antibiotic-, traveler's-, and HIV/AIDS-associated diarrheas.

23. Where are the molds used in food fermentation? What precautions are needed while using a mold strain in food fermentation?

Answer: The three main types of cheese that rely on molds are blue cheese, soft ripened cheese (such as camembert and brie) and rind-washed cheese (such as limburger). Blue cheese is treated with a mold, usually *P. roqueforti*, while it is still in the loosely pressed curd form. As the cheese matures, the mold grows, creating blue veins within it which gives the cheese its characteristic flavor, methylketone. Soft ripened cheese such as brie and camembert are made by allowing *P. camemberti* to grow on the outside of the cheese, which causes them to age from the outside in. The mold forms a soft white crust, and the interior becomes runny with a strong flavor. Also Inoculations of sausages with molds were done with the indigenous biota of the slaughters. Different molds (such as *P. chrysogenum* and *P. nalgiovensis*) can be used to ripen surfaces of sausages. The mold cultures develop the aroma and improve the texture of the sausages; some mold strains can produce mycotoxins, and thus selection and developments of strains need careful consideration. In the past, soy sauce has been made by mixing soybeans and other grains with a mold (*A. oryzae* or *A. sojae*) and yeast. This mixture was then left to ferment in the sun. Today soy sauce is made under controlled conditions.

24. What is the role of LAB in fermented milk products? What are the predominant species in fermented milk products?

Answer: Fermented milk by LAB was first made in order to increase the shelf life of dairy products, as well as to make milk easier to digest and enhance the flavor and texture of dairy foods; the specific chemical reaction and product that results from fermentation depend upon the type of bacteria used and the process by which it is combined with the milk. It is commonly used to create dairy products such as yogurt, kefir, cheese, and sour cream, and so on. The predominant species are listed in Table 4.1.

25. What is the current classification of LAB? Why lactic bifidobacteria are now not classified as LAB?

Answer: Read: Björkroth and Koort (2011). Lactic Acid Bacteria: Taxonomy and Biodiversity. In: Encyclopedia of Dairy Sciences (Second Edition), 2011, Pages 45–48. Sonomoto and Yokota (2011). Lactic Acid Bacteria and Bifidobacteria: Current Progress in Advanced Research. Caister Academic Press. Bifidobacteria, which also produce lactate and acetate as the end products of carbohydrate metabolism, have a unique pathway of hexose fermentation, a fructose-6-phosphate shunt that differs from Embden–Meyerhof–Parnas (EMP), and 6-phosphogluconate metabolic routes of LAB. They also have a G+C content of over 50 mol%, and are not related to LAB but to actinobacteria.

26. What are the primary events that occur during the fermentation of milk products? Do we know the secondary metabolism of fermented dairy products and its effect on cheese?

Answer: Glycolysis (lactose to lactic acid), proteolysis (protein to amino acids), and lipolysis (fat to fatty acids) are the primary events that happen during fermentation of milk products. Only primary biochemical changes are well known, but the secondary events are known only in general terms. Numerous secondary changes, which occur simultaneously, are mainly responsible for the final aspects of cheese flavor as well as for the modification of the cheese texture.

27. How can insoluble milk protein, casein be metabolized by LAB? List the possible proteolytic and peptidolytic enzymes involved in casein metabolism.

Answer: Proteolysis of native proteins to peptides by extracellular serine proteinases located on the cell envelope. Further hydrolysis is carried out by peptidases (up to 13 *exo*- and *endo* peptidases such as pepC, N, O, X, T, Q, V, DA) in *L. lactis* localized in or on the cell wall or cytosol, eventually to individual amino acids, and transportation into the cell by peptide transport system(s) (e.g., ATP hydrolysis by a proton-translocating ATPase or an end-product efflux).

28. Why does the hard type of cheese, Cheddar cheese, ripen at a lower temperature (4–6 °C) and not at a higher temperature (over 15 °C)? What are the current methods used for accelerating Cheddar cheese ripening and which one is the most common one?

Answer: Temperature increases will certainly speed up the ripening, but it will produce the down-graded cheese, due to the growth of spoilage microorganisms; that's why lower temperatures (4–6 °C) are used to retard the growth of contaminants; elevated ripening temperatures, addition of enzymes, addition of cheese slurry, attenuated starters, adjunct cultures, genetically engineered starters, recombinant enzymes and microencapsulation of ripening enzymes are traditional and modern methods used to accelerate cheese ripening, but the enzyme addition is the most common method.

29. What is a major problem of the phage infection in cheese industry? Why does the cheese industry use multi-strain starters than single-strain starters? Why is strain rotation used?

Answer: Phage infection is a major cause of poor growth and acid production by starter cultures; the purpose of multi-mixed starters is to prevent bacteriophage infection. Also alternate use of strains having different phage susceptibility profiles (strain rotation) is common in cheese making.

30. How was the cheese-coagulating enzyme produced in the past? How is this enzyme currently produced?

Answer: Natural calf rennet was extracted from calf stomachs; now recombinant rennet (chymosin) is produced by recombinant DNA technology by the expression of the gene into yeast (*K. lactis*) and fungi (*A. niger*).

31. What is EMC? Where is EMC used?

Answer: The enzymes of LAB, when added to fresh cheese slurry and incubated in a controlled environment, produce within a few days an intense Cheddar cheese flavor without bitterness and off-flavors; This slurry, so-called EMC, can be used directly as a flavoring agent in snack foods and crackers, Pizza topping and pizza sauce formulations and other enzyme-modified butter fats, creams, and cultured creams. Other novel flavors are also available for use in formulated foods.

32. What are the types of fermented sausages used in the United States and Europe? What are the procedures to make sausages?

Answer: Sausages are classified as dry or semidry on the basis of the final moisture content of the product in the United States, but the European system is based on the temperature treatment that the product receives. Other classification systems often used in the United States are based on the ethnic origin of the sausage (Germanic,

Italian, or Lebanese), the moisture-to-protein ratio, and the composition (moisture, fat, protein, salt, sugar, pH, total acidity, and yield). After the meat is formulated by grinding, chopping, and mixing with the fat, to give the desired fat content, the spices, flavorings, curing salts, carbohydrate, nitrite, and starter culture are mixed in, and the mixture is stuffed into the proper sausage casings (cellulose, collagen, or natural) at a temperature of -2.2 to 1.1°C . In the traditional process, nitrite is now added directly; this step is no longer required, and the characteristic pink color of cured meats (nitroso-hemochrome) is formed without pan curing. After stuffing, the sausages are hung in a maturing room. Traditionally, fermentation takes place in a room which is at a temperature of 15.6 – 23.9°C and relative humidity from 80% to 90%. The temperature is raised over the course of fermentation: for dry sausages, up to 37.8°C ; for semi-dry sausages, up to 43°C . Following fermentation, sausages are fully cooked, partially cooked, and/or placed in a drying room. Fermentation may continue during this process depending on the growth characteristics of the particular bacteria, temperature, pH, carbohydrate level, and degree of heat penetration. Ready-to-eat sausages are dry, semidry and/or cooked. Dry sausages may be smoked, unsmoked or cooked. Semidry sausages are usually heated in the smokehouse to fully cook the product and partially dry it. Cooked sausages (e.g., bologna and frankfurters) are cooked and may also be smoked.

33. What are the examples of dry and semidry sausages?

Answer: Dry sausages include Sopressata (a name of a salami), pepperoni (not cooked, air dried), Genoa Salami (Italian, usually made from pork but may have a small amount of beef; it is moistened with wine or grape juice and seasoned with garlic.)

Semidry sausages include summer sausage, Lebanon bologna, Cervelat, Thuringer.

34. Are there any sausages that are shelf stable?

Answer: Some dry sausages are shelf stable (in other words, they do not need to be refrigerated or frozen to be stored safely). Dry sausages require more production time than other types of sausages and result in a concentrated form of meat. If the product is shelf stable and ready to eat, the product is not required to have a safe handling statement, cooking directions or a “keep refrigerated” statement.

35. Should people “at risk” eat dry sausages?

Answer: Because dry sausages are not cooked, people “at risk” (older adults, very young children, pregnant women and those with immune systems weakened by disease or organ transplants) might want to avoid eating them. The bacterium *E. coli* O157:H7 can survive the process of dry fermenting, and in 1994, some children became ill after eating dry cured salami containing the bacteria. After the outbreak, dry sausages had to follow the guidelines or the product had to be heat treated.

36. What is fermented fish? What is biogenic amine of fermented food in general?

Answer: Traditional fermentation is a method that attacks the ability of the microbials to spoil fish; this is carried out by the combined action of fresh fish enzymes and microbial enzymes in the presence of salt. It does this by making the fish muscle more acidic; bacteria usually cease multiplying when the pH drops below 4.5. A modern biopreservation, adds LAB to the fish to be fermented. This produces active antimicrobials such as lactic and acetic acid, hydrogen peroxide, and peptide bacteriocins. Fermented fish preparations can be notable for their putrid smell, but fish

is still fermented because some people enjoy the taste; biogenic amine is a group of naturally occurring, biologically active amines, such as norepinephrine, histamine, and serotonin, which act primarily as neurotransmitters. The amines are reported to be toxic to humans. Common toxic symptoms of biogenic amines in humans are nausea, respiratory distress, hot flushes, sweating, heart palpitation, headache, a bright red rash, oral burning, and hypertension as well as hypotension.

37. Why should one eat lacto-fermented foods? What are the benefits?

Answer: Most cultures around the world have some sort of fermented food as a staple food in their diet. We should be following in their footsteps because their health is more stable than those of the United States. It is becoming widely known that 80% of our immune system is in our gut. The immune system deals with aging, infection, disease, and general health. Consuming these probiotic and enzyme rich foods help build the immune system and aide digestion, taking a load off our system.

38. Can one suffer from food poisoning by eating fermented foods?

Answer: Fermented foods that are properly made are considered very safe to eat.

39. What are the common pickling problems? What types of microorganisms are involved in this problem? How can this problem be prevented?

Answer: The major problem with pickles is due to fermentative yeasts, which may cause gas production inside the cucumbers resulting in *bloaters*. The major yeast species responsible for this are *Brettanomyces*, *Hansenula*, *Saccharomyces*, and *Torulopsis*; *Lb. brevis* also has been observed. Another type of spoilage caused by bacteria is blackening of pickles. Organisms, such as *B. nigrificans*, which are found on cucumbers, are able to produce hydrogen sulfide if the process water contains high concentrations of iron or copper ions. Pickles may be preserved by refrigeration or pasteurization (internal temperature of 74 °C for 15 min).

40. Why are unfermented soy products harmful? What are the benefits of fermented soy products?

Answer: Unfermented soy contains phytates, protease inhibitors, hemagglutinin, isoflavones, saponin, spyatoxin, oxalates, goitrogens and esterogens and also soy proteins are allergenic. However, soy is loaded with the antioxidant isoflavones, genistein, and daidzein. Soy isoflavones behave such as mild estrogens and may reduce hot flashes in women, leading to breast cancer. Soy isoflavones have a direct correlation with increased thyroid disease. Soy phytoestrogens are known to disrupt endocrine function, may cause infertility, and may lead to breast cancer in women. Genistein and daidzein, the soy phytoestrogens have been shown to possess anticancer properties, but more studies are needed to better understand and elucidate all pathways mobilized by genistein and daidzein. During the fermentation process, the enzymes produced by the bacteria and yeast break down and become easier to digest and also good compounds such as isoflavone, genistein, and daidzein are more easily available. This also makes the protein content of unfermented soy protein easier to digest.

41. Can you name four major fermented soy products popular in Asia? Which undesirable compound is produced during soy fermentation and fermented foods in general?

Answer: Soy sauce, miso, natto and tempeh; EC, known as urethane, is a genotoxic carcinogen in animals and classified as a possible human carcinogen. This toxicant occurs naturally in alcoholic beverages and most fermented foods.

42. What are organic acids? What are the uses of organic acids?

Answer: An organic acid is an organic compound with acidic properties. In general, organic acids are weak acids and do not dissociate completely in water, whereas the strong mineral acids do. Organic acids have been utilized for a long time by the food industry as food additives and preservatives to prevent deterioration and extend the shelf life of perishable food ingredients. The production of different organic acids (acetic acid, lactic acid, succinic acid, etc.) through fermentation of biomass sugars can be a primary or secondary coproduct in a bio-refining system. They have been widely used in foods, beverages, pharmaceuticals, cosmetics, detergents, plastics, resins, and other biochemical or chemical products.

43. What are the main disadvantages of the fermentation process compared with chemical synthesis? Why is a sudden interest shown in the production of organic acids by the fermentation process?

Answer: Fermentation reactions frequently produce a variety of products in dilute aqueous solutions. The expense of separating the chemicals from each other and from the large volumes of water has been so great that production of chemicals by fermentation has not been able to compete with the production of the same chemicals from fossil fuel sources; However, the gradual depletion of petroleum fossil fuel with the resultant increase in prices of petrochemical feedstocks has revived interest in such fermentation reactions, which can convert carbohydrates renew raw materials into simple organic chemicals.

44. Which useful products can be produced from lactic acid?

Answer: Biodegradable plastics (bioplastics) such as PLA and ethyl lactate can be produced from the fermentation produced by lactic acid, which is commercially available.

45. Can you list any company that produces SCP (MBP)? How can SCP be produced using methane gas?

Answer: UniBio A/S (Denmark) converts natural gas or methanol into SCP (highly rich protein of 71%). Using a microbial culture by mixing only nitrogen source such as ammonium salts or urea in methane gas, SCP can be produced and used in the making of animal and aquaculture feeds.

46. What is *S. acidophilum*? What is the advantage of growing this fungus in waste carbohydrates?

Answer: Acid-tolerant fungus isolated from peat moss can grow in very low pH, that is, in a pH below 1. This advantages of this fungus is that it can be used as a low aseptic condition (there is no need to sterilize the media), and can reuse acids.

47. What are the major problems in producing SCP? Is there a problem with humans consuming it?

Answer: SCP is more expensive than soybean meal or fish meal protein; the essential amino acid, methionine, is very low in SCP. A high content (RNA from 6% to 15%)

of microbial nucleic acids is a limiting factor for human consumption (2 g/day) due to the lack of the enzyme uricase. Uric acid produced by nucleic acid is insoluble, causing gout as well as kidney stone formation.

48. Why do microorganisms produce gums (polysaccharides)? What are uses of microbial gums?

Answer: Gums provide a mechanism for storing carbon or energy source for the cell. They also protect phagocytosis and are used in firmly attaching things to the surface. For applications of microbial gums, see Table 4.19.

49. How are xanthan gums commercially produced? Why are most of the microbial gums produced by the pathogenic bacteria?

Answer: See Figure 4.11 for the production process of xanthan gums. Because good producers of microbial gums are mostly pathogenic bacteria, a plant pathogen, *X. campestris* is used in the production of commercial xanthan gum. The gum can be easily separated out by solvent (propanol) precipitation and purified partially; thus, it is safe without any toxicity in the gum itself.

50. Which gum sources of LAB are commercialized? Why will the extremophilic bacteria-derived gum have advantages?

Answer: Dextran gum produced by food grade LAB (e.g., *Lc. mesenteroides*). EPSs and polyhydroxyalkanoates by extremophilic *Archaea* are also of biotechnological importance as their biopolymers possess unique properties (e.g., thermoplastic) that offer insights into biofilm (bioplastic) production.

51. Name some plant-derived gums. What are the advantages of microbial gums over plant-derived gums?

Answer: Starch, guar, locust bean gum, gum arabic, carrageenan, pectin, carboxymethylcellulose, and so on. Microbial gums have the advantages of controlled cost, continuous supply, and diverse chemical structure and functionality from diverse microbial sources.

52. What are catalysts?

Answer: Catalysts are substances that reduce the activation energy of a chemical reaction, facilitating it or making it energetically viable. The catalyst increases the speed of a chemical reaction.

53. What amount of catalyst is consumed in the reaction it catalyzes?

Answer: Catalysts are not consumed in the reactions they catalyze.

54. Is there a difference between the initial and the final energy levels in catalyzed and noncatalyzed reactions?

Answer: The catalysis does not alter the energetic state of reagents and products of a chemical reaction. Only the energy necessary for the reaction to occur, that is, the activation energy, is altered.

55. What are enzymes? What is the importance of enzymes for living beings?

Answer: Enzymes are proteins that are catalysts of chemical reactions. Catalysts are nonconsumable substances that reduce the activation energy necessary for a chemical reaction to occur.

They are of vital importance for life because most chemical reactions of the cells and tissues are catalyzed by enzymes. Without enzymatic action those reactions would not occur or would not happen in the required speed for the biological processes in which they participate.

56. What are the main theoretical models that try to explain the formation of the enzyme–substrate complex?

Answer: There are two main models that explain the formation of the enzyme–substrate complex: the lock and key model and the induced fit model.

In the lock and key model, the enzyme has a region with specific spatial conformation for the binding of the substrate. In the induced fit model, the binding of the substrate induces a change in the spatial configuration of the enzyme for the substrate to fit.

57. On what structural level of the enzyme (primary, secondary, tertiary, or quaternary) does the enzyme–substrate interaction depend?

Answer: The substrate binds to the enzyme in the activation centers. These are specific three-dimensional sites and thus they depend on the protein tertiary and quaternary structures. The primary and secondary structures, however, condition the other structures and so they are equally important.

58. What is the activation center of an enzyme? Is it the key or the lock of the lock and key model?

Answer: The activation center is a region of the enzyme produced by its spatial conformation to which the substrate binds. In the lock and key model, the activation center is the lock and the substrate is the key.

59. Why can it be said that the enzymatic action is highly specific?

Answer: The enzymatic action is highly specific because only specific substrates of one enzyme bind to the activation center of that enzyme. Each enzyme generally catalyzes only a specific chemical reaction.

60. The serine protease subtilisin derived from protein engineered *Bacillus subtilis* is an important industrial enzyme as well as a model for understanding the enormous rate enhancements affected by enzymes. Explain how it was improved by protein engineering.

Answer: Read Bryan N. Philip. 2000. Protein engineering of subtilisin. *Biochim. Biophys. Acta* 1543:203–222.

61. What are the applications of enzymes?

Answer:

- (a) Pulp/paper industry by amylase, lipase, esterase, etc.
- (b) Detergent industry (protease: subtilisin), lipase, cellulase, etc.
- (c) Textile industry (amylases, cellulases, catalase, pectinase and protease).
- (d) Feed industry (α -galactosidase, phytase, protease, xylanase (hemicellulase).
- (e) Food and beverages (see Table 5.1).

62. Give other enzyme sources and enzyme examples.

Answer: Animal (lysozyme from egg, rennet from calf stomach) and plant sources (papain from papaya; bromelin from pineapple).

63. List a few important therapeutic enzymes.

Answer: See Table 5.4.

64. Which enzymes are useful for bioethanol production? Why ethanol production should be directed toward renewable feedstocks than corn-based crops?

Answer: α -Amylase, cellulase, hemicellulase (xylanase), glycoside hydrolase; as corn is food, bioethanol production will compete with food, and this could result in food shortage in the future.

65. What are the sources of commercial enzymes? What advantage does this source have?

Answer: Microorganisms; rapid growth, easily find robust strain, lower production costs, and easy of genetic manipulation.

66. Current enzymes used in food processing are not ideal catalysts. What techniques can one use to improve enzyme properties and production?

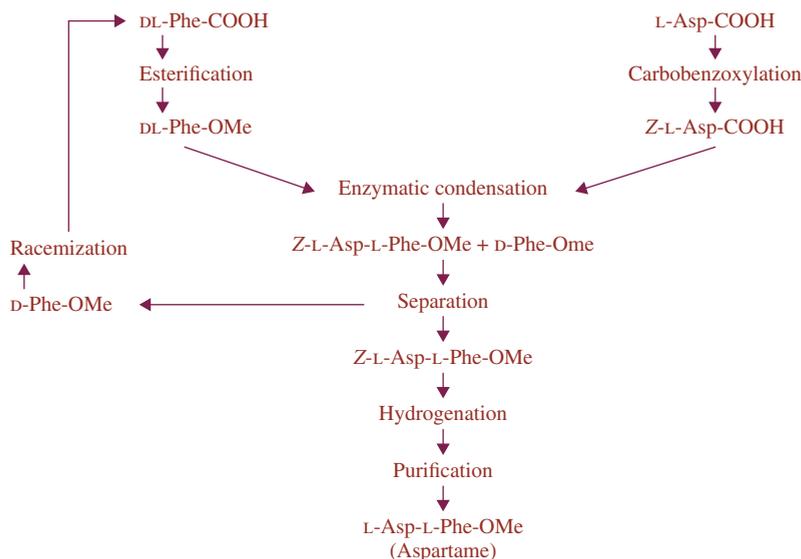
Answer: Recombinant DNA technique and protein engineering.

67. Biotechnology has played an important role on development of the nutritive sweeteners market and will have its impact on recent development on nonnutritive, high-intensity sweeteners. Discuss the manufacturing processes of (a) high fructose corn syrup (HFCS) 55, (b) high-intensity sweetener (aspartame), and (c) low-calorie sugar (xylitol).

Answer:

- (a) Discuss the manufacturing processes of HFCS 55: Figure 5.6.
- (b) Aspartame: NutraSweet[®] (aspartame) is made through fermentation to produce amino acids (l-aspartic acid and l-phenylalanine by *B. flavum* and *C. glutamicum*, and then synthesis processes. Phenylalanine is reacted with methanol resulting in a compound called l-phenylalanine methyl ester. Aspartic acid is also modified in such a way to shield various portions of the molecule from the effects of further reactions. After the amino acids are appropriately modified, they are pumped into a reactor tank, where they are allowed to mix at room temperature for 24 h. The temperature is then increased to approximately 65 °C and maintained for another 24 h. After cooling to room temperature, it is diluted with an appropriate solvent and cooled to about -18 °C, causing crystallization. The crystals are then filtrated and dried. The intermediate is converted to aspartame by reacting it with acetic acid. This reaction is performed in a large tank filled with an aqueous acid solution, a palladium metal catalyst, and hydrogen and thoroughly mixed and allowed to react for about 12 h. The metal catalyst is removed by filtration and the solvent is distilled, leaving a solid residue. This residue is purified by dissolving it in an aqueous ethanol solution and recrystallizing. These crystals are filtered and dried to provide the finished, powder aspartame.

Aspartame manufacturing process.



(c) Xylitol: Xylitol is a natural sweetener containing 75% less carbohydrates and 40% less calories than sugar, and has a myriad of oral health benefits including the prevention of tooth decay and is safe for diabetics. Xylitol is manufactured from xylose through a chemical hydrogenation using nickel as a catalyst that has some disadvantages, including a high energy requirement, extensive purification steps and a high cost of product. In the manufacturing process of xylitol, the xylan molecules are first hydrolyzed into d-xylose. The latter is chemically reduced to xylitol which can be separated by large-scale column chromatography. Xylitol is finally crystallized. The entire process is complicated and demands great engineering skills and experience. Xylitol can also be made by means of bacterial fermentations, which utilize d-xylose, d-glucose, or other suitable raw materials as substrates. These processes have not been economically feasible.

68. What are the names of the plant-derived non-nutritive sweeteners? Which one is commercially available plant sweetener?

Answer: Thaumatin and stevioside. The stevioside extracted from the leaves of *Stevia rebaudiana* is commercially available, approved by FAO and WHO, and is used widely in Japan, China, Korea, Europe, and North America.

69. Nowadays, flavors represent over a quarter of the world market for food additives; how do you think these flavor compounds are produced?

Answer: Most of the flavoring compounds are produced via chemical synthesis or by extraction from natural materials.

70. Do you think flavors produced by chemical transformation of natural substances can be labeled as natural? What are the problems of chemical synthesis?

Answer: Chemically transformed flavors cannot be labeled as natural. Chemical method is environmentally unfriendly production processes and lacks substrate selectivity, which may cause the formation of undesirable racemic isomer mixtures, which are harmful for humans; this process also reduces process efficiency and increases downstream costs. Also, the consumer has developed a “chemophobia”-attitude toward chemical or synthetic (even nature-identical) compounds, especially when related to food and products used at home.

The production of natural flavors by direct extraction from plants is also subject to various problems on low concentrations of the desired compounds, making the extraction expensive. Moreover, their use depends on factors difficult to control such as weather conditions and plant diseases.

71. What are the alternatives for natural flavors? Give some disadvantages of the microbial natural flavors?

Answer: An alternative route for flavor synthesis is based on microbial biosynthesis or bioconversion. The most popular approaches involve the use of microbial culture or enzyme preparations. Microorganisms can synthesize flavors as secondary metabolites during fermentation. Microbial or enzymatic conversion for flavors from materials of vegetal or animal origin has been clearly defined as natural flavors in the United States as well as in Europe (EC). As the yields of microbial flavors are low, the costs for downstream processing are high, but a few products are on the market.

72. What are the natural flavor enhancers derived from yeasts? Give the commercial names and production processes derived from yeast extracts. How are they different than monosodium glutamate (MSG) derived from bacterial fermentation?

Answer: Flavor enhancers or potentiators do not possess any aroma on their own, but are capable of enhancing flavors already present. Such compounds from yeast autolysates include inosine 5'-monophosphate (5'-IMP) and guanosine 5'-monophosphate (5'-GMP). Production process: See Figure.

MSG is salt of glutamic acid, which is mostly produced by bacterial fermentation using *Corynebacterium glutamicum*. Now MSG must be labeled by law for some susceptible individuals and also some safety concerns have recently been raised on MSG.

73. What are the uses of amino acids in industry?

Answer: Amino acids are used for a variety of applications in industry, but their main use is as additives to animal feed, since many of the bulk components of these feeds, such as soybeans, either have low levels or lack some of the essential amino acids: lysine, methionine, threonine, and tryptophan are most important in the production of these feeds. In this industry, amino acids are also used to chelate metal cations in order to improve the absorption of minerals from supplements, which may be required to improve the health or production of these animals.

The food industry is also a major consumer of amino acids, in particular, glutamic acid, which is used as a flavor enhancer and aspartame (aspartyl-phenylalanine-1-methyl ester) as a low-calorie artificial sweetener. The chelating ability of amino acids has been used in fertilizers for agriculture to facilitate the delivery of minerals to plants in order to correct mineral deficiencies, such as iron chlorosis. These fertilizers are also used to prevent deficiencies from occurring and improving the overall health of the plants. The remaining production of amino acids is used in the synthesis of drugs and cosmetics.

74. Which main amino acids are commercially produced for human diet, food additives, and animal feeds?

Answer: Glutamic acid (monosodium glutamate) is an important amino acid that is used as an additive in the human diet and l-aspartate and l-phenylalanine are used as materials for the peptide sweetener (intensive sweetener, aspartame), while l-lysine and dl-methionine, l-threonine, and l-tryptophan are used almost entirely in the supplementation of animal feeds as animal feed meals such as soybean have low levels or lack some of the essential amino acids.

75. Do humans synthesize amino acids?

Answer: No, that is why supplementation through diet is important.

76. Which methods are used to produce amino acids? Which method is the most common production method?

Answer: Extraction from natural sources, chemical synthesis, fermentation, and enzymatic catalysis. The fermentation method is the most common one to produce amino acids.

77. How metabolically engineered *Corynebacterium glutamicum* became the best lysine producer (110 g/L) known so far, as compared with the previous yields (about 40 g/L) from *C. glutamicum* mutant?

Answer: Describe how the authors did? Read Becker et al., 2011. *Metab. Eng.* 13:159–168.

78. What is the difference between water-soluble and fat-soluble vitamins? Why can fat-soluble vitamins cause harm when ingested in excess?

Answer: Water-soluble vitamins are those that are soluble in water. Fat-soluble vitamins are those that are soluble in oil (lipids, fat). Vitamin C and the vitamins of the B complex are examples of water-soluble vitamins. Vitamins A, D, E, and K are examples of fat-soluble vitamins.

Fat-soluble vitamins, since they are not soluble in water, cannot easily be excreted by the body. So they tend to accumulate in tissues with toxic effect when they are ingested in amounts over what is necessary.

79. How are a majority of vitamins produced and which ones are produced by the fermentation process?

Answer: Mostly vitamins are synthesized by chemical methods, except for a few vitamins, such as vitamin B complex and vitamin C.

80. What are the vitamins that make up the B complex? What problems does the lack of these vitamins cause?

Answer: Vitamins of the B complex are thiamin, or vitamin B1; riboflavin, or vitamin B2; and niacin (B3), essential for the constitution of the hydrogen acceptors flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide phosphate (NADP) of the energetic metabolism; pyridoxine, or B6; and cyanocobalamin, or vitamin B12.

Deficiency of vitamin B1 causes beriberi, loss of appetite, and fatigue. The lack of vitamin B2 causes mucosal injuries in the mouth, tongue and lips. Deficiency of niacin causes nervousness, digestive disturbances, loss of energy and pellagra. Lack of vitamin B6 causes skin lesions, irritation, and convulsions. Vitamin B12 acts

together with folic acid and its deficiency causes cell division disruptions leading to pernicious anemia (a type of megaloblastic and nutrient deficiency anemia). The absorption of vitamin B12 depends on another substance called the intrinsic factor secreted by the gastric mucosa.

81. From which food can you get vitamin B1, vitamin B2, and vitamin B12?

Answer:

B1: Pork, legumes, peanuts, and whole grains.

B2: Dairy products, meats, enriched grains, and vegetables.

B12: Meats, eggs, and dairy products.

82. How do microflora in gastrointestinal affect the vitamin production in your body?

Answer: Many of the bacteria in our body, especially in the large intestine, are responsible for the production of vitamins. Our body then utilizes the vitamins for all sorts of things.

83. Does the human body produce vitamin B12? How is vitamin B12 produced commercially?

Answer: Human beings or plants do not synthesize vitamin B12 (cobalamin). The species *Propionibacterium shermanii* and *Pseudomonas denitrificans* are more commonly used today for the fermentation process.

84. How is vitamin C (ascorbic acid) manufactured?

Answer: The Reichstein chemical process was developed in the early 1930s and uses a short fermentation process, followed by chemical processing: Glucose > Sorbitol + Fermentation > Sorbose > Diacetone-sorbose > Keto-gulonic acid > Keto-gulonic acid methylester > Ascorbic acid.

An improved method using a two-step fermentation process was developed in China in the 1960s: Glucose > Sorbitol + Fermentation > Sorbose + Fermentation > Keto-gluconic acid > Ascorbic acid. Most vitamin C is currently produced in China using this method; there are only a couple of manufacturers outside of China producing vitamin C.

A new one-step fermentation method is being developed to compete against the two-step process. The Scottish Crop Research Institute (SCRI) is successfully scaled-up for commercial production by a yeast-based single-step process. This could prove to be a better alternative to the Chinese technology of vitamin C production. The US-based Genentech Inc has also patented methods for producing ascorbic acid using recombinant strain combining one or more enzymes in the metabolic path converting glucose to 2 keto-l-gulonic acid.

85. Why do you think natural pigments from microbial sources are potentially good alternative ones to synthetic pigments? What are the drawbacks of plants and what are the advantages of microbial sources?

Answer: Many artificial synthetic colorants, which have widely been used in food-stuff, dyestuff, cosmetic, and pharmaceutical manufacturing processes, comprise various hazardous effects. To counter the ill effect of synthetic colorants, there is worldwide interest in process development for the production of pigments from natural sources (plants and microorganisms). Also the accessible authorized natural pigments from plants have numerous drawbacks such as instability against light,

heat, or adverse pH, low water solubility and are often unavailable throughout the year. The latter are of great interest owing to the stability of the pigments produced and the availability of cultivation technology. The advantages of pigment production from microorganisms include easy and fast growth in the cheap culture medium, independence from weather conditions, and possibility to get colors of different shades.

86. What type of carotenoid receives substantial attention for provitamin A activity? What are the types and benefits of microbial carotenoids?

Answer: β -Carotene; microbial carotenoids are b-carotene, lycopene, lutein, zeaxanthin, canthaxanthin, astaxanthine, rhodoxanthin; carotenoids have strong antioxidant effects.

87. Where is astaxanthine present? What are the applications of astaxanthine?

Answer: Astaxanthin is present in many types of seafood, including salmon, trout, red sea bream, shrimp and lobster, as well as in birds such as flamingo.

88. Where is astaxanthine used the most? How is this pigment produced today?

Answer: Salmon feed industry; commercial sources of this pigment are a red yeast, *Pharffia rhodozyma* and microalga, *Hematococcus pluvialis*.

89. Which mushroom is the most grown species in the world? What are the major steps in cultivation?

Answer: *Agaricus bisporus* (bottom or white mushroom); two major steps are preparation of suitable composts and germination of spores, and frutification.

90. What are the major problems in cultivating mushroom?

Answer: Preparation of uniformly good compost, and the prevention of infection by viruses, bacteria, fungi worms, and insects.

91. What conditions are important in cultivating mushroom? Describe the stages in growing *A. bisporus*?

Answer: See Figure.

92. What is medicinal mushroom? What are the known medicinal effects of mushroom?

Answer: Mushrooms are currently used in medicine or medical research. Folklore medicinal practice of mushroom has been since ancient time. Few species have been thoroughly studied to demonstrate the ability to treat diseases and to promote well-being. Yet, there are not any approved uses of these compounds.

93. How is chocolate produced? Describe the process of cocoa production.

Answer: Chocolate is the cocoa bean fermented products. Cocoa production process: See Figure 5.14 for the process.

94. Why is fermentation important in cocoa processing? What is the role of microorganisms in the fermentation process?

Answer: The chocolate flavor of cocoa develops during this stage. The first major fermentation is by alcohol fermentation done using yeasts; this results in ethanol production, but acetic and lactic acid bacteria fermentation take place simultaneously. The temperature can reach to 50 °C and thus heat and acetic acid in the fermentation heap kill yeasts, and other bacteria, darkening cocoa beans. Many chemical reactions

take place, and it is during this fermentation process that the chocolate acquires its color and flavor. The beans are dried thoroughly in the sun after fermentation to drive off all moisture and prevent further chemical and microbial activity. At this time, the flavors are locked in and ready for transporting to the manufacturer for roasting, grinding, and making into chocolate coatings and bars.

95. What is the difference between black tea, green, oolong, white tea, and dark tea?

Answer: All teas are products of *Camellia sinensis* leaves and buds; the only difference is how they are processed: non-fermented tea (e.g., green tea), semi-fermented tea (e.g., oolong tea), fermented tea (e.g., black tea), non-oxidized tea (e.g., white tea), and post-fermented tea (e.g., dark tea). Black tea is processed in a way that allows for fermentation, whereas green tea's processing avoids the fermentation process. As a result, green tea retains a maximum amount of antioxidants and the rich nutrients and grassy flavors of these light teas. To avoid the oxidation of green tea, white tea, and the rare yellow tea, growers immediately apply heat to and dry the tea leaves soon after picking. Oolong is a traditional Chinese tea (*Camellia sinensis*) produced through a unique process including withering under the strong sun and oxidation before curling and twisting. White tea is minimally processed and not oxidized, meaning it retains the natural antioxidants, but does not develop as much flavor, color, or caffeine. For post-fermentation of dark tea mainly produced in China, the tea leaves undergo a microbial fermentation process after they are dried and rolled. This tea is then left to age and ferment for long periods of time until the tea leaves turn a dark black color.

96. Are tea's health claims conclusive?

Answer: Black and green teas contain flavonoids, which are antioxidants. One of these, epigallocatechin gallate (EGCG) is thought to help combat the free radicals that can contribute to cancer, heart disease, and clogged arteries. The fermentation process that makes black tea converts EGCG into other compounds, but there are compounds in black tea that promote heart health and fight cancer as well. Many health claims remain cloudy and inconclusive.

97. What is bacteriocin?

Answer: Bacteriocin is a small molecule, produced by bacteria, that inhibits closely related strains. It is usually a peptide or a protein – varying sized chains of amino acids.

98. How are bacteriocins currently classified?

Answer: See Table 5.7.

99. Which ones are the commercialized bacteriocins? What are their importance in food, agriculture, and medicine?

Answer: Among many LAB bacteriocins, nisin is the most extensively characterized and second one is pediocin, that are used in food preservation, agriculture (animal feed, aquaculture, etc.). Applications for pharmaceutical and medicine are forthcoming in dental caries treatment (mutacin-producing strain) and in controlling vaginal microbiota with infection of the urogenital pathogen *Staphylococcus aureus*.

100. What is the difference between functional foods and nutraceuticals?

Answer: Functional foods are food products eaten as part of the usual diet that have health beneficial effects. Functional foods look like foods, whereas nutraceuticals will have the same health-promoting substances packaged as pills, powders, or capsules.

101. Can you name of one or two known functional foods and nutraceuticals on the market?

Answer: Probiotic containing yogurt and bread made with omega-3 fatty acid containing flaxseed are functional foods, whereas capsule forms of omega-3 fatty acid and capsule containing antioxidant lycopene are nutraceuticals.

102. Do you think that nutraceuticals must undergo clinical trials similar to those that potential drugs must go through before being sold to the public?

Answer: Normally no, but depending on the jurisdiction, if products claim to prevent chronic diseases, improve health, delay the aging process, etc., one must go through clinical trials to prove the effects.

In US law, depending on its ingredients and the claims with which it is marketed, a product is either a drug, a food, a food ingredient, or a dietary supplement, and in US law, a dietary supplement does not need clinical trials. As a nutraceutical is a food or part of a food that has a medical or health benefit, such as the prevention and treatment of a disease, the effectiveness of a nutraceutical is tested through clinical studies. In Canadian law, one must go through clinical trials.

103. What is difference between probiotics, prebiotics, and synbiotics?

Answer: Probiotics: live microorganisms that, when administered in adequate amounts, confer a beneficial effect on the hosts (some strains of *Lactobacillus* and *Bifidobacterium*, with minimum viable count of 10⁹ cells/g product); Prebiotics:

104. Is there any known beneficial effect of probiotics?

Answer: Some like alleviation of lactose intolerance, improvement of diarrhea and inhibition toward pathogenic bacteria in the gut are recognized, but probiotics validity and health claims have continuously been rejected on the basis of “biomarker deficiency” by European Food Safety Authority (EFSA) and probiotic word has been banned from Europe since 2012. Despite the difficulty, bile salt hydrolase active probiotic, *Lactobacillus reuteri* (Cardioviva™, Montreal, Canada) has been approved for cholesterol reduction and marketed by the health authority in the North America since 2012.

Part III

Other Potential Applications of the New Technology

6

Plant Biotechnology, Animal Biotechnology, and Safety Assessment

6.1 Plant biotechnology

6.1.1 Introduction

Conventional plant breeding has been going on for hundreds of years and is still commonly used today by artificially mating or cross-pollinating to increase yields. Desirable characteristics from different parent plants could also be combined in the offspring. Almost half of the increased crop yields and quality has come about through conventional genetic improvements (Day and Lichtenstein, 1992). The successes of plant breeders in generating rice and wheat genotypes with high yield potentials stimulated a significant increase in investment in crop research, particularly since 1971, through the Consultative Group on International Agricultural Research (CGIAR) consortium. Thus attempts to improve productivity or product quality by the alteration and selective propagation of the producing organism are not new. Since the beginning of agriculture 8000–10,000 years ago, farmers have been altering the genetic makeup of the crops they grow. Early farmers selected the best looking plants and seeds and saved them to plant for the next year. Then once the science of genetics became better understood, plant breeders used what they knew about the genes of a plant to select for specific desirable traits to develop improved varieties. When the science of plant breeding was further developed in the 20th century, plant breeders understood better how to select superior plants and breed them to create new and improved varieties of different crops. This has dramatically increased the productivity and quality of the plants we grow for food, feed, and fiber.

The *in vitro* culture of cells, tissues, and organs of plants is one of the growth areas of biotechnology because of its potential to produce high value metabolic products, and to generate improved crops and ornamental plants. These applications include plant propagation, germ plasm maintenance and storage, production of useful chemicals, and plant genetic engineering. The cultural techniques of plant cell, tissue, and organ culture,

together with recombinant DNA (rDNA) technology, may generate genetic variants and lead to the selection and propagation of desired mutant cell lines. In the past 30 years or so, techniques such as rDNA, cell fusion, and plant tissue culture have been developed, which extend the capabilities of these efforts. However, progress is slow in this area of research because plants are genetically and physiologically more complex than single-cell organisms such as bacteria and yeasts. Genetic engineering does not displace conventional plant breeding but simply permits more rapid progress. Plant productivity will be enhanced by genetic technologies in the areas of increased yield, improved fertilizer and photosynthetic efficiencies, better disease and herbicide resistance, greater tolerance to saline soils, higher nutritional quality, and reductions in undesirable plant constituents. The most intense concerns in plant genetic research today are the development of techniques to introduce DNA into plants and the study of the nitrogen fixation reactions, whereby atmospheric nitrogen is converted into the forms required for metabolism. Improvements in the efficiency of nitrogen fixation by plants would greatly reduce the need for added fertilizers. Plant cells are *totipotent*, which means that any plant cell should be able to regenerate the phenotype of the complete and differentiated organism from its parents under the appropriate nutritional and physical atmosphere. The interchangeable terms *plant tissue* and *cell culture* designate all forms of plant cultures grown *in vitro*, ranging from single cells and disorganized proliferations of cells to highly organized multicellular masses (tissue) and organ cultures. Plant tissue or cell culture *in vitro*, which is the propagation of plant cells in aseptic nutrient media, offers tremendous potential in the production of high value biochemicals and naturally derived food ingredients. The ability to select and regenerate single totipotent cells into whole plants offers notable opportunities for the rapid and large-scale propagation of many species. The tools of plant biotechnology, which shorten the time for crop improvement, offer the food industry the opportunity to modify their raw materials. Although plant cells, which have slow growth rates and aseptic conditions for scale up, are unlikely to compete with microbial systems with respect to cost of production, certain unique compounds synthesized exclusively by plant cells may have commercial value. Despite significant efforts in this field, only a few products of a secondary metabolites *in vitro* such as *ginseng saponins*, *shikonin*, and *berberine* have been commercialized (Weathers et al., 2010). Success largely depends on either high productivity in shikonin or high market value in *paclitaxel*. *Shikonin* has been traditionally used in Japan as both a medicine (antibacterial and anti-inflammatory) and a dye. *Lithospermum erythrorhizon* species is grown for 5–7 years before the concentration of shikonin in the roots rises to 1–2%. The pure natural product is valued at about \$5000/kg and was an attractive commercial target for plant cell culture. An overproducing cell line was established that accumulated up to 15% dry weight as the product, and the product was extracted by chemical means and industrial production of shikonin has now been established (Yamamoto et al., 2002). Other well-known compound is *paclitaxel* to treat patients with lung, ovarian, breast, head, and neck cancer and advanced forms of Kaposi's sarcoma (Taura et al., 2014). This was isolated from the bark of the Pacific yew tree (*Taxus brevifolia*) and sold under the trademark *Taxol* by Bristol-Myers Squibb (BMS). Other semi-commercial status is on *vanillin*.

Other subject on *genetically modified organisms (GMOs)* or *genetically modified crops* has also been discussed in details. In 2011, the global area of biotech crops continued to increase for the sixteenth year at a sustained growth rate of 8% or 12 million ha (30 million acres), reaching 160 million hectares or 395 million acres. Biotech crops have set a precedent in that the biotech area has grown impressively every single year for the past 16 years, with almost a remarkable 94-fold increase since commercialization in 1996. The major concern has been on the safety and potential impact of GMO on human health. However, the future of biotech crops looks encouraging as the global area of biotech crops

continue to increase yearly and commercialization of *drought-tolerant maize* is expected in 2013, *golden rice* is already produced.

6.1.2 Plant cell and tissue cultivation

Plant tissue culture is the process by which small pieces of living tissue (*explants*) are isolated from an organism and grown aseptically for indefinite periods on a nutrient medium (Figure 6.1). It is usually started with an explant rich in undetermined cells, such as the cortex or meristem, because such cells can proliferate rapidly. The usual explants (buds, root tips, nodal segments, germinating seeds, etc.) are placed on suitable culture media, where they grow into an undifferentiated mass known as a *callus*. The callus may then be propagated indefinitely by subdivision. Plant tissue culture was introduced to facilitate the clonal propagation of horticultural species. In clonal propagation, plants are reproduced asexually so that the new individuals are all identical to the original plant (i.e., they are all members of the same clone). Many plants, especially hybrids, cannot reproduce sexually or, if they do, lose the desirable characteristics that have been bred into them. Based on the material used the technology of plant tissue culture may be differentiated into five classes:

1. Callus culture, in which masses of unorganized cell clusters are grown on nutrient agar.
2. Cell suspension culture in liquid media.
3. Organ culture from excisions of roots, shoots, embryos, anthers, ovaries or ovules seeded on nutrient agar.
4. Meristem tip culture, in which meristemic tissue inoculated onto nutrient agar generates single plants or numbers of shoot buds.
5. Protoplast culture, in which the cell walls are removed by digestion with a mixture of enzymes followed by culture in liquid media to achieve cell wall regeneration and cell division.

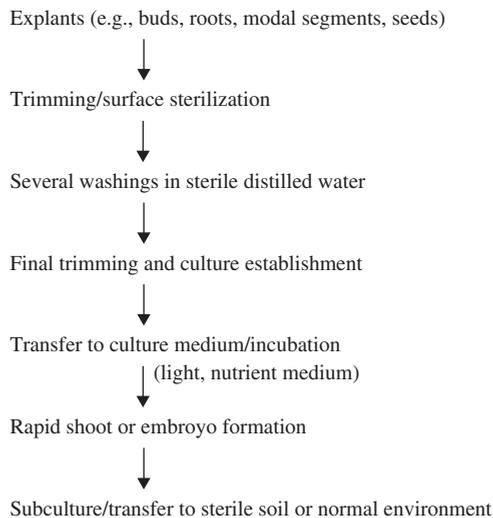


Figure 6.1 Basic procedure for obtaining a culture of plant tissue.

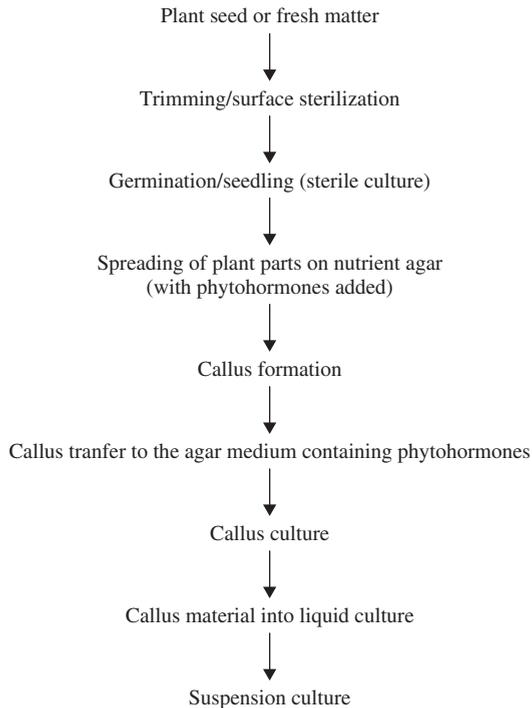


Figure 6.2 Basic procedure for obtaining a culture of plant cell.

When a callus is transferred to a liquid medium and agitated, the cell mass breaks up to give a suspension of isolated cells, small clusters of cells, and much larger aggregates that are called a plant cell culture (Figure 6.2). Such suspensions may be maintained indefinitely by subculture but are extremely heterogeneous because there is a high degree of genetic instability in the aggregates. Plant cell and tissue culture is a valuable adjunct to traditional plant breeding because of time savings and the potential for obtaining superior crops. Two modes of cell culture are used: cultivation of clusters of cells on a solid medium and cultivation of cell suspensions in a liquid medium. Plant tissue dissected from the interior of plant organs, after washing and disinfection, may be cultured on agar containing a suitable growth medium, with a mixture of inorganic salts and glucose or sucrose as a source of carbon and energy.

Plant cells are usually propagated chemoheterotrophically rather than photoautotrophically. The basal medium is usually supplemented with particular plant growth regulators, phytohormones, vitamins, amino acids, and the sugar. The medium composition will vary according to species and type of culture. Since plant cells also require oxygen for division, aeration is necessary, either by diffusion from the top surface of the culture or by sparged gas.

Various species of plants may be maintained in callus or cell culture, and it is possible to isolate new genotypes by means of somaclonal variation, protoplast fusion or mutagenesis. Such cell variants, however, are not useful unless they can be induced to develop into whole plants. While callus, suspension, and meristem cultures have been demonstrated with many

hundreds of plants, regeneration of whole intact plants from these cultures yields seed or vegetable progeny that express the genetic traits desired. It may take several years for traits of plants to be stabilized and inherited by subsequent progeny. Also, significant variability can be expected among different species. The practice of plant cell and tissue culture offers unique techniques to plant breeders.

6.1.3 Plant breeding

Most conventional plant-breeding efforts have aimed to increase yield and crop quality and have occurred by means of the breeding and selection of lines resistant to pests and diseases. The most exciting developments in plant breeding are centered on rDNA technology, but a few nonrecombinant DNA techniques are just as useful and are already bringing new crops to the marketplace (Brown and Thorpe, 1994)

6.1.3.1 Nonrecombinant DNA techniques Basically, there are two nonrecombinant approaches: the exploitation of spontaneous or induced variation in cultured plant cells or tissues – that is, somaclonal/gametoclonal variation, and the use of intra- and interspecific protoplast fusion to mediate genetic exchange.

Somaclonal variation In contrast to clonal propagation, which produces multiple clones, the regeneration of plants from callus, tissue explants, or plant protoplasts results in the recovery of somaclonal variants. In other words, plants regenerated from a cell culture often show a great deal of genetic variability in their characteristics. Now the term *somaclonal variation* is universally used for all forms of tissue culture derived variants, though other names such as protoclonal, gametoclonal, and mericlinal variation are often used to describe variants from protoplast, anther, and meristem cultures, respectively (Bairu et al., 2011). Other types of variation arise by specific culture of cells or tissues, which include (protoclonal), anthers and microspores (gametoclonal), callus (calliclinal), and apical meristem (mericlinal). Gametoclonal variation is obtained by regenerating plants from cultured microspore or pollen cells still contained in the anther set. There is now much interest in variability as a potential way of producing desirable new characteristics for crop improvements. Although the causes have not yet been fully elucidated, somaclonal variation is likely to arise through a sexual cycle. It is, however, not always possible to demonstrate heritability due to complex sexual incompatibilities, seedlessness, polyploidy, or long generation cycles (Bairu et al., 2011). Uncontrolled and random spontaneous variation occurs during the culture process, which is the most undesired phenomenon. Despite these negative effects, this technique has been very useful in crop improvement through creation of novel variants. Thus the major likely benefit of somaclonal variation in plant is improvement. Somaclonal variation leads to the creation of additional genetic variability. Characteristics for which somaclonal mutants can be enriched during *in vitro* culture are resistance to disease pathotoxins, herbicides, and tolerance to environmental or chemical stress, as well as for increased production of secondary metabolites. Micropropagation can be carried out throughout the year independent of the seasons and plants. A serious disadvantage of somaclonal variation occurs in operations which require clonal uniformity, as in the horticulture and forestry industries where tissue culture is employed for rapid propagation of elite genotypes. The genetic diversity of plants emerging from disorganized callus tissues provides the breeder with a means of introducing variability into established cultivars without the use of sexual crosses. Some useful plant variants developed for food industry are listed (Table 6.1).

Table 6.1 Some useful plant variants generated through somaclonal variation for the food industry

Plant	Improvement
Banana	Resistance to Fusarium wilt, high yield, heavier fruit bunches
Blackberry	Thornless
Carrots	Improved sweetness, crunchiness, crispness
Celery	Improved sweetness, crunchiness, crispness, <i>Fusarium</i> resistance
Finger millet	High yield, rapid seed germination at low temperature (15–20 °C), early maturity
Flax	Salt and heat resistance
Mustard	High yield, early maturity, seed boldness
Potato	Resistance to late blight fungus (<i>Phytophthora infestans</i>) and early blight fungus (<i>Alternaria solani</i>), reduced tuber browning
Rice	<i>Picularia</i> resistance
Sugar cane	Resistance to eyespot disease (<i>Helminthosporium sacchari</i>), downy mildew, and Fiji disease (leafhopper-transmitted virus); increased sucrose content
Tomato	Increased solids content

Source: Lankin (2004) and Bhojwanii and Dantu (2013).

The variability present in cell cultures is ultimately visible in populations of regenerated plants. Simple visual examination can be used to identify gross morphological changes in plant height, growth pattern, leaf size or shape, flower morphology and pigmentation, and so on. However, most of the desired phenotypic traits are not associated with morphological changes, and screening procedures must be used. So far, the application of mutagenesis to the selection of plant mutants has had limited success. Many traits of agronomic importance, such as grain quality and pest resistance, cannot be selected directly. Somaclonal variation may be expected to generate mutations of direct use in genetically tailoring plants for the food industry. Somaclones have been selected with desirable changes in fruit color and increased solids content to facilitate harvesting, distribution, and processing. This technology has also been successful in selecting new breeding lines of carrot and celery suitable for the so-called vegi-snax market, which emphasizes color, texture, and sweetness suitable for prepackaged, uncooked vegetable snacks.

Protoplast fusion (somatic hybridization) Protoplast fusion has been proposed as a means of developing unique hybrid plants that cannot be obtained with conventional sexual hybridization. Protoplast fusion is a procedure in which cells with enzymatically degraded cell walls are crossed, leading to the production of new hybrid plants. The production of interspecific hybrids by the fusion of the protoplasts of two species may be of great importance for the pharmaceutical and agricultural industries. Two basic methods, chemicals, and electrofusion, are used. Chemicals, such as polyethylene glycol, act as a fusogen in that it promotes protoplast fusion. In electrofusion, protoplast adhesion occurs in a nonuniform electrical field, and fusion occurs when a short pulse of direct current is applied.

When the two protoplasts first fuse and then grow in the culture medium, a new hybrid cell, which contains both nucleus and cytoplasm, is produced. If the parent cells are not identical, the product is a *heterokaryon*. If the two nuclei subsequently fuse, a mononuclear cell known as a *synkaryon* is produced. Though fusion brings the plastids (chloroplasts and mitochondria) together, they are not compatible, and eventually the *plastids* from only one plant predominate (Figure 6.3). This procedure is limited to species that are capable of plant regeneration from protoplasts. The process is time consuming, but it opens the door to the transfer of new traits that are not accessible using conventional breeding approaches.

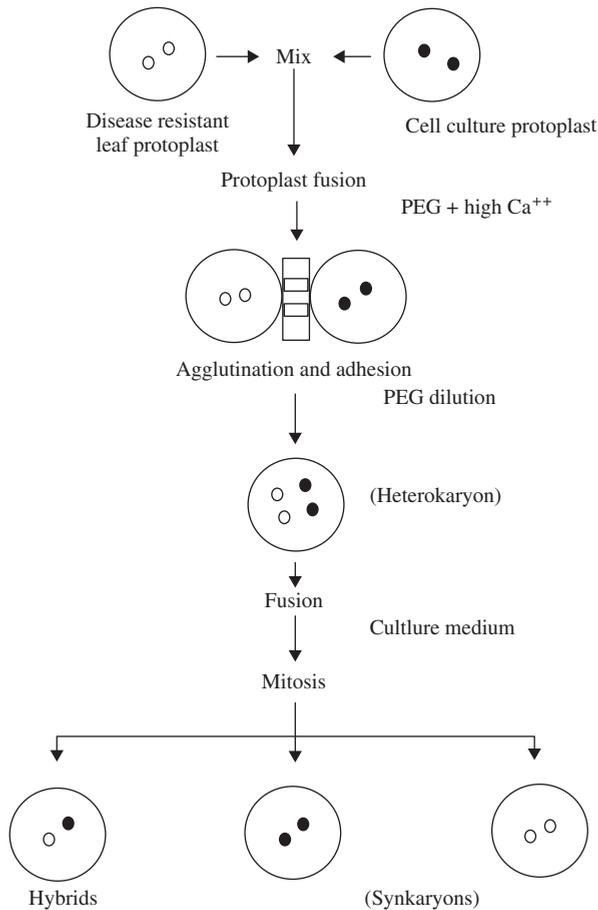


Figure 6.3 Basic procedure for obtaining hybrids.

Nonetheless, several new hybrids exist between domestic tobacco and wild tobacco species with resistance to the tobacco mosaic virus. Another use of protoplast fusion is the transfer of cytoplasmic genetic information. Since protoplast fusion combines both parent cytoplasm into a single protoplast, fusion can produce unique nuclear–cytoplasmic combinations not possible using conventional breeding.

6.1.3.2 Recombinant DNA techniques While early studies were directed toward the introduction of exogenous bacterial or plant DNA into seed or isolated pollen grains by means of microscopic needle (i.e., *microinjection*) set, difficulty in analyzing the fate of the exogenous DNA has hampered the widespread application of this method of DNA incorporation. rDNA techniques have proven to be successful in genetically engineering tobacco, corn, and other plants not presently responsive to *Agrobacterium* gene transfer methods. Ballistic methods have also been developed to deliver genes into target plant cells (Miller and Lauritis, 1989). The microscopic particles are coated with DNA and are

accelerated to high speed in a partial vacuum using a gun for transformation. Marker genes, such as chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase (NPT II), β -glucuronidase (GUS), and luciferase (LUC) have been studied (Miller and Morrison, 1991).

Great hopes are now being placed on the routine transfer of genes through vectors (plasmids) by rDNA techniques. Gene vectors may facilitate the uptake, stabilization, and replication of foreign DNA into a plant host. Two different types of cloning vector, one based on *plant viruses* and the other on *Ti (tumor-inducing) plasmid* of the plant pathogen *Agrobacterium tumefaciens*, are involved in the formation of crown gall tumors in dicotyledonous plants. However, handling protoplasts is technically tricky, and the most promising method uses a bacterial Ti plasmid. When the bacterium infects plant cells, a small fragment of this DNA, called *T-DNA*, is transferred to the plant genome. The *Vir* region of the Ti plasmid encodes diffusible products that facilitate DNA transfer to the plant. This plasmid codes for enzymes that are responsible for the utilization of one of a range of amino acid derivatives termed *opines*. Opines are not normally encountered in plant tissues, and thus Ti plasmid-bearing strains of *A. tumefaciens* can infect plants, causing a segment of the Ti plasmid to be integrated into the plant genome. The ability to catabolize the opine is retained by the plasmid in the infecting bacterium. The T-DNA has now been modified in such way that the transformation of plant cells can be achieved even when the DNA sequences coding for the tumor characteristics are no longer present in the fragment.

Modified Ti plasmids have been used successfully as host vectors to introduce desired new traits into plant cells. One of the modified plasmids is an *octopine plasmid* which brings about transposon inactivation of the auxinlike functions of the T-DNA. Tumors induced on tobacco plants with this plasmid produced numerous shoots. The gene for the storage protein *phaseolin* was transferred via a T-DNA vector from beans to tobacco plantlets and was expressed. Further improved vectors and the development of efficient promoters for better expression will make this the most promising area in modern plant biotechnology. New plant binary vectors of Ti plasmid in *Agrobacterium tumefaciens* with a broad host-range replicon of pRK2, pRi, pSa, or pVS1 are also reviewed (Murai, 2013).

By cloning the β -galactosidase gene into the nonpaline synthetase gene to construct an in-frame fusion, constitutive expression of an active β -galactosidase was obtained in sunflower and tobacco cells. The practical implications for plant breeding of the successful selection for resistance of new somatic hybrids, as well as of genetically transformed cells, will depend on the feasibility of regenerating plants. This technology will have greatest application to crops such as tobacco, tomato, potato, carrot, rapeseed, lettuce, and alfalfa, which are able to regenerate intact plants.

One example of genetic engineering that has been used in the food industry is transgenic tomatoes. Flavr Savr™ was developed by Calgene Fresh, a California-based agricultural biotechnology company. Genetically modified (GM) tomatoes with reduced levels of two cell wall-softening enzymes, *polygalacturonic acid (PG)*, and *pectinesterase (PE)*, were generated (Hall et al., 1993). The Flavr Savr™ gene is the antisense polygalacturonase (PG) gene. The gene produces an APH(3')II protein, which causes tomato cells to be resistant to kanamycin. The Flavr Savr™ gene functions in tomatoes by producing an *antisense RNA*, which is complementary to the normal messenger RNA (mRNA) producing the PG enzyme. When the two RNAs come together, they bind very tightly so that mRNA cannot make the PG protein. Analysis of transgenic tomatoes reveals notable reduction of PG. PG-reduced tomatoes are firmer than unmodified tomatoes and can last on the vine for longer periods. It was first sold in 1994, but ceased in 1997. Calgene made history, but mounting costs prevented the company from becoming profitable.

6.1.4 Application of plant cell and tissue culture

The aims of plant cell and tissue culture in agricultural and food applications are improvement of crops and increased food production.

6.1.4.1 Agricultural applications

Clonal propagation and genetic engineering crops A wide variety of plant species can be clonally propagated *in vitro* from plant tissue, using carefully defined culture media that are sufficient to induce the formation of multiple buds. Clonal propagation is particularly useful where seed production is difficult. Shoot tip clonal propagation has already been used commercially to propagate a large number of marketable ornamentals, as well as tissue-culture-propagated crops such as strawberry, asparagus, and oil palm. It has also been economically possible to develop *in vitro* surface-sterilized explants capable of producing virus-eradicated plants. This technology resulted in a significant business worldwide (Acquaah, 2012). Although clonal propagation may appear to be uneconomical for agronomic crops that are readily propagated from seed, considerable optimism is growing for the future of this technology as applied to nursery propagation for woody species. Advantages of clonal propagation for plants are speed of plant multiplication and the quality and uniformity of plants produced within a controlled environment, independently of season and climate. One set of examples of the genetic improvement possible through clonal propagation consists of increases in the resistance of plants to chilling, fungal toxins, ions, salts, pests, and disease.

The nutritional quality of plants has potential for genetic improvement by means of augmentation of certain nutrient levels (e.g., amino acids) and by lessening antinutritional factors (e.g., tannin, trypsin inhibitors). The genetically achieved increase in the solid materials portion of the potato is a good example of a commercial application. By means of protein engineering, it may be possible to alter the amino acid composition of plant proteins. The genes for a number of plant proteins and pharmaceutical proteins have already been cloned (Table 6.2). The enzyme ribulose biphosphate carboxylase (RuBPCcase), which mediates carbon dioxide fixation and photorespiration, has been cloned to increase the efficiency of photosynthesis. Also many transgenic proteins including vaccines, antibodies, antigens, immunomodulators, other therapeutic proteins, and enzymes over 10 have been cloned by *in vitro* plant cell cultures (Weathers et al., 2010; Huang and McDonald, 2012). Although more costly in terms of capital investment, *in vitro* plant cultures can provide some benefits as compared with field production because of year round production without weather and pathogen problems, and also, the potential to secrete the products into the culture medium, that can result in reducing purification costs and avoiding release of GM field crops. Both plant suspension cells and hairy roots using bioreactor systems are often used to produce functional proteins.

A new technique, known as *transwitch* was developed by DNA Plant Technology Corporation (New Jersey, USA) for controlling the expression of specific genes in cells. This technique may provide the complete shutoff of gene expression, typically achieved with antisense DNA or RNA techniques. This process begins with the identification and cloning of a specific gene in the plant cell, whereupon the duplicate gene is inserted back into the chromosome by any transformation method. The insertion of a chalcone synthetase gene into petunias or other plants caused different pigmentation modification.

Genetically engineered (GE) plants are often generated in a laboratory by altering their genetic makeup by cloning one or more genes to a plant's genome using the *biolistic method* (particle gun) or by *A. tumefaciens* mediated transformation. In research,

Table 6.2 Some useful plant proteins and pharmaceutical proteins whose genes have been cloned in plant cell and tissue cultures

Protein	Source	Function/use
<i>Plant Proteins</i>		
β -Conglycinin	Soybean	Seed storage protein
Glycinin	Soybean	Seed storage protein
Hordein	Barley	Seed storage protein
Legumin	Pea	Seed storage protein
Phaseolin	French bean	Seed storage protein
RuBPCase, large subunit	Tobacco, wheat	CO ₂ fixation
RuBPCase, small subunit	Pea	CO ₂ fixation
Thaumatin	<i>Thaumatococcus</i>	Sweet-tasting protein
Vicillin	Pea	Seed storage protein
Zein	Maize	Seed storage protein
<i>Pharmaceutical Proteins</i>		
α -Interferon	Human	Anti-cutaneous melanoma
Erythropoietin	Human	Hormone for red cell production
IL-12	Human	Suppression of tumor growth
Hepatitis B surface antigen	Human	Vaccine
Monoclonal antibodies	Human	Cancer treatment, diagnostic
Aprotinin	Bovine	Inhibition of fibrinolysis
Alpha-1-antitrypsin	Human	Inhibitor for neutrophil elastase
Glucocerebrosidase	Human	Treatment of Gaucher's disease

Source: Primrose (1987), Weathers et al. (2010) and Huang and McDonald (2012).

tobacco and *Arabidopsis thaliana* are the most common plant species because of their well-developed transformation methods, easy propagation, and well-studied genomes (Koorneef and Meinke, 2010).

As of 2012, about 35 commercial (i) herbicide-resistant or (ii) insecticidal *Bt* (*Bacillus thuringiensis*) protein containing crops including soy, maize/corn, canola, alfalfa, wheat, potato, and cotton have been developed (Table 6.3). (i) Herbicide-resistant crops incorporate a recombinant gene that results in resistance to the herbicide glyphosate (trade name Roundup), and simplifies weed control by glyphosate application (Table 6.3). These crops are in common commercial use in several countries. Glyphosphate (active ingredient in roundup) kills plants by interfering with the shikimate pathway in plants, that is essential for their synthesis of the aromatic amino acids (phenylalanine, tyrosine, tryptophan). Glyphosphate simply inhibits the enzyme, 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS). This shikimate pathway is absent in animals, and thus animals obtain aromatic amino acids from their diet. (ii) Transgenic plants such as maize contain the expressed *B. thuringiensis* (*Bt*) toxin. *B. thuringiensis* is a bacterium that naturally produces a protein (*Bt toxin*) with insecticidal properties, as an insect-control strategy. Recently, plants have been developed that express a recombinant form of the bacterial protein, which may effectively control some insect predators.

GMO industry has grown exponentially worldwide and many developing nations are looking to GM foods as the solution to increased food productivity and food security (Figure 6.4). About 1.6 billionth hectare planted in 2012 – same area as the United States and GMO crops have increased from 25 to 29 countries with strong 10% growth rate (Bawa and Anilakumar, 2013; James 2013). By 2013, 88% of corn (maize) and 94% of soy grown

Table 6.3 Commercially grown genetically modified crops and food species

Crop	GM properties	Modified % (US)	Modified % (World)
Alfalfa	Resistance to glyphosate or glufosinate	ND	ND
Canola/rapeseed	Resistance to glyphosate or glufosinate	ND	ND
Corn/maize	Resistance to glyphosate or glufosinate	86	26
Cotton (cottonseed oil)	Kills susceptible insect pests	93	49
Papaya (Hawaiian)	Resistance to papaya ringspot virus	80	ND
Potato (food)	Bt resistance to the Colorado beetle	0	0
Potato (starch)	Amflora: resistance gene to an antibiotic for selection and better starch production	0	0
Rice	Golden Rice modified to contain β -carotene (vitamin A precursor)	Market expected (2014/2015)	0
Soybeans	Resistance to glyphosate or glufosinate	93	77
Squash (Zucchini/Courgett)	Resistance to yellow mosaic virus	13 (2005 data)	0
Sugar beet	Resistance to glyphosate, glufosinate herbicides	95	9
Sugar cane	Resistance to certain pesticides, high sucrose content	ND	ND
Sweet peppers	Resistance to cucumber mosaic virus	ND	Small scale in Canada
Tomatoes	Antisense RNA to inhibit softening enzyme, polygalacturonase	Stop production	ND
Wheat	Resistance to glyphosate herbicide	ND	ND

Abbreviation: ND, not determined.

Source: From Wikipedia and Author's compiled data.

in the United States were GM (United States Department of Agriculture, USDA). The main players are US firms Monsanto, DuPont, and Dow Chemical, as well as Germany's Bayer and Syngenta of Switzerland. Since their introduction in the 1990s, GM products have conquered agriculture in the United States and hold a large share of the food on Americans' plates.

Though most GMOs are not directly involved in human consumption, about 60–70% of processed foods have ingredients derived from GMOs (<http://phys.org/news/2013-06-gmo-corn-soybeans-dominate.html>).

Of all the GM crops grown worldwide, only four, soybean, maize, cotton, and canola, dominate the market: Herbicide-tolerant soybean continued to be the dominant crop in 2011, occupying 75.4 million hectares or 47% of global biotech area. It was grown commercially in the United States, Argentina, Brazil, Paraguay, Canada, Uruguay, Bolivia, South Africa, Mexico, Chile, and Costa Rica. The second most dominant crop was GM maize with stacked traits, which occupied 37.3 million hectares or 23% of the global biotech area. It was grown commercially in the United States, Brazil, Argentina, South Africa,

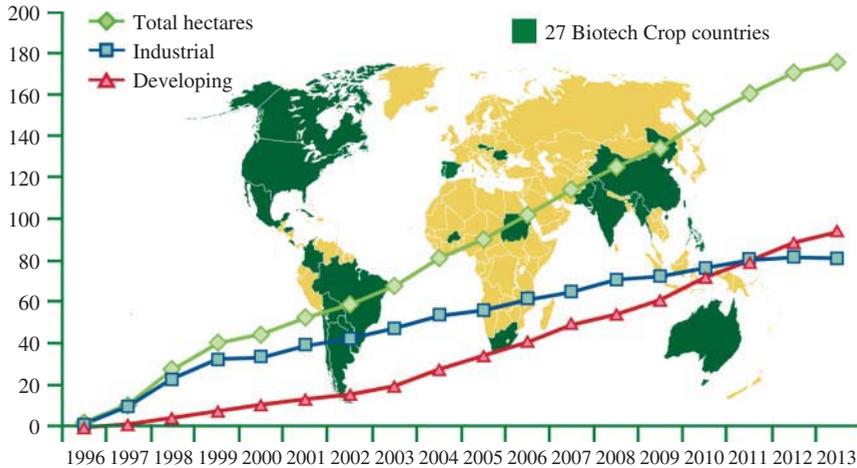


Figure 6.4 Global area of biotech crops from 1996 to 2013 (million hectares). *Source:* James, C. 2013. Global Status of Commercialized Biotech/GM Crops for 2013: ISAAA Brief 46. ISAAA, Ithaca, NY. Reproduced with permission.

Canada, the Philippines, Uruguay, Honduras, and Chile. Biotech Cotton was the third most dominant crop grown in 2011. Bt cotton was planted in more than 179 million hectares in India, China, Pakistan, Myanmar, Burkina Faso, Brazil, the United States, Argentina, Australia, Colombia, and Costa Rica, that is equivalent to 11% of the global biotech area. Cottonseed contains about 22–23% protein, and the cotton already produced worldwide has enough protein to meet the requirements of 500 million people. But it also contains the toxin gossypol, making it poisonous to animals, including human. The plants are modified by RNA interference (RNAi), shutting down the genes for gossypol production in the seed, while leaving them unaffected in the rest of the plant (Rathore et al., 2012). The resulting gossypol-free cottonseed is then suitable as a high-quality protein source suitable for consumption not only by cattle but also by humans.

Europe has relatively few GE crops with the exception of Spain where one-fifth of GM maize grown, and smaller amounts in five other countries. The EU had a “de facto” ban on the approval of new GM crops, from 1999 until 2004; in a controversial move. GM crops are now regulated by the EU. Developing countries grew 50% of GE crops in 2011. In recent years, there has been rapid growth in the area sown in developing countries. A total of 29 countries worldwide grew GM crops in 2011 by approximately 16.7 million farmers and 50% of GM crops grown worldwide were grown in developing countries. For example, the largest increase in crop area planted to GM crops in 2011 was in Brazil (303,000 km² vs 254,000 km² in 2010). There has also been rapid and continuing expansion of GM cotton varieties in India since 2002 (106,000 km² in 2011). An additional 31 countries, totaling 60 have granted regulatory approvals for biotech crops for import for food and feed use and for release into the environment since 1996. Thus, biotech crops are accepted for import for food and feed use and for release into the environment in 60 countries, including major food importing countries such as Japan, which do not plant biotech crops. Of the 60 countries that have granted approvals for biotech crops, the United States tops the list followed by Japan, Canada, Mexico, South Korea, Australia, the Philippines, New Zealand, the European Union, and Taiwan. Maize has the most events approved followed by

cotton, canola, potato, and soybean (14 each). Most countries is herbicide-tolerant soybean event GTS-40-3-2 with 25 approvals (EU=27 counted as 1 approval only), followed by insect-resistant maize MON810 with 23 approvals, herbicide-tolerant maize NK603 with 22 approvals each, and insect-resistant cotton (MON1445) with 14 approvals worldwide.

Since then, *Golden rice*, a recombinant variety of rice that has been engineered to express the enzymes responsible for β -carotene (Vitamin A precursor) biosynthesis. Golden rice was created by transforming rice with only two β -carotene biosynthesis genes: *psy* (phytoene synthase) from daffodil (*Narcissus pseudonarcissus*) and *crtI* (carotene desaturase) from the soil bacterium *Erwinia uredovora* (Ye et al., 2000). β -Carotene derived from Golden Rice was effectively converted to vitamin A in humans (Tang et al., 2009). Golden Rice could supply 50% of the Recommended Dietary Allowance (RDA) of vitamin A from a very modest amount if consumed daily. Thus, Golden Rice containing provitamin A could possibly solve the problem of malnutrition in millions and save thousands of children from blindness in the developing countries.

6.1.4.2 Plant germ plasm banks The numbers of wild species and their natural habitats are disappearing rapidly as a result of growing urbanization and the concomitant decrease in the amount of land being cultivated. There is a general fear that potentially valuable germ plasm is being lost irretrievably. As a consequence, *germ plasm storage* (or *gene banks*) is being used to maintain an inventory of plant genetic resources. Although seed is the most convenient form for storing germ plasm at reduced temperature and humidity, the stocks lose viability under such conditions, and plants propagated vegetatively may be conserved more economically by tissue culture.

Plants propagated vegetatively (e.g., bulbs and tubes) cannot be conserved for long periods of time. As a result of recent advances, cell, tissue, and organ cultures are being considered the most convenient form of germ plasm storage. Two techniques are adapted: one is slow growth, involving the depression of metabolism by physical or chemical means (this is applicable only to organized shoot cultures); the other is cryopreservation, which has proved to be the most successful for cell culture and shoot tips. It is essential that genetic variability not occur during regeneration of the intact plant, but with present tools, this cannot be guaranteed. Recently 12,000 economically important vascular plants used for a wide range of human activities are published (Wiersema and León, 2013). Such plants provide food and food additives, materials, fuels, medicines, forage, raw genetic material for plant breeding or environmental and social effects.

6.1.4.3 Biochemicals and foods

Production of native plant constituents Native plant constituents produced by cell cultures range from alkaloids to antimicrobial compounds, such as reserpine, steroids, and sugar derivatives (Table 6.4). Extensive reviews have been on production of secondary metabolites (Karuppusamy, 2009; Weathers et al., 2010) and transgenic proteins (Huang and McDonald, 2012) by *in vitro* plant cultures. They are secondary metabolites, not directly involved in cell growth. These compounds may contribute protection to the plants from animal or microbial invasion, or they may provide one plant species with the ability to compete with another in a particular habitat. In principle, the synthesis of any natural product can be expected; but not all cell cultures produce the natural compounds, and those that are produced accumulate in very small amounts. This is because cultured cells are different from the intact plant in terms of morphology, cytology, and physiology. Also, the poor production of secondary metabolites is due to culture conditions inadequate

Table 6.4 Substances reported from plant cell cultures

Alkaloids	Lipids
Allergens	Naphthoquinones
Anthraquinones	Nucleic acids
Antileukemic agents	Nucleotides
Antitumor agents	Oils
Antiviral agents	Opiates
Aromas	Organic acids
Benzoquinones	Proteins
Carbohydrates (including polysaccharides)	Peptides
Cardiac glycosides	Perfumes
Chalcones	Pigments
Dianthrone	Phenols
Enzymes	Plant growth regulators
Enzyme inhibitors	Reserpine
Flavonoids, flavones	Steroids and derivatives
Flavors (including sweeteners)	Sugars
Furanocoumarins	Tanning
Hormones	Terpenes and terpenoids
Insecticides	Vitamins

Source: Adapted from Fowler (1987) and Weathers et al. (2010).

to support the synthesis of the product. Currently, consumers tend to choose natural products rather than the synthetic ones and to prefer low calorie foods. The many problems associated with the accumulation of minute amounts of secondary products in cell cultures notwithstanding, plant cultures are thought to be capable in principle of forming considerable amounts of natural products.

Sufficient biomass production is a prerequisite for effective production of desired biochemicals, and a large amount of biomass can be produced by submerged cultures of plant cells. Biomass production can be maintained in a bioreactor for the process of immobilization. The secondary metabolites from plant cells may be continuously released and recovered by the development of complex polysaccharide gel matrices that suppress cell growth. The future challenges of immobilizing plant cells include achieving sufficient mechanical stability, maintaining diffusivity in the gel matrix, and ensuring sufficient mass transfer of oxygen, nutrients and plant metabolites. In addition to improvements of product yields by the immobilization method, supplements of metabolic precursors have resulted in an increase of desired compounds. For example, ferulic acid, an immediate precursor of vanillin, affected the accumulation of flavor components of cultured *Vanilla planifolia* cells. Three classical examples of commercially viable secondary metabolites *in vitro* are (i) ginseng saponins, (ii) shikonin, and (iii) berberine. Saponins are phytochemicals consisted of a sugar group linked to either a steroid or a triterpene group. They have a bitter flavor and are found in oats, legumes, potatoes, spinach, tomatoes yucca, ginseng and alfalfa sprouts. A few saponins are toxic in large doses, but most saponins are safe and may be beneficial for (i) reducing elevated cholesterol levels by forming complexes with cholesterol and

bile acids and (ii) colon, breast, uterine, and prostate cancer, (iii) preventing oxidation of cholesterol in the colon, which may also help to reduce colon damage, and (iv) stimulating antibody production, inhibiting viruses and inducing the response by lymphocytes, which are white blood cells that fight infection (Francis et al., 2002). Shikonin is a traditional Oriental medical herb extracted from *Lithospermum erythrorhizon* and it seems to possess anticancer ability against many different cancers, including hepatocellular carcinoma (Wei et al. 2013). Shikonin derivatives also have wound-healing, anti-inflammation, antibacterial, antidiabetes, antiviral activities, as well as beneficial proliferation of the granulation tissue. Due to these various beneficial effects, shikonin derivatives are used in pharmaceutical preparations, as food colorants and additives, and as ingredients in cosmetic formulations. Berberine, a compound found in plants including barberry and Oregon grape, has also been used as an herbal medicine to treat bacteria-associated diarrhea and intestinal parasitic infections. Recent studies have found that berberine inhibits the growth of a range of tumor cell lines (Li et al., 2014).

Biotransformation Plant cells can metabolize a wide range of exogenously supplied compounds through various reactions (reduction, oxidation, hydroxylation, glycosidation, esterification, methylation, demethylation, isomerization, etc.). Plant cells are much more susceptible to shear forces and grow much more slowly than microbial cells; thus stringent aseptic conditions need to be observed. Moreover, plant cells tend to aggregate; hence oxygen transfer is a problem in large-scale cultures. Plant cells also retain the products intracellularly, leading to increased overall costs of production. Attempts have been made to induce excretion of the products by control of external pH or nondestructive permeabilization of immobilized plant cells by organic chemicals. While plant cultures have been shown to produce various secondary metabolites (Table 6.5), the production levels are extremely low and are quite variable. Unfortunately little success has been at commercialization with biotransformation with native plant cells until recently, but Jay Keasling et al. (Noorden, 2010) had successfully co-expressing genes into *Escherichia coli* and then yeast that are part of a metabolic pathway that produces a precursor to the antimalarial drug artemisinin. Cycle-based combination therapy (artemisinin-based combination therapy, ACT) is currently the most commonly used treatment for malaria. The pharmaceutical company Sanofi will launch the large-scale production of a partially synthetic version of artemisinin.

Since the first commercial process that used *L. erythrorhizon* cell cultures was achieved for the production shikonin (Curtin, 1983), many efforts have been directed at obtaining secondary metabolites from plant cell suspension cultures. The production of secondary metabolites from plant cell cultures involves two different strategies: two-stage and one-stage culture systems. In *two-stage systems*, cells are placed in a medium that encourages growth. Once a large quantity of biomass is obtained, the medium is replaced with one that promotes secondary metabolism. The *one-stage system* allows cells to grow and produce secondary metabolites simultaneously. A mathematical model described by Guardiola et al. (1995) explains the growth and production of secondary metabolite (anthocyanin) by grape cells, *Vitis vinifera*, in batch and semicontinuous modes. The major efforts to overcome the problem of low yield focus on the selection of high-yielding cell lines. Some instances of high-producing cell lines, especially for vitamins, alkaloids, and pigments (e.g., anthocyanin) are known in various plant species. Somaclonal variation technology has been used in conjunction with cell aggregate cloning techniques to select high-producing cell lines.

Table 6.5 Secondary plant metabolites with potential commercial value

Product	Use	Species
<i>Drugs/Chemicals</i>		
Anthraquinones	Laxative, dye	<i>Morinda citrifolia</i>
Ajmalicine	Tranquilizer	<i>Catharanthus roseus</i>
Camptothecin	Antitumor agent	<i>Camptotheca acuminata</i>
Cephalotoxine	Antitumor agent	<i>Cephalotaxus harringtonia</i>
Digitoxin	Cardiac drug	<i>Digitalis lanata</i>
Diosgenin	Hormone	<i>Dioscorea deltoidea</i>
Ginseng saponins	Herbal drug	<i>Panax ginseng</i>
Thebaine	Codeine	<i>Nicotiana tabacum</i>
Ubiquinone-10	Cardiac drug	<i>Catharanthus roseus</i>
Vinblastine	Antitumor agent	<i>Catharanthus roseus</i>
<i>Flavors/Fragrances</i>		
Vanilla	Flavoring	<i>Vanilla planifolia</i>
Cocoa	Flavoring	<i>Theobroma cacao</i>
Rose oil	Fragrance	<i>Rosa damascena</i>
Menthol	Flavoring	<i>Mentha piperita</i>
Jasmine	Flavoring	<i>Jasmin grandiflorum</i>
<i>Color/Pigments/Sweeteners</i>		
Anthocyanin	Color	<i>Vitis vinifera</i>
Peruvian annatto	Color	<i>Bixa orellana</i>
Saffron	Color/spice	<i>Crocus sativus</i>
Shikonin	Dye	<i>Lithospermum erythrorhizon</i>
Rosmaric acid	Spice, antioxidant	<i>Coleus blumei</i>
Stevioside	Nonnutritive sweetener	<i>Stevia rebaudiana</i>
Thaumatococin	Nonnutritive sweetener	<i>Thaumatococcus danielli</i>

Source: Author's compiled data.

Summary

As a result of recent advances in the areas of plant cell and tissue culture, the future should bring developments in crop improvement, unique germ plasm, biotransformation, and novel products. The potential for biotransformation using immobilized cells or enzymes also exists. While plant cells cannot compete with microbial cells for cost of production, certain unique plant-derived compounds that are not synthesized by microorganisms may have commercial value.

It is possible to modify plants by means of genetic manipulation techniques. However, we know less about plant biochemistry and the enzymology of natural product synthesis than about microbial systems. Nonetheless, many GM crops are already commercialized, but only a few products produced by *in vitro* plant cultures have been commercialized. Heterologous therapeutic proteins such as antibodies, vaccines, and enzymes have successfully been expressed in *in vitro* plant cell including suspended dedifferentiated plant cells, moss, and hairy roots.

6.2 Animal biotechnology

6.2.1 Introduction

In the past 40 years or so, a number of modern techniques for improving animal lines have been developed. Artificial insemination has already had an enormous impact on the dairy industry. Other techniques such as *in vitro* fertilization, embryo cloning, and nuclear transplantation are becoming more prevalent. Developments in biotechnology that most likely will affect food animals are the use of exogenous, GE substances administered to the animals or used in foods derived from the animals and the transfer of new genetic material into animal genes to produce transgenic animals. Transgenic technology offers some direct medical benefits, including the availability of valuable therapeutic proteins. This technology also has the potential to provide a precise genetic route to developing disease-resistant strains of farm animals as well as strains that produce lean meat or grow more efficiently.

Over the past 20 years, the prerequisites for the maintenance and propagation of animal cells in cultures have been systematically compiled. In particular, the ability to produce, say, vaccines, routinely and cheaply from cell cultures, has led to an extensive improvement in public health. The type and range of such commercially valuable products, termed biologicals, is rapidly expanding, and this technology has spawned a new industry to exploit the possibility of large-scale culture.

GM animals are also an important category of genetically modified organisms. Transgenic mice, rats, rabbits, sheep, and pigs have been developed in the early 1980s, but the process is a slow, tedious, and expensive process. However, new technologies are making genetic modifications easier and more precise, among which some are already in use. GM animals currently being developed can be placed into six different broad classes for the intended purpose of the genetic modification to: (i) investigate human diseases, (ii) produce industrial or consumer products, (iii) produce human therapeutic proteins, (iv) enrich or enhance the animals' interactions with humans (hypoallergenic pets), (v) enhance production or food quality traits for faster growing fish, pigs that digest food more efficiently, and (vi) improve animal health for disease resistance. This chapter is about transgenic animals and animal cell culture, which can be included in food biotechnology.

6.2.2 Transgenic animals

6.2.2.1 Techniques Conventional breeding program using artificial insemination, embryo transfer, sexed semen, and *in vitro* fertilization have been applied to improve genetic merit of livestock for many generation. Cloning as the most recent advance in breeding selection programs does not alter the genetic makeup of the animal. Production of cloned animals can be accomplished by few methods such as embryo splitting or nuclear transfer (Seidel, 2000). Splitting of preimplantation embryo from selected females was one of the old cloning techniques used to genetically create identical animals, and thereafter, the first sheep was cloned using embryonic stem cells by Willadsen (1989). Since the 1990s, meat and milk produced from cloned sheep and cattle by embryo splitting or embryonic cell nuclear transfer entered in the market without food safety concern. However, production of a sheep, Dolly from an adult somatic cell has been the source

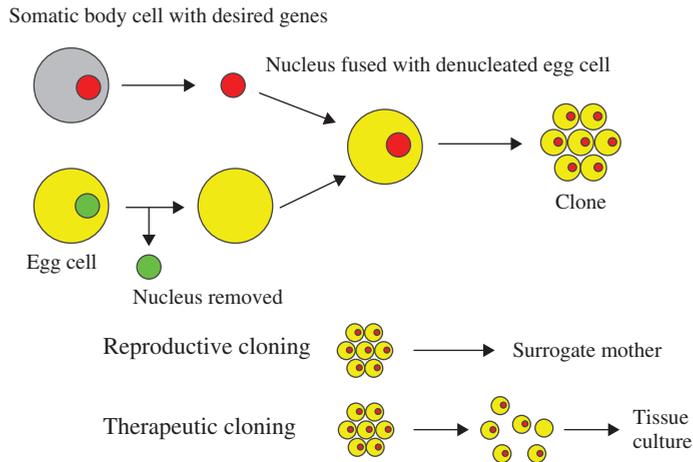


Figure 6.5 Somatic-cell nuclear transfer. *Source:* http://en.wikipedia.org/wiki/Somatic-cell_nuclear_transfer. Reproduced under a Creative Commons License. (See inserts for color representation of this figure.)

of the overwhelming public concern about such cloning. Somatic-cell nuclear transfer (SCNT) involves the introduction of DNA from the nucleus of an adult somatic cell into an enucleated oocyte (Hernandez Gifford and Gifford, 2013). Somatic-cell nuclear transfer can create clones for both reproductive and therapeutic purposes. The diagram depicts the removal of the donor nucleus for schematic purposes; in practice usually the whole donor cell is transferred (Figure 6.5).

Gene therapy, which is the correction of an inborn error of metabolism by the insertion of a normal gene into the afflicted organism, has been achieved in a number of animal systems. The same techniques being used for gene therapy could be developed into sophisticated animal breeding programs to introduce new or existing characteristics more quickly than is possible by conventional breeding. In animal breeding, modification of germ cells and improvements of the animal are the desired goals. In the modification of somatic or germ cells by gene manipulation *in vitro*, or transgenesis, the goal is to produce animals with a heritable change in their genotype that permits the benefits of the gene manipulation to be passed onto the offspring. Economically, current interest is in the modification of farm animals. Many of the complex biological processes that determine the production traits of farm animals, including fertility, growth rate, and milk yield, are regulated by protein hormones. The genes encoding some of these hormones have been cloned, providing an opportunity to manipulate the physiology of farm animals.

Gene transfer is mostly carried out by microinjection of recently fertilized ova or early embryos; but other procedures, such as electroporation and sperm-mediated gene transfer, may be more appropriate in the future. For example, to make a transgenic mouse, eggs are surgically removed from a mature female and fertilized with sperm in a test tube. A recombinant plasmid carrying a recognizable new gene is microinjected into the fertilized eggs with a thin glass needle, whereupon the manipulated ova are implanted into a foster mother. Whatever the means employed to produce transgenic animals, there is no control over where the donor DNA will integrate in the recipient genome. A major limitation is lack of knowledge regarding which genes control various physiological processes and how they are regulated at the developmental and tissue-specific levels. Genes put back into

microorganisms may be transformed, and the resulting transformants grow with a doubling time of less than 2 h. Only 5–10 successful microinjections into the fertilized eggs of mouse can be done in a day, however, and not all of these will necessarily express the foreign gene.

While transgenic mice are of current interest as an experimental tool, the technique needs to be applied to farm animals if any commercial benefit is to ensue. Working with large domestic animals is much more difficult than working with mice because cattle and hogs do not produce as many eggs and reimplantation of manipulated embryos is more difficult. Animal-derived food products resulting from the new technologies are considered to be safe scientifically, but introducing these products into the US marketplace will require US Food and Drug Administration (FDA) clearance with respect to safe levels of any pharmacologically or toxicologically active residues. New technologies, such as embryo cloning and nuclear transplantation, and their potential effects on the environment, genetic diversity, animal production, and consumers have been reviewed elsewhere (<http://www.fda.gov/AnimalVeterinary/NewsEvents/FDAVeterinarianNewsletter/ucm106070.htm>).

While the potential benefits of using the gene transfer technique for the direct modification of animal production traits are immediately apparent, the greatest contribution to the food industry probably will be through more subtle exploitation of transgenic animals to confer resistance to parasites or disease, to improve feed digestion, and to produce novel or modified proteins for the food and medical industries. The most obvious example is that of milk produced by cows given recombinant bovine somatotropin (rBST). The protein hormone somatotropin is produced by the pituitary gland of all farm animals and humans. In the United States, the companies Eli Lilly and Monsanto have developed recombinant BST produced by GE microorganisms. Cows injected with rBST produced more milk, and milk containing rBST went on sale in 1994. Although milk produced with the hormone was declared safe by federal agencies (Gillespie et al., 2010), many consumers feared that it was a health hazard (Lloyd-Evans, 1994). From 2000 to 2005 the USDA National Agricultural Statistics Service (NASS) survey of dairy producers found that about 17% of producers used rBST. BST has not been allowed on the market in Canada, Australia, New Zealand, Japan, Israel, and all European Union countries (currently numbering 27), by 2000 or earlier.

About 90% of the world's adult population is lactose intolerant (malabsorption) and thus the milk of transgenic animals that produce lactase (β -galactosidase) in the mammary gland might show reduced lactose content (Chen et al., 2009). Mammary gland of milk cow was transformed with a gene encoding an extracellular lactase-hydrolyzing enzyme cloned from a human small intestinal complementary DNA (cDNA) library. The invention also provides a new extracellular lactase-phlorizin hydrolase (ecLPH) gene that can express human lactase-hydrolyzing enzyme in the mammary gland of animals. Alternatively, the lactose content could be reduced by disruption of the gene for lactalbumin, which is essential for lactose synthesis, or by disruption using the antisense RNA mechanisms (Mercier, 1986). To produce low-fat milk, transfer and expression of the 12-desaturase gene, normally found in plants, may reduce milk fat content by about 30% of the fatty acids to polyunsaturated forms (Peterson et al., 2003).

6.2.2.2 Applications of transgenic animals There are many examples of transgenic animals and primates for the purpose of research, but few examples are listed for agricultural production in use or in process (Hernandez Gifford and Gifford, 2013). Within the field known as biopharming, transgenic goat produced few biotherapeutics, among which the drug, ATryn, that is an anticoagulant was extracted from the goat's milk. The FDA approved ATryn[®] (Antithrombin [Recombinant]) for reducing the probability of blood clots during surgery or childbirth in 2009 (www.gtc-bio.com).

In 2000, goats have been engineered to produce milk with spiderweb-like silk proteins (BioSteel®) in their milk by Nixia Biotechnology (Montreal, Canada), and later by the University of Wyoming and the University of Utah for many applications (<http://phys.org/news/194539934.html#jCp>). The Nixia Biotech company was sold in 2005 to Pharmathene. The frame silk of spiders is an ultra-strong material that can hold 400,000 pounds per square inch without breaking and is acknowledged to be world's strongest material. Because this silk is highly prized, yet cannot be cultivated commercially, scientists have sought other ways to replicate the material. The genes spider silk have been identified; since they are large and contain many repetitive units, it is better to use mammary gland to produce these proteins (e.g., in milk of transgenic goat) than in microbial or cellular production systems. BioSteel® will be used in a variety of medical device products, such as superfine ophthalmic applications and prostheses. It can also be used in industrial applications, including light weight, flexible body armor for military and law enforcement and high-performance sporting equipment (Lazaris et al., 2002).

A pig was also engineered to produce omega-3 fatty acids through the expression of a roundworm gene (Lai et al., 2006). The rationale for creating omega-3 transgenic livestock is well supported. Two omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are increasingly being recognized as important modulators of multiple biological pathways that affect health and disease (Kang and Leaf, 2007).

Enviropig™, the engineered Yorkshire pigs with phytase enzyme by microinjection in Canada (University of Guelph), could digest plant phosphorus more efficiently than conventional Yorkshire pigs (<http://www.uoguelph.ca/enviropig/commercialization.shtml>). The use of these pigs would reduce the potential of water pollution since they excrete from 30 to 71% less phosphorus in manure. In 2011 and 2012, several scientists in China, Argentina, and New Zealand also generated dairy cows GE with genes for human beings to produce milk that would be the same as human breast milk. In 2012, researchers from New Zealand developed a GE cow that produced allergy-free milk by using RNAi technique for “knocking out” the cow’s gene for β -lactoglobulin (Jabed et al., 2012). The RNAi technique uses a natural method for switching off genes without the need to generate DNA mutations within the genes. However, all these technologies will take a long time to produce this desirable milk, after getting approval, and accepting by the population as a substitute for regular cow’s milk. Gene therapy that uses genetically modified viruses to deliver genes can cure disease in humans using transgenic animal models. Although gene therapy is still relatively new, some successes have been reported in treating genetic disorders such as severe combined immunodeficiency, and Leber’s congenital amaurosis, cystic fibrosis, sickle cell anemia, Parkinson’s disease, cancer, diabetes, heart disease, and muscular dystrophy (Chicoine et al., 2013). These recent clinical successes have led to a renewed interest in gene therapy. This technology only targets the nonreproductive cells meaning that any changes introduced by the treatment cannot be transmitted to the next generation. Gene therapy targeting the reproductive cells – so-called “germline gene therapy” – is very controversial and is unlikely to be developed in the near future.

Meeting the increasing demand for fish while protecting marine fish supplies, GM fish have considerable potential to further increase the yield of fish farms. Some have developed various transgenic fish, but none of them have been approved for aquaculture. The first GE fast-growing salmon has come a step closer to the dinner table by the FDA. Aqua Bounty Technologies, Inc. (MA, USA) initially developed by Canada successfully cloned a growth hormone gene from Chinook salmon and an antifreeze protein into an Atlantic salmon that grows four to six times faster as a juvenile than wild-type salmon (Ledford, 2013). Environmental Canada approved for salmon egg production for commercial purposes in Canada, but other relevant regulatory bodies still need to provide approval before

the eggs and salmon can be sold. Engineered tilapia can grow and put on weight up to 300% faster (Muir, 2004). A mud loach developed in Korea can grow up to 35-fold faster than normal (Nam et al., 2001). Other various GM fish species provide better resistance to bacterial diseases or make the fish more tolerant to cold temperatures. Research to control introduced carp, which have become a major problem in Australian rivers and lakes, is now close to being implemented (Nowak, 2002).

The only transgenic fish commercially available today is a zebrafish that glows when illuminated under the brand name GloFish™. It is not meant for human consumption but for aquariums pet marketed by Yorktown Technologies LP (Austin, TX, USA). To produce GloFish, the green fluorescent protein (GFP), originally extracted from a jellyfish, that naturally produced bright green fluorescence, was inserted into a zebrafish embryo, allowing it to integrate into the zebrafish's genome (Gong et al., 2000). This caused the fish to be brightly fluorescent under both natural white light and ultraviolet light and the original goal was to develop a fish that could detect pollution by selectively fluorescing in the presence of environmental toxins.

Engineered zebra fish was used for enhanced sensitivity to different categories of environmental pollutants, including aromatic and halogenated hydrocarbons, electrophile response elements (quinines), and metal response elements (mercury, copper, etc.) (Carvan et al., 2000). Shortly thereafter the same team developed a line of red fluorescent zebra fish by adding a gene from a sea coral, and orange-yellow fluorescent zebra fish, by adding a variant of the jellyfish gene. Later, a medaka (rice fish) with a fluorescent green color was developed. A variety of different GloFish are now sold in bright red, green, orange-yellow, blue, and purple fluorescent colors. Recently “Electric Green,” “Sunburst Orange,” “Moonrise Pink,” and “Galactic Purple” colored tetra (*Gymnocorymbus ternetzi*) and an “Electric Green” tiger barb (*Puntius tetrazona*) have been added to the lineup. Although not originally developed for the ornamental fish trade, it is one of the first GM animals to become publicly available as a pet and only sold in the United States. In addition to commercial GM fish, transgenic fish are widely developed and used in many laboratories all over the world as models for understanding the mechanisms of growth and development, and disease resistance, or for studying human diseases.

Scientists have also applied transgenic techniques to create fish that act as biosensors. By incorporating an easily detectable “reporter” gene such as luciferase, a light-emitting enzyme (Legler et al., 2000) or GFP (Chen et al., 2000). Such fish could be used as early detection environmental monitors. Fish with sensitivity to estrogen and retinoic acid are also being developed (De Coster and Larebeke, 2012). GE dairy cows were created with human genes to produce genes that would be the same as human breast milk (www.youtube.com/watch?v=rQCF5m-3SbA). This could be used by mothers who lack the ability to produce milk or produce insufficient amounts to feed infants instead of formula foods. Researchers from New Zealand in 2012 also developed a GE cow that produces allergy-free milk (Jabed et al., 2012). They used combined two tools: nuclear transfer and RNAi, a technique relatively new for livestock used to knock out β -lactoglobulin gene and produce milk free of β -lactoglobulin protein that can cause allergic skin, digestive, and respiratory reactions predominantly in infants. Milestones of successful transgenic animals and fish for production are summarized in Table 6.6.

6.2.3 Animal cell culture

Originally the large-scale culture of animal cells was developed for the production of animal viruses to be used as vaccines. In 1962, baby hamster kidney (BHK) cells were adapted to grow in suspension and have been used industrially for the production of

Table 6.6 Milestones of successful transgenic animals and fish for production

	Trangenic trait	Gene cloned	Year
<i>Animals</i>			
Pig, sheep	Enhanced disease resistance	IgA	1991
Pig	Influenza resistance	Mx protein	1992
Pig	Increased growth rate with less body fat	GH	1999
Pig	Increased growth rate with less body fat	IGF-1	1999
Pig	Phosphate removal	Phytase	2001
Pig	High lactose in milk	α -Lactalbumin	2001
Pig	Increased polyunsaturated fatty acids in pork	Desaturase from spinach	2004
Pig	Omega 3-fatty acids from roundworm <i>C. elegans</i>	Desaturase (fat-1 gene)	2006
Pig (Environ™)	Phosphate removal	Phytase	2012
Sheep	Wool growth	IGF-1	1996
Sheep	Visna virus resistance	Visna virus envelop	1994
Goat	Milk fat composition	Stearoyl desaturase	2004
Goat	Blood thinner	Human antithrombin protein	2009
Goat	Spider silk protein	Dragline silk gene	2010
Cattle	Increased lactoferrin	Human lactoferrin	1994
Cattle	Increased whey protein	β - and k-caseins	2003
Cattle	Mastitis resistance	Lysostaphin	2005
Cow	Low-lactose milk	Lactase-phlorizin hydrolase	2009
Cow (Rosita)	Human breast milk	Two human milk genes	2011
Cow (Daisy)	Allergy-free milk	Knock out β -lactoglobulin	2012
<i>Fish</i>			
GloFish	Fluorescent pet fish	GFP of jelly fish	2010
Salmon	Enhanced growth	GH gene/antifreeze protein	2013

Abbreviations: GH, growth hormone; IGF, Insulin growth factor.

Source: Author's compiled data.

foot-and-mouth disease (FMD) vaccines in production operations having capacities of up to 10,000 L. Since animal cells of most types are suitable for *in vitro* utilization, the annual demand of 280 million experimental animals worldwide in 1990 will be reduced as additional animal cell cultures develop. More recently the aim has been the production of human proteins of potential therapeutic value, such as hormones, lymphokines, enzymes, antibodies, and interferons. Like plant cell culture, animal cell culture deals with the study of parts of organs, tissues, or individual cells *in vitro*. The recombinant proteins such as human insulin and growth hormone approved for human use were produced by *E. coli* but the bacteria have some serious shortcomings on inability of posttranslational modifications. Thus, among the 58 therapeutic products approved from 2006 to 2010 by FDA, 32 are produced by animal cells (Walsh, 2010) using several large reactors up to 10,000 L, and this situation will continue as more therapeutic proteins have complex glycosylation that cannot be practically produced in prokaryotes or lower eukaryotes. However, successful production in animal cell culture must overcome many hurdles such as the cellular fragility, complex and expensive media, expensive equipment, and increased risk of contamination of human pathogens.

6.2.3.1 Establishing cell lines The cultivation of animal cells begins with the dissociation of the tissue fragment into its component cells by treatment with trypsin or chelator. Animal cells come in two types: anchorage-dependent cells, which require a surface for growth such as the human fibroblast cells, and suspension cells, which are anchorage-independent cells such as HeLa cells (a tumorigenic cell line) and hybridomas. Irrespective of the cell line, all animal cell cultures require an adequate supply of oxygen, only moderate changes in pH, a carbon and energy source (usually glucose), and supplementation of amino acids, trace metals, specific growth factors, and hormones.

Anchorage-dependent cells The cell suspension is placed in a flat-bottomed glass or plastic container that contains a suitable liquid medium. After a lag period, the cells attach to the container and start dividing mitotically. This type of culture, arising from differentiated tissue, is called a primary culture. The bottom of the culture vessel becomes thick with a continuous layer of cells, which is called a monolayer. The primary culture cells may be detached from the vessel by trypsin digestion or the addition of the chelating agent ethylenediamine tetraacetic acid (EDTA). These cells can be used to initiate secondary cultures by reincubation in fresh media at high cell density. The term *cell line* is applied to the generations obtained after the first subcultivation and all subsequent ones. The cell strain may be used only when cells with specific and stable properties have been obtained, through selection or cloning. Cell strains do not have an infinite life and divide only a finite number of times before they die. Human cells generally divide only 50–100 times before dying. Using conventional techniques, it is usually possible to obtain cell densities of approximately a million suspension cells per milliliter. For example, only 3×10^7 cells can be obtained from a bottle with a surface area of 500 cm², since the surface area for growth is only a small percentage of the total bottle volume. Also the variation in the cell cultures obtained from a series of individual bottles makes it practically impossible to monitor cellular kinetics and to change the growth environment.

Many methods have been suggested to solve the anchorage culture problem of low ratios between surface area available for cell growth and the total culture volume. These include dextran microcarrier beads, the multiple propagator, the spiral film, and plastic bags. The microcarrier system is the preferred methodology for scale up when it can be made to work well and reliably, provided the problem of oxygen transfer limitation is solved.

Another type of adherent culture is organotypic culture, which involves growing cells in a three-dimensional (3D) environment as opposed to two-dimensional culture dishes. This 3D culture system is biochemically and physiologically more similar to *in vivo* tissue, but is technically challenging to maintain because of many factors (e.g., diffusion).

Suspension cells (anchorage-independent) Suspension cultures systems are preferred for the large-scale manufacturing processes because of easy homogeneous suspension, which allows efficient monitoring and control of process parameters. Since 1950s, many different systems have been developed up to pilot-scale reactors for hundreds of liters. Very large scale up to 3000 L using BHK cells in stirred-tank reactors was used for the production of vaccines against FMD virus (Pullen et al., 1985).

HeLa cells, lympholactoids, and many other tumor cell lines do not require a surface to grow on and may be cultured in fermentors that are used for bacteria. However, mammalian cells lack a cell wall and are easily damaged by shear forces. Therefore, novel techniques have been developed to avoid creating high shear stresses. The basic modification of the conventional agitator-driven system is the introduction of air bubbles

at the bottom. Such a system has been scaled up to 8000 L for the commercial production of FMD virus from BHK cells or from a different cell line derived from hamsters (IFFA-3). Also, α -interferon produced from the Namalwa lymphoblastoid cell line is currently in production. The system can be modified for use in technologically less developed countries for production of vaccine. Cells also may be protected from shearing by the creation of artificial cell walls by encapsulating the cells in calcium alginate. Encapsulation is currently used commercially to produce hybridoma cells and their products.

The Chinese hamster ovary (CHO) line is the most commonly used cell type and about 70% of all licensed biotherapeutic proteins (hormones, growth factors, thermobolytics, blood clotting factors, immunoglobulins) are produced by this cell line. The CHO cell line is safe and has compatibility with (i) efficient gene expression systems and (ii) excellent post-translational modifications of proteins with freedom of pathogen agents that can grow in large-scale suspension bioreactors. Several anchorage-independent sublines of CHO cells can also grow in suspension even in static flasks or in chemically defined medium (Birch and Racher, 2006). The yield improvement can be adjusted by clone selection, cell engineering, medium optimization, environmental factors (temperature, pH, osmolality, CO₂), and culture mode.

6.2.3.2 Continuous cell lines (hybridoma) Some cells become altered through subcultivation generation, acquiring a different morphology, growing faster, and gaining the ability to start a continuous cell line. Such cell lines are said to be transformed, and generally they are neoplastic – that is, they produce cancer if transplanted into related animals. The transformed cell lines can also be obtained from normal primary cell cultures by infecting them with oncogenic viruses or by treating them with carcinogenic chemicals. Continuous cell lines circumvent the potential for unlimited subcultivation *in vitro* (Figure 6.6). This immortalized cell line (hybridoma) is used to produce monoclonal antibodies (mAbs). In brief, lymphocytes isolated from the spleen (or possibly blood) of an immunized animal are combined with an immortal myeloma cell line (B cell lineage) to produce a hybridoma which has the antibody specificity of the primary lymphocyte and the immortality of the myeloma. When selective growth medium, HA or HAT (hypoxanthine–aminopterin–thymidine) is used to select against unfused myeloma cells; primary lymphocytes die quickly in culture and only the fused cells survive. These are screened for production of the required antibody, generally in pools to start with and then after single cloning. To meet the demand of the mAbs up to hundreds of kilograms or tons per year, bioreactors with working volumes up to 20,000 L are currently used (Birch and Racher, 2006). Rodent mAbs are typically produced in suspension culture of hybridoma cells that are used in diagnostic and research applications, but for the majority of therapeutic applications, antibodies are now GE and produced in CHO cells or mouse lymphoid cell types, NS0 and SP2/0 in large suspension culture. Immobilized human cell lines such as PER.C6[®] technology are also being developed for the production of recombinant proteins in large-scale such as vaccines, mAbs, and gene therapy products. Thus, the continuous hybridoma culture is a practicable and useful method either for generating cells or for gathering information about those cells.

At present, for large-scale cultivation of animal cells culture system has two, microcarrier bioreactors, typically a combination of both for the host cell culture systems, more often using a stirred-tank bioreactor with the spherical microcarriers. Although stirred bioreactor combined with spherical microcarrier culture process has the training process can be monitored with the mass production process is stable, easy to carry out the expansion, can reduce pollution occur between batches is easy to control product quality, improve cell culture density. The advantage, however, as these two systems own limitations, there is such a drawback Culture Process: stirred reactor easy to produce shear forces on the cell which

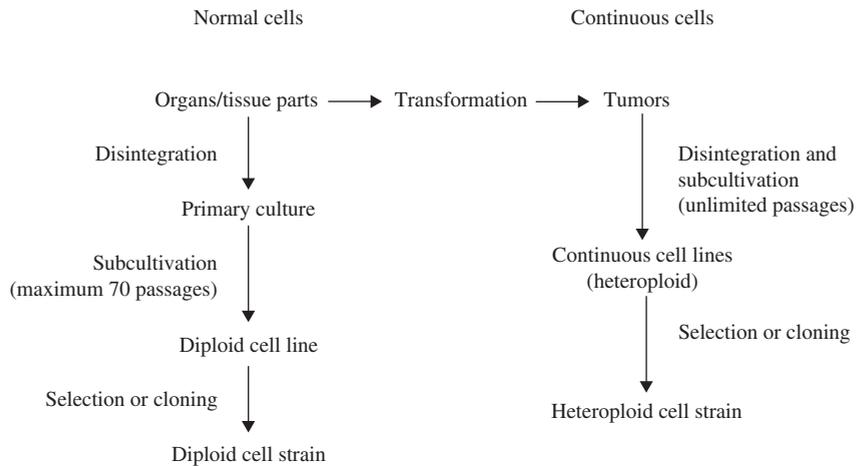


Figure 6.6 Correlation between tissue and cell types.

ultimately affect the efficiency of adsorption influence cell yield, the reactor tank cleaning, disinfection time consuming, the number of small spherical microcarriers use, inexpensive production costs.

6.2.3.3 Suspension culture reactors The majority of products produced using large-scale animal cell culture are (i) stirred reactor, that was described details of process flow from 20,000-L scale reactor with height to diameter ratios (1:11 to 3:1), (ii) air-lift reactor, which is used less commonly than stirred reactor, due to lack of flexibility in working volume, though this reactor is and low shear characteristic; air-lift reactors have been used for the BHK and human lymphoblastoid cells, insect cells to scale up to 5000 L, and (iii) micro-carrier bioreactor (porous and non-porous), (iv) roller-bottle reactor, and (v) hollow fiber reactor. Bioreactor design for animal cell should consider: surface adherence properties of the cells, increased demand on aseptic procedures, the shear sensitivity of the cells, potential foaming in the medium, potential mass transfer limitations, control and automation, ease of scale up, and removal of toxic waste products. Production methods are based on batch, repeated batch, fed-batch, and continuous (perfusion) systems, among which batch culture and fed-batch culture are simple, reliable, flexible, and shorter time to develop and validate as compared with the continuous systems. Batch, fed-batch, and perfusion methods are compared in Table 6.7. The production economics of continuous versus fed-batch are compared for a choice (Lim et al., 2006). After batch system was successfully operated for several months for mAb production in a 1300-L reactor, the reactor was harvested at every 84 h, and then leaving about 10% of the culture for next culture.

6.2.3.4 Applications of animal cell cultures Animal cell cultures produce a wide range of biological products of commercial interest, including immunoregulators, antibodies, polypeptide growth factors, enzymes, and hormones. They are already used in the manufacture of virus vaccines, tissue plasminogen activators (enzymes that facilitate the destruction of blood clots), α -interferon, mAbs, and tumor-specific antigens (for inclusion in diagnostic kits).

Table 6.7 Comparisons of batch, fed-batch, and continuous (perfusion)

	Batch	Fed-batch	Perfusion
Cell density	Low ($\sim 4 \times 10^6/\text{mL}$)	High ($\sim 1.5 \times 10^7/\text{mL}$)	High (3 to $4 \times 10^7/\text{mL}$)
Labor intensity	Severe	Less severe	Less severe
Operation time	5–7 d	15–25 d	30–180 d
Others	Contamination low	Byproducts accumulation (high osmolality, cell inhibition, need optimal feeding strategy)	High cell density (due to removal of byproducts)

Source: Authors' compiled data.

Table 6.8 Some examples of industrial scale animal cell suspension culture

Product	Cell line	Reactor
Foot-and-mouth	BHK21	3,000 L disease vaccine
Interferon	Namalwa	8,000 L
Tissue plasminogen activator	CHO	10,000 L
Factor VIII	BHK 21	500 L (perfusion), 2,500 L (repeated batch)
Urokinase	CHO	–
Monoclonal antibody	Hybridoma/myeloma	500 L (perfused spin filter)
Monoclonal antibody	NS0 myeloma	2,000 L (fed-batch)
Monoclonal antibody	Hybridoma	1,300 L (repeated batch)
Monoclonal antibody	Myeloma	2,000 L (air lift)
Recombinant proteins/antibodies	Various	20,000 L (stirred)

Source: Author's compiled data.

Another potential application of cultured cells is in the evaluation of new drugs and toxic chemicals. In conjunction with conventional animal models, cultured cells could reduce much of the time and cost as well as the total number of animals required for a given study. Many human proteins known to have therapeutic potential are in short supply. Animal cell lines may be used to synthesize the desired proteins such as interferons (α -, β -, γ -), interleukins, plasminogen activator, acetyl-cholinesterase, and urokinase. However, such cell cultures produce only small amounts of these proteins. One solution to the problem of low yields is to produce the protein in bacteria by using rDNA technology, but animal cells may be more suitable because many mammalian proteins undergo posttranslational modifications such as glycosylation, in which a protein is correctly processed and secreted into the culture medium. There are problems, however, relating to slow growth rate, low cell densities, and low product yield. If animal cell culture is the only route to obtaining certain therapeutic proteins, cells that are overproducers are required. Some examples of industrial scale from animal cell suspension culture processes are shown in Table 6.8.

Another potential application of cultured cells is in the evaluation of new drugs and toxic chemicals. In conjunction with conventional animal models, cultured cells could reduce much of the time and cost as well as the total number of animals required for a given study. Two major alternatives to *in vivo* animal testing are *in vitro* cell culture techniques and *in silico* computer simulation, but they cannot replace animals completely as they are unlikely to ever provide enough information about the complex interactions of living systems (Lipinski and Hopkins, 2004). Other alternatives include the use of humans for skin irritancy tests

and donated human blood for pyrogenicity studies. Another alternative is the so-called microdosing, in which the basic behavior of drugs is assessed using human volunteers receiving doses well below those expected to produce whole-body effects (Festing and Wilkinson, 2007). While microdosing produces important information about pharmacokinetics and pharmacodynamics, it does not reveal information about toxicity or toxicology.

6.2.3.5 Strain development of animal cell cultures The advance to GE animal cells began in the 1960s, when the viability of a fusion product of a human cell and a mouse cell was demonstrated. Kohler and Milstein (1975) produced a fused lymphocyte–myeloma mouse cell line that retains its viability and excretes antibody molecules. This hybridoma technology was then developed for the production of mAbs. The increased demands for mAbs in such diverse applications as diagnostic reagents, tumor imaging, immunopurification, and potential therapy on a large scale has led to the development of air-lift and stirred-tank reactors as well as immobilization systems. In addition to animal cell fusion method of producing GE cell lines, there are many different techniques for the production of new cell lines for proteins, some of which are commercially successful. Some proteins that are produced from GE animal cells are listed in Table 6.8. In some instance the levels of expression are too low to be of commercial significance, but these have been undoubtedly improved significantly in recent years.

Although many simpler proteins can be produced using GE bacterial cultures, more complex glycosylated proteins must be made in animal cells. An important example of such a complex protein is the hormone erythropoietin. The cost of growing mammalian cell cultures is high, and thus intensive works are underway to produce such complex proteins in insect cells or in higher plants, use of single embryonic cell and somatic embryos as a source for direct gene transfer via particle bombardment, transit gene expression, and confocal microscopy observation is one of its applications. It also offers to confirm single cell origin of somatic embryos and the asymmetry of the first cell division, which starts the process.

Almost all cell culture manipulation during cell-line generation is carried out by transfection, selection, limiting dilution cloning, cryopreservation, and recovery, and cells are mostly optimized for serum-free medium. Chemically defined serum-free media (SFM) with no animal components has become industry standard, because of a high risk adventitious agent in the serum and serum proteins (albumin, transferrin, insulin). Invitrogen's Gibco CHO-S cells were the first suspension adapted CHO derivative available commercially and cell-line specific media are commercially manufactured for superior performance (Agrawal and Bal, 2012).

Despite a number of available animal cell lines, such as BHK, mouse myeloma-derived NSO, human embryonic kidney (HEK)-293, and human retina-derived PER.C6, almost 70% of all recombinant therapeutic proteins are produced in recombinant Chinese hamster ovary (rCHO) cells (Jayapal et al., 2007), due to the (i) safe hosts, and low specific productivity, but low yield can be overcome by (ii) gene amplification systems such as dihydrofolate reductase (DHFR)-mediated or glutamine synthetase (GS)-mediated that are available for efficient posttranslational modification. These cell lines are now easily adapted growth in large scale of serum-free suspension culture. Over the past decades, higher than 100-fold titer improvement in rCHO cell culture more than 10 g/L (Kim et al., 2012). All cell lines can be transfected using polyethylenimine (PEI) or calcium phosphate. PEI-mediated transfection showed 50–80% cells expressing GFP that is currently used for production of recombinant therapeutic proteins by both transient transfection and formation of stable cell lines (Khan, 2013).

Many vector systems can be used to transform animal cells. Both the simian virus 40 (SV 40)- and polioma-based vectors have been used as lytic vectors in cells. However, naked

DNA, presented as a calcium phosphate precipitate, is a popular method of transfection, particularly when coupled with genes, which can act as selective markers, and carrier DNA, which increases the efficiency of the process. The vectors bind to the cell surface and are ingested endocytotically. Direct methods in DNA-mediated gene transfer involve microinjection into the nucleus of a somatic cell or zygote.

Production of clinical stage products by stable cell lines is prevalent because it offers high yields, consistent products quality, and safe regulatory status, but milligram to gram levels of proteins are often required in early development. Because stable cell-line generation is time consuming and resource-intensive, transient transfection in animal cells is frequently used instead. Several cell lines have been used for transient expression of proteins, but only a few of them have advantages of (i) high transfectability with common methods, (ii) easy of adaptation to suspension cultivation in serum-free conditions, and (iii) cost-effective scalability (Baldi et al., 2007). HEK 293 is the most commonly used cell line for large-scale transient gene expression (TGE). TGE is a relatively new technology that was only recently considered for recombinant therapeutic protein production. TGE is defined as the production of a recombinant protein over a short period (1–14 days) following DNA transfer into single-cell suspension cultures. The recombinant gene(s) is usually cloned in a nonviral expression vector and transfected into cells with a chemical delivery agent such as CaPi or PEI. In contrast to stable gene expression from recombinant cell lines, genetic selection is not applied to the transfected cells during the protein production phase, mainly with CHO and HEK-293 cells. They are easily transfected, are grown in single-cell suspension, and have been used for the production of therapeutic proteins which have gained regulatory approval. TGE is typically performed in stirred-tank bioreactors or agitated containers including shake flasks, wave-type bioreactors, and plastic or glass bottles. As HEK-293 productivities of 1 g/L have been achieved in a bioprocess lasting 14 days, significant quantities of recombinant protein can be obtained within a few days of transfection in 100 L (Jesus and Wurm, 2011). However, there has not been a therapeutic protein produced by TGE that has gained regulatory approval. The application of high-throughput culture systems will help to alleviate existing problems with TGE. Also disposable plastic bioreactors have proven to be well-suited for TGE, due to inexpensive and easy operation.

Baculovirus expression in insect cells is also a robust method for producing recombinant glycoproteins and baculovirus-produced proteins are currently under study as therapeutic cancer vaccines with several immunologic advantages over proteins derived from mammalian sources (Griffin et al., 2007). Baculoviruses possess rod-shaped capsids with large double-stranded DNA genomes. They productively infect arthropods, particularly insects. Two baculoviruses have been extensively developed as vectors, namely the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) that is used for protein expression in insect cell lines, and the *Bombyx mori* nuclear polyhedrosis virus (BmNPV), that normally infects the silkworm for the production of recombinant protein. Construction and application of a recombinant baculovirus containing a bicistronic expression cassette were used for stable protein expression in mammalian cells, and expression of a secreted protein in mammalian cells using baculovirus particles was also studied (Jardin et al., 2012). Baculoviruses are incapable of infecting mammals and plants and thus have a restricted range of hosts that they can infect. Because baculoviruses are not harmful to humans, they are considered a safe option for use in research applications.

Monoclonal antibodies Mammalian cells are currently the main hosts for commercial production of therapeutic proteins, including mAb. One animal cell product that attracts ever-increasing attention is the mAb produced by the class of lymphocyte known as *B cells* (plasma cells). This is a specific antibody produced from a normal, short-lived,

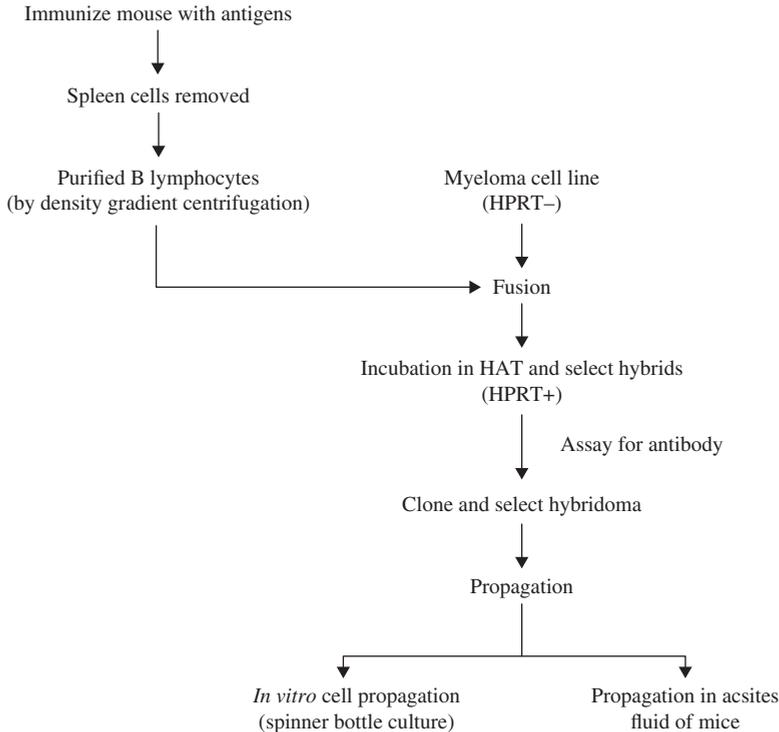


Figure 6.7 Steps in the production and selection of monoclonal antibody/secreting hybridoma cell lines. HAT, hypoxanthine–aminopterin–thymidine; HPRT, hypoxanthine phosphoribosyltransferase.

antigen-activated B cell that has been immortalized by hybridizing it with a myeloma cell (neoplastic transformation). These hybrid cells (hybridomas) retain the ability of the B cell to secrete antibody and the ability of the myeloma cell to grow indefinitely. When compared with traditional polyclonal antisera, which derive from many cells and contain heterogeneous antibodies, the mAb is derived from a single cell that comprises a uniform breed of antibody specific for a single antigen site. The preparation of hybridoma cell lines capable of secreting mAbs is outlined in Figure 6.7.

The first step involves the isolation of B lymphocytes from the spleen of animals (usually mice) that have been injected with the desired antigen. The spleen is removed when a high titer has been achieved. The B lymphocytes are fused with myeloma cells (which are tumor cells derived from malignant lymphocytes) by incubating the mixture with polyethylene glycol. The washed cells in fresh media comprise a mixture of hybridomas, unfused myeloma cells, and unfused lymphocytes. At best only about one myeloma cell in a thousand fuses with a spleen cell, and it is thus necessary to select against the unfused cells. B lymphocytes die *in vitro*, and to select against myeloma cells, lines defective in hypoxanthine phosphoribosyltransferase (HPRT) or thymidine kinase (TK) are used. HPRT- and TK-deficient strains are unable to use hypoxanthine and thymidine, respectively; rather, they rely on *de novo* synthesis of nucleotides, the pathways for which are sensitive to aminopterin (A). Therefore the addition of aminopterin in the presence of hypoxanthine

and thymidine kills the original myeloma cells and selects for hybrids that have received the HPRT (or TK) gene from the spleen cells.

The hybrids are then cloned such that each well of a microtiter plate receives one or a few clones, and the hybrids are screened for the desired antibody. Positive clones are subcloned by dilution and redistribution in microtiter plates, and positive clones are characterized. Thus the hypoxanthine–thymidine medium is growth inhibitory to the HPRT– cells but growth promoting to the HPRT+ cells. However, production yield of mAbs *in vitro* is often low ($\approx 5\text{--}10\ \mu\text{g/L}$ specific antibody). One solution is to grow the hybridoma to high cell density in a suspension culture with or without the use of microcarriers. In this way, antibody levels of 10–100 mg/L may be obtained. Large-scale culture in air-lift fermentors up to 2000 L is now a commercial process. An alternative way of obtaining a high concentration of antibody is to grow the hybridoma as ascites (tumor cell suspensions within the peritoneal cavity of an isogenic species). Mice bearing ascitic tumors can accumulate as much as 20 g/L of mAbs, a level superior to that obtained by the best *in vitro* culture. The mAbs produced usually are purified before use by precipitation with ammonium sulfate and by ion exchange chromatography (diethylaminoethyl cellulose (DEAE)-based matrix) or protein A/G affinity chromatography.

After mAb could be generated, scientists have targeted the creation of “fully” human antibodies to avoid some of the side effects of humanized or chimeric antibodies using transgenic mice and phage display. Transgenic mice technology is by far the most successful approach to making “fully” human mAb therapeutics. Repertoire cloning or phage display rely on rapid cloning of immunoglobulin gene segments to create libraries of antibodies with slightly different amino acid sequences from which antibodies with desired specificities can be selected. The phage antibody libraries are a variant of the phage antigen libraries first invented (Pieczenik, 1999). These techniques can be used to enhance the specificity with which antibodies recognize antigens, their stability in various environmental conditions, their therapeutic efficacy, and their detectability in diagnostic applications. Fermentation chambers have been used to produce these antibodies on a large scale. High levels well above the levels seen from parental hybridomas of chimeric antibodies and recombinant antibody fragments have been achieved by using low copy number cell lines.

Additional applications of monoclonal antibodies mAbs, with their specificity and high purity, are far more versatile than polyclonal antisera, which are produced by bleeding immunized animals and are used for diagnostic purposes. Thus mAbs are used in many diverse applications such as human therapy, commercial protein purification, bone marrow and organ transplantation, immunotoxins as therapeutic agents, and chimeric antibodies.

In particular, mAbs can be used to identify or isolate minute quantities of specific antigens. This property has proved particularly useful in medical diagnosis and in the biosensor field. Immunodiagnostic tests for blood typing and diagnosis of a variety of diseases can now be performed routinely within 15–20 min. However, this application for therapy is limited by the availability of mAbs, which must come from human–human hybridomas. mAb affinity columns are also particularly valuable for the purification of proteins. The columns are usually prepared by coupling mAbs to a cyanogen bromide activated chromatography matrix (e.g., Sepharose). The immunoaffinity methods have several advantages over conventional purification methods. Since the mAbs have a unique specificity for the desired protein, the level of contamination by unwanted proteins is usually very low. The mAb–antigen complex has a single binding site, and thus the protein is eluted in a single peak. Also, the capacity of mAbs remains unchanged, regardless of the concentration of a total crude protein in a mixture. Thus immunoaffinity chromatography is being used commercially to purify recombinant interferon-2 with

a 5300-fold purification. Both monoclonal, mAbs (homogenous isotype and antigen specificity) and polyclonal (heterogeneous isotype and antigen specificity) antibodies are available from industry vendors and in individual labs. Polyclonal antibodies are isolated from the sera of animals that have been immunized against a target antigen. Although polyclonal antibodies are often specific for an antigen, they contain a mix of different antibodies against different epitopes of the same antigen, which can affect assay results. In contrast, mAbs are grown from hybridomas, which are immortalized clonal cell lines that can produce a continuous supply of homogenous antibody and are the current standard for antibody production technology. However, mAbs are expensive, but the cost of mAb is dropping. The large number of immunocompromised people who could benefit from mAbs; the growing recognition of the microbiome, which is disrupted by antibiotics; and the increased availability of diagnostic tests that may make mAbs more feasible to administer.

For the application of antibody in food industry, the FDA since 2005 has required manufacturers to label their products with regards to eight specific allergens: milk, eggs, fish, shellfish, peanuts, wheat, soybeans, and tree nuts, that are responsible for over 90% of the documented food allergen-related cases. In food industry, the two most common and preferred methods for the detection of allergens are the enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR). Testing methods have been developed that can now detect these allergens in finished products at very low levels. These techniques detect the food allergens at the molecular level and provide a quick and definitive result that allows manufacturers to dispose of or relabel contaminated products before they are released. However, some matrices can interfere with the ELISA method, such as chocolate, or can cause cross reactivity as seen between different types of nuts. This method is also not the most suitable for cooked or heated products because the protein molecules are denatured. PCR methods, which are more sensitive and detect the DNA molecules of these allergens, can be used in raw and cooked products and are not affected by the heating process because DNA typically remains intact after being exposed to the cooking temperatures of most foods. PCR, however, cannot be used on all products including none of DNA-containing products, such as milk or egg whites. These products must instead be tested using ELISA-based methods for detection. Also antibacterial polyclonal antibodies and positive controls for identifying Shiga-toxin-producing *E. coli*, *E. coli* O157:H7, *Salmonella*, and so on, are available for foods such as ground beef, lettuce, spinach. Various bacterial, fungal pathogens, and toxins are the primary agents of concern in the food industry; thus, ELISA, PCR, and other methods are already well described (Mandal et al., 2011; Sharma and Mutharasan, 2013). The development of mAbs greatly enhanced the field of immunology by providing a constant and reliable source of characterized antibodies. A highly sensitive sandwich ELISA based on two mAbs to measure the content of the major peanut allergen Ara h 1 in foods has been developed (Peng et al., 2013). mAbs has also been used to develop highly specific and sensitive tests for the detection of plant pathogens in soil and in plants (Thornton and Wills, 2013).

Summary

The novel aspect of transgenesis is the ability to transfer genes across species lines. Inter-species crosses have proven to be very helpful in improving animals and in biomedical and genetic research, as well as in the development of new lines of plants. In the past, species-specific proteins and peptides for human clinical use could be isolated only from human glands or serum or urine. However, the advance of animal cell culture is now

opening up entirely new sources for the production of commercially valuable biologicals (human hormones, lymphokines, enzymes, antibodies, interferons, etc.). Such biologicals could also be used in evaluating new drugs and toxic chemicals as a replacement for or in conjunction with conventional animal testings.

The problem of low yields by animal cell cultures may be overcome by cloning therapeutic proteins into bacteria. The best prospect for obtaining a correctly processed and posttranslationally modified protein is the introduction of the gene into an immortal cell line. Despite large-scale processes of animal cells, transgenic animals have been developed for the production of a number of compounds of diagnostic and therapeutic interest. More recently, mAbs and a number of rDNA proteins have been added to a growing list of commercial and valuable cell culture products.

6.3 Food safety issues of new biotechnologies

6.3.1 Introduction

Food legislation in all countries is intended to ensure the safety of human and animal foods, including food ingredients produced by both traditional and modern methods in both short- and long-term usage. However, judgments with respect to the degree of risk-benefit involved must be based on the data of expensive chemical, toxicological, and nutritional tests. Such data, supplemented with detailed experimental data, are then submitted to the appropriate regulatory agency. The commercial success of a new technology depends on its ease of entry into the marketplace. Obtaining regulatory clearances to manufacture and market products, however, results in long delays in the delivery of useful products. Extensive safety testing is an impediment in the food industry, in particular, with its traditionally low margins of profit. Governments of all countries are trying to adhere as closely as possible to internationally agreed food standards developed through the Codex Alimentarius Commission jointly administered by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations. Each government agency usually sets standards not only for the composition and microbiological standards of foods, but also for their labeling and advertising. Government agencies regulate the use of food additives such as preservatives, coloring agents, and antioxidants and restrict claims of therapeutic or prophylactic value and “naturalness” of foods. Many regulations have been promulgated that are applicable to the manufacture of foods, drugs, and medical devices, the so-called Good Manufacturing Practices regulations (GMPs), which pervasively govern nearly all aspects of manufacturing processes.

Within the United States, the FDA and the USDA are the agencies mainly responsible for the safety of all human and animal foods, including ingredients or additives that are produced by both modern and traditional means. The principal statute administered by FDA is the federal Food, Drug and Cosmetic Act (as amended in 1982). There is also the federal Meat and Poultry Products Inspection Act. Genetically altered plants may be subject to the federal Plant Pest Act and other laws depending on how they are constructed and their intended use. In Canada, novel food additives are required to meet the existing data requirements outlined in the Canadian Food & Drug Regulations, in addition to questions related to genetic modification. Also on the national level, Agriculture and Agri-Food Canada regulates and inspects agrifood products of biotechnology, and Health Canada undertakes the development of regulations that would require pre-market notification for novel foods produced by biotechnology. Since 1973, the safety aspects of genetic engineering have been the subject of considerable debate in many countries.

Each government has taken different approaches to assess the risks associated with the use of genetic engineering technology and the development and release of GMO, including genetically modified crops and genetically modified animals/fish. Regulation varies in a given country depending on the intended use of the GM products. Some of the most marked differences occur between the United States and Europe. One of the key issues concerning regulators is whether GM products should be labeled. Labeling can be mandatory up to a threshold GM content level or voluntary. In Canada and the United States, labeling of GM food is voluntary, while in Europe all food (including processed food) or feed that contains greater than 0.9% of approved GMOs must be labeled. However, there is broad scientific consensus that foods on the market derived from GM crops pose no greater risk than conventional foods and the consumption of approved GM foods has no detrimental effect on human health. Some scientists and advocacy groups such as Greenpeace and World Wildlife Fund have, however, called for additional and more rigorous testing for GM food.

6.3.2 Safety evaluation of novel food products

The safety of rDNA or hybridoma technologies in place of conventional processes should not be a major reason for concern, although one cannot assume that these technologies will not present dangers in the future. The long history of traditional biotechnology may be cited as evidence of its benign nature, because organisms have been manipulated by natural selection for centuries in growing crops, breeding domestic animals, and effecting the bioconversion of agricultural products into food and drink (cheese, yogurt, wine, beer, etc.). The existing regulatory framework outlined in connection with the evaluation of the safety of foods (Table 6.9) seems to be sufficient to cope with GE organisms and their products. Such regulation does not apply to organisms obtained by means of the techniques of genetic modification through mutagenesis and cell fusion, since these specific applications have been used in a number of applications and have a long safety record. Although there are national differences, the general principles used are similar all over the world.

Table 6.9 Summary of criteria used in the safety evaluation of foods

Items	Description
Basic information	Food or additive source, intended market or use, consumption levels
Chemical composition	Proximate analysis, nucleic acid, vitamins, fatty acids, amino acids, toxic metals, antinutritional factors
Specification of product	Provided product clearance is granted for novel food, pilot-scale specification of chemical and microbiological purity must exactly match larger scale production of food both nutritionally and toxicologically
Nutritional/metabolic data	Animal results, verification by humans, nutritional consequences, macro- and micronutrient equivalency, effect of novel food determined for the whole population including children and elderly, effect on nutrition after processing, storage, cooking, etc.
Toxicological data	Acute and subacute toxicity tests, metabolic fate, carcinogenicity, mutagenicity, embryotoxicity, allergenicity using laboratory animals and cell cultures

Source: Adapted from Edwards and Fleet (1989), *Biotechnology and the Food Industry*. Gordon and Breach Science Publishers, New York. Reproduced with permission of Taylor & Francis Group.

Most recently, the FDA Food Safety Modernization Act (FSMA), the most sweeping reform of our food safety laws in more than 70 years, was signed into law on January 4, 2011. It aims to ensure that the US food supply is safe by shifting the focus from just responding to contamination to taking efforts of preventing it. The FDA intends to provide regular updates to inform the public and Congress on its progress in implementing the FSMA (<http://www.fda.gov/Food/GuidanceRegulation/FSMA/ucm255893.htm>). Oversight of GMO in the United States is conducted by a combination of FDA, primarily the USDA, and the US Environmental Protection Agency (USEPA).

In Canada, a *novel food* is defined as any food that has not been used previously to any significant degree for human consumption. A similar definition exists for a novel food process. Of most immediate concern are (i) products that contain GMOs or result from production by GMOs, (ii) products and processes that have never been used before as food or to process food, (iii) microorganisms that have never been used as food or in the processing of food, and (iv) substantially modified traditional products and processes. In other cases, the manufacturer is requested by the Canadian Food Directorate to seek further advice with respect to notification requirements. Determination of the need for notification and the safety assessment of each novel product is done on a case-by-case basis, and the novel substance is compared to an analogous traditional food. For example, a new microorganism in a food product might be considered to be a food additive. Notification may not be required if a high degree of similarity exists. This concept of substantial equivalence is similar to the policy developed by the Organization for Economic Cooperation and Development (OECD, 2010). Most OECD members are high-income economies with a very high Human Development Index (HDI) and are regarded as developed countries (<http://en.wikipedia.org/wiki/OECD>), composed of 34 members of the OECD. The list includes 21 of the 28 European Union member states, all except Bulgaria, Croatia, Cyprus, Latvia, Lithuania, Malta, and Romania in 2013.

The Food Directorate in Canada and the FDA in the United States do not intend to establish new procedures or requirements to govern the new products of biotechnology intended for food use. GRAS food substances are sufficient. It is generally agreed that the application of genetic modification does not inherently increase or decrease the risk associated with an organism. However, a wide variety of modifications is possible through genetic manipulation, and the potential for the introduction of toxic compounds, unexpected secondary effects, and changes in nutritional and toxicological characteristics gives rise to safety concerns. In the case of food constituents consisting of single chemical products or well-defined mixtures, procedures for safety assessment are well developed and internationally accepted. Specifications for identity and purity developed for products from traditional sources may not be entirely adequate to ensure the safety of products derived from genetically modified organisms, however, and additional specifications may need to be drafted.

The safety issue of whole foods derived from genetically modified microorganisms, plants, and animals is more difficult than that of single food constituents or defined chemical mixtures that will be used in food. The basis for the safety assessment of whole foods is the comparison of molecular, compositional, toxicological, and nutritional data for the modified organism with that of its traditional counterpart. If the genetic modification is well defined, the safety evaluation can be limited to information on the development and production of the modified organism and comparison of the compositions of the modified and unmodified cases. If a genetically altered organism is found to be significantly different from a traditional one, a more comprehensive review on toxicological and nutritional tests, including *in vitro* and *in vivo* assays, is required for the novel product. Where there are potential concerns related to the allergenicity of the product, limited human volunteer

Table 6.10 Some areas of safety consideration in the regulation of foods, food ingredients, and processing aids derived from new biotechnologies

Microorganisms

Production of fermented foods and beverages by genetically engineered starter cultures
 Application to extend shelf life and use as biological control agents by genetically engineered strains
 Direct consumption of engineered strains or their derived components as foods (e.g., SCP)
 Use of engineered microbial metabolites as food ingredients or processing aids

Plants

Products derived from recombinant DNA products
 Products derived from tissue cultures by somaclonal/gametoclonal variation
 Products derived from bioreactor plant cell cultures

Animals

Use of genetically engineered substances administered to the animal and fish and used in foods derived from this animal
 Use as food of transgenic animals developed by gene technology

Abbreviation: SCP, single cell protein.

Source: Adapted from Edwards and Fleet (1989), *Biotechnology and the Food Industry*. Gordon and Breach Science Publishers, New York. Reproduced with permission of Taylor & Francis Group.

studies and limited marketing trials may be required after a review of data from comprehensive animal and laboratory tests. Table 6.10 summarizes some topics for consideration in the regulation of foods and food ingredients derived from new biotechnologies.

6.3.3 Genetically modified microorganisms and their products

Bacteria, yeasts, and fungi have been employed in fermented foods and drinks for millennia, mostly to yield secondary metabolites such as flavoring aromas of butter, cheese and yogurt, desirable metabolites (ethanol, glutamate), preservatives (acetate, lactate, etc. in pickles and sauerkraut), or leavening agents. Other enzymes and flavor ingredients have been used in food and food processing. Microorganisms such as SCP have also been consumed directly as foods and probiotic cultures, and as vitamin supplements in animal feeds. The genetically modified microorganisms (GMMs) included here are those developed by rDNA technology and other methods of DNA introduction such as protoplast fusion, microinjection, and electroporation. Microorganisms developed by deletion, rearrangement or suppression of native DNA are also subject to consideration. Organisms that have undergone genetic modification by artificially induced mutagenesis using chemical or ultraviolet irradiation may also be included. Special concern is focused on modified microorganisms when a parent or vector originates from a species that is known to produce toxic compounds.

The use of GMMs in the food industry is well established and around 40% of the food enzymes marketed in Europe and North America are produced by recombinant bacteria, yeasts, and fungi (AMFEP, 2009). All the additives such as amino acids, vitamins, and gums (polysaccharides) are also derived from recombinant strains and future ingredient products include biomass-derived products, intact microbial cells such as GM microalgae enriched oil (Franklin et al., 2011), GM baker's yeast, GM wine yeast, GM lactic and probiotics in

dairy foods such as yogurt and cheese (Aguilera et al., 2013), or omega-3 fatty acid enriched GM *E. coli* or lactic acid bacteria (Amiri-Jami and Griffiths, 2010).

International standard on the safety of GMOs, including GMMs have been developed by OECD (2010) and the safety of GMM produced food enzymes is well described by Olempska-Beer et al. (2006).

Antibiotic-resistant markers should not be present in the final product. The decision to accept such markers is made on a case-by-case basis. In any case, the prospective seller must prove that the foreign DNA did not introduce detrimental properties that would be expressed during fermentation. These concerns would be minimized by choosing the DNA source only from microorganisms of GRAS organisms. For example, genes responsible for the strong lipolytic and proteolytic enzymes of *Rhizopus* and *Aspergillus* species used in soy sauce fermentation can be cloned into lactic acid bacteria for accelerated cheese ripening. The procedures for the safety assessment of certain processing aids or ingredients (e.g., enzymes, amino acids, vitamins, polysaccharides, sweeteners produced by genetically modified microbial fermentation) are well established by government authorities. Any new ingredient or processing aid containing either traditional or GE microorganisms must be evaluated according to the accepted criteria before approval can be granted. However, special consideration can be given for certain ingredients (enzymes, amino acids, etc.) that have been approved in their traditional forms but can now be produced more efficiently and more economically using engineered strains. Emphasis is given in this case for the evidence supporting the purity and chemical authenticity of the final product rather than the novelty of the process by which it is derived.

Another recent example submitted by Novozyme (USA) and approved by FDA (May 30, 2013) is a new recombinant enzyme, an asparaginase preparation produced by submerged fermentation of a genetically modified *Bacillus subtilis* microorganism expressing a gene coding for an asparaginase from *Pyrococcus furiosus*. The recombinant plasmid DNA was integrated into the *B. subtilis* host strain chromosome by homologous recombination without using antibiotic marker(s). Toxicological testing, including 13 week oral toxicity in rats, Ames test and human lymphocyte cytogenetic or micronucleus assay confirmed the safety of recombinant enzyme preparations derived from these six *B. subtilis* production strains.

The food, food products, and feed products derived from the use of GMMs fall under different EC (the European Parliament and the Council) legislative categories in Table 6.11.

Table 6.11 Regulatory framework for food, food products, feed products derived from GMMS under EC (the European Parliament and Council)

Categories	EC number
Food enzymes	1332/2008
Food additives	1333/2008
Food flavors	1334/2008
Products of cell biomass (containing GMM cells such as biomass-based food ingredients, bread or feed)	1829/2003
Products of GMMs (not destroyed or inactivated such as yeast for baking, yogurt, and lactic starters for cheese, etc.) or	1829/2003
Deliberate release into environment of GMOs	18/EC/2001

6.3.4 Genetically modified plants and their products

New varieties of plant crops may be consumed as food or used to produce natural food ingredients that are used in food or food processing. The variety of ways by which plants can be modified and the extent of genetic modification that can be produced determine the type and quantum of information required to make a safety assessment. For many novel plants, the final product is the result of repeated backcrosses between the initially modified organism and the host. Some data generated in the initial stages (e.g., method of modification, stability of transformed plants, molecular genetics) must be accepted for the final assessment. Of course, the detailed chemical and toxicological data from the final product should be generated. New varieties of plant crops selected from the somaclonal variants do not contain foreign DNA but reflect other changes in chemical composition, organization, and/or expression of existing genetic material. Thus, it would be important to ascertain whether the variant produces new or decreased concentrations of nutritional components such as protein and essential amino acids. Regulatory authorities might also be concerned with the degree of altered physicochemical properties such as color, shape, and flavor of the variant.

The best characterized somaclonal variation program has been developed for tomato. Somaclonal variants have been observed for many secondary metabolites including terpenes, alkaloids, phenolics, anthocyanins, carotenoids, and chlorophyll. Whether novel plants are developed using mutagenesis, somaclonal/gametoclonal variations, protoplast fusion or DNA procedures, sufficient data on the process used to effect genetic modification should be provided. Detailed information should also be provided on the source, purity, and stability of all inserted materials. The introduction of foreign DNA genes into plants to confer specific resistance traits, such as ability to withstand herbicides, pests, microbial diseases, and environmental stresses (cold, drought, contaminants, etc.) has been advantageous for crop yield. However, the safety of a crop as a food should be taken into account even though an additional property inserted would not be toxic to the consumer.

6.3.4.1 Whole GM crops Due to high regulatory and research costs, the majority of GM crops in agriculture consist of commodity crops, such as soybean, maize, cotton, and rapeseed. Some research and development has been targeted to enhancement of crops that are locally important in developing countries, such as insect-resistant cowpea for Africa and insect-resistant brinjal (eggplant) for India.

The GM food controversy over the use of food and other goods derived from GM crops instead of from conventional crops, and other uses of genetic engineering in food production is nothing new and debates are intensive by various groups. By 2030, world population is expected to hit 8.3 billion, causing a 50% increase in the global demand for food and energy and a 30% increase in the demand for fresh drinking water – a resource that is already in short supply for about a third of the world's people. Climate change will complicate things even further, and in unpredictable ways (<http://environment.about.com/b/2009/03/23/population-growth-expected-to-cause-a-food-water-and-energy-crisis-by-2030.htm>).

The key areas of controversy related to GMO food are many on the labeling issue, the effect of GM crops on health and the environment, the effect on pesticide resistance, the impact of GM crops on farmers, and the role of GM crops in feeding the world population. The pros and cons of GM crops are summarized in Table 6.12.

In Canada alone, as many as 30,000 different products of GM ingredients are estimated to be on grocery store shelves, and this is largely because many processed foods contain soy, a crop of which more than 90% is GE in North America (www.responsibletechnology.org).

Table 6.12 Some of pros and cons for genetically modified crops*Pros*

Crops

- (a) Improved resistance to disease, pests, and herbicides
- (b) Reduced maturation time
- (c) Increased nutrients, yields, and stress tolerance
- (d) Enhanced taste and quality
- (e) New products and growing techniques

Animals

- (a) Increased resistance, productivity, hardiness, and feed efficiency
- (b) Better yields of meat, eggs, and milk
- (c) Improved animal health and diagnostic methods

Environment

- (a) Friendly “bioherbicides and bioinsecticides”
- (b) Conservation of soil, water, and energy

Society

- (a) Increased food security for growing populations

Cons

Food safety

- (a) Transfer of unintended toxin, allergens, antibiotic marker,
- (b) Allergenic reaction: appropriate testing of allergen, long-term safety effect

Economic concerns

- (a) Trade restrictions among different countries
- (b) For profit only multinational companies due to termination technology used

Environmental impact

- (a) Transgenic organism dominates and thus loss of diversity
- (b) Unintended transfer of traits to wild or “undesirable” species by cross-pollination, that will create superweeds, etc.
- (c) Unintended effect on non-target species
- (d) The risks of “tampering with Mother Nature”

Source: Author’s compiled data.

The method for assessing the safety of all GM food is to evaluate its *substantial equivalence* to the nonmodified version. The concept of substantial equivalence implied that the GM crop be compared with an isogenic counterpart, that is the same genotype without the transgene(s) to assess agronomic, morphological, and chemical characteristics, such as macro- and micronutrients, anti-nutrients, and toxic molecules. Further testing is then carried out on a case-by-case basis to ensure that concerns over potential toxicity and *allergenicity* are addressed before a GM food is marketed. There is broad scientific consensus that food on the market derived from GM crops poses no greater risk than conventional food and no reports of ill effects have been documented in the human population from GM food (Nicolia et al., 2013). Although labeling of GMO products in the marketplace is required in many countries, it is not required in the United States or Canada and no distinction between marketed GMO and non-GMO foods is recognized by the FDA. Advocacy groups such as Greenpeace, The Non-GMO Project and Organic Consumers Association insist that risks of GM food have not been adequately identified and managed, and have questioned the objectivity of regulatory authorities. They have expressed concerns about the objectivity

of regulators and rigor of the regulatory process, about contamination of the non-GM food supply, about effects of GMOs on the environment and nature, and about the consolidation of control of the food supply in companies that make and sell GMOs.

Genetic use restriction technology (GURT), colloquially known as *terminator technology* or *suicide seeds*, is the methods for restricting the use of genetically modified plants by causing second generation seeds to be sterile. The technology was developed under a cooperative research and development agreement between the Agricultural Research Service of the USDA and Delta and Pine Land company in the 1990s, but it is not yet commercially available. Since 2001, there has been a de facto worldwide moratorium on the use of terminator technology (UN Convention on Biological Diversity). The genetic engineering regulation concerns the approaches taken by governments to assess and manage the risks associated with the development and release of GM crops. The GM regulation differs among countries, with some of the most marked differences between the United States and Europe (Table 6.13). Also regulation varies in a given country depending on the intended use of the GM products. For example, a crop not intended for human food is generally not reviewed by food safety authorities. There are differences in the regulation of GM crops between countries, with some of the most marked differences occurring between the United States and Europe. Regulation varies in a given country depending on the intended use of the products of the genetic engineering. For example, a crop not intended for food use is generally not reviewed by authorities responsible for food safety. The European Union cannot meet its goals in agricultural policy without embracing GM crops, but consumers and governments in Europe have rejected GM food more widely than those in other parts of the world, including North America. The opposition to GMOs stems from a fear of potential health risks and the rise of agricultural monocultures, which are perceived as being bad for the diversity of local agriculture and the food supply.

Table 6.13 Safety consideration and regulation of genetically modified foods in the United States, Canada, and Europe

United States	(a) The FDA currently requires labeling of GE foods if the food has a significantly different nutritional property; if a new food includes an allergen that consumers would not expect to be present or if a food contains a toxicant beyond acceptable limits (b) Mandatory labeling of GE foods in the United States has been proposed, but not enacted, at the national, state, and local levels.
Canada	(a) Health Canada indicates that it is a seven- to ten-year process to research, develop, test and assess the safety of a new genetically modified food, and manufacturers and importers who wish to sell or advertise a GM food in Canada, must submit data to Health Canada for a pre-market safety assessment, as required under Division 28 of Part B of the Food and Drugs Regulations (Health Canada "The Regulation of Genetically Modified Food", http://www.hc-sc.gc.ca/sr-sr/pubs/biotech/reg_gen_mod-eng.php) (February 2012) (b) Labelling for GMOs is currently not mandatory. A 2003 poll indicated that 88% of Canadians believe that labels for GE foods should be made mandatory
Others	(a) Mandatory labeling is currently enforced throughout Europe, Australia, New Zealand, China, and Japan

Abbreviation: FDA, US Food and Drug Administration; GE, genetically engineered.

Source: Ahmed 2002. Reproduced with permission of Elsevier.

6.3.4.2 Safety concern of GM crops and cell cultures GE maize crops that produce a Bt-based toxin have been commercially cultivated since 1996. After the introduction of Bt seeds, some problems were encountered in large-scale cultivation due to pest insects resistant to adapted to Bt cotton and maize crops (Edgerton et al., 2012). In 2010, around half of all arable land in the United States, that is, 17.8 million hectares, was planted with insecticide-producing, triple-stack maize crops. *Triple-stack crops* are produced by crossing GE crops and have distinct traits: (i) tolerance to herbicide, (ii) toxic to pest insects which damage the crop above the soil, and (iii) toxic to rootworm which attacks the crops in the soil. By October 2012, the emergence of 13 new resistant weeds in 31 states of the United States has been reported. Either these weeds can no longer be eradicated using glyphosate, or greater quantities have to be applied to eradicate. Thus, concerns have been raised about possible outbreak of resistant population of target species because of the high selection pressures produced by repetitive sowing of GE herbicide and pest-resistant crops (Nicolia et al., 2013). Glyphosate-resistant weeds (Shaner et al., 2012), and Bt-resistant pests (Gassman, 2012) have been reported, and thus proper management of weed control is necessary. Herbicide-resistant weeds have already blighted around 20–25 million hectares of arable land in the United States. These will include using larger quantities of glyphosate, applying additional pesticides, more regular ploughing and hand weeding, which are time consuming and costly counter measures.

These controversies have led to litigation, international trade disputes, protests, and restrictive legislation in most countries. The previously stated advantages of GM crops, however, far outweigh the disadvantages. GM crops have proven to be a viable and very real solution among many others, which can help secure future food production. Canada changes to GM food safety regulation to permit imports contaminated with levels of GM foods if they are approved in at least one other country in 2013 (www.cban.ca).

Plant cell cultures in bioreactors also offer tremendous opportunities to produce a wide range of food ingredients or additives such as pigments, amino acids, flavors, and enzymes. It should be determined, however, whether the cell line used in the reactor is a product of rDNA, or whether abnormal physiology has developed under prolonged culture, resulting in the production of toxic compounds. Currently, regulatory emphasis is on specification of identity, purity, and safety of the final product, rather than on the modification process. Thus, extensive testing of the final criteria would demonstrate acceptability.

Gene flow defined as the movement of genes, gametes, individuals, or groups of individuals from one population to another occurs both spatially and temporally (Mallory-Smith and Sanchez Olguin, 2011). As GM plants may be capable of surviving through seed or asexual propagules for many years in the field, or to fertilize sexually compatible non-GM plants, this may lead to the spread and persistence of transgenes into the environment or the product. Among the possible three subgroups, (i) for gene flow to wild relatives, few formation of hybrids have been reported in GM canola with multiple herbicide resistance genes (Schafer et al., 2011). Other cases on a new herbicide-tolerant intergeneric hybrid of transgenic creeping bentgrass (Zapiola and Mallory-Smith, 2012), but there is no evidence of negative effects of transgene introgression and this appears in wild population due to natural mutation (Kwit et al., 2011); (ii) for gene flow to a non-GM crop, it is not only involving the movement of pollen, but also remaining the seeds in the field. This coexistence cases have been actively investigated for several species such as maize, canola, soybean, flax, wheat, potato, cotton, and sugar beet, but these are extra economic costs due to the containment practices (Nicolia et al., 2013); (iii) for *horizontal gene transfer* (HGT) of selective antibiotic marker gene in soil microorganism, soil bacteria can uptake exogenous DNA at very low frequency (10^{-4} to 10^{-8}) in laboratory experiments, but there was no evidence in the field (Ma et al., 2011).

6.3.4.3 Safety of GM crops- and GMM-derived ingredients These GMO-derived processed ingredients from soy, and so on, and additives (vitamins, enzymes, live cultures) generally undergo several processing steps and cannot be differentiated from their conventionally produced counterparts. This holds true for processed ingredients or additives made from GM plants and for additives or vitamins produced with the help of genetically modified microorganisms. For the products to be marketed into the European Union or other countries, an authorization is required and as a prerequisite to gain the authorization, a risk assessment of the product must be carried out to make sure of its safety. Those who intent to commercialize a product must prepare an application containing all the necessary scientific data conducted for the risk assessment and forward to the European Food Safety Authority (EFSA), which performs an independent evaluation in Europe. EFSA has a series of Guidance Documents for the methodology for the safety evaluation, the data requirements for food enzymes, flavors, additives, and GMMs (Table 6.3). In category 1, both GMMs and newly introduced genes have been removed as in case of crystallized vitamins or amino acids and other highly purified molecules. This category has the least data requirements due to its high purity. In category 2, both GMMs and newly introduced genes are no longer present, but still contain rests of GMM cells as shown in preparation of commercial GM enzymes derived from byproducts of fermentation and other impurities (DNA, toxins, etc.). In category 3, viable GMM cells are not present, but still contain recombinant genes derive from biomass food supplements such as beers and cheeses or potential horizontal transfer of the newly introduced genes to other microbes from the gut or environments. In category 4, the most complicated level of assessment in those consisting or containing viable GMMs such as yeasts for baking, starter cultures from fermented foods and yogurt. This category requires assessment on the safety impact of any change in biology of GMMs with respect to its wild type, its potential adverse effects to the consumer (toxicity, pathogenicity, effect in the gut microbiota), and to the environment (persistence, ecosystem, etc.).

Currently GMMs derived additives and enzymes added to animal feed are mostly produced with the help of GMMs. For example, vitamins (B2, B12, biotin, C-E300, riboflavin-E101), various amino acids (improved feed quality; glutamate-E621, cysteine E-921, etc.), enzymes (improved breakdown efficiency by phytase, etc), coloring pigments such as β -carotene (e.g., for salmon), thickener (xanthan-E 415), acidity regulator (citric acid-E 330), preservative (natamycin-E 235, nisin-E 234, lysozyme-E 1105). Details on food ingredients, additives, feed, and so on from GMMs in Europe are published elsewhere (Aguilera et al., 2013) or GMO Compass (<http://www.gmo-compass.org/eng/home/>). As legally specified safety standards set purity requirements for vitamins and other additives, the production method by chemical, biological, or with GMOs is not a factor. All systems that use GMMs must be reported to governmental agencies. Additives that are produced with the help of GMMs do not require labelling because GMOs are not directly associated with the final product. Because the final product is carefully purified and does not contain any GMMs, vitamins and additives made in this way are not subjected to special regulations or labelling requirements. In some cases, amino acids and enzymes are not legally considered foods, but rather, they are known as processing aids. This is why there is no legal requirement to declare these additives on the list of ingredients.

6.3.5 Genetically modified animals and their products

There are two major ways in which new biotechnologies may affect the supply of food presently obtained from unmodified animals. One way features exogenous, GE substances such as vaccines, bovine growth hormones, and bovine somatotropin (BST), administered

to the animals or used in foods derived from the animals. The second way entails the transfer of new genetic material into the animal genome to produce *transgenic animals*. Transgenic technology has new direct benefits including such production advantages as rapid growth, disease resistance, lean meat, and the availability of valuable therapeutic proteins (e.g., vaccines, drugs, hormones, mAbs, other pharmaceuticals). In any case, it would be necessary to establish (i) that the product injected into the animals was pure and did not itself adversely affect the composition of foods derived from the transgenic animals and (ii) that the particular property engineered into animals did not inadvertently alter the safety or wholesomeness of foods derived from particular tissues of those animals. For example, a transgenic chicken resistant to avian leukosis virus (retrovirus) has been developed in Canada. Even if the transgenic animal is resistant to a disease, the authority of Health of Animals in Canada must determine that the transgenesis is safe, not only for the animal but also for the people who handle it. Its safety must be established at the abattoir level, and at the same time it will need to be established how many generations of animals will be resistant and how the change will affect the quality of the animal. To bring transgenic animals to the marketplace, a person or company must prove the safety, efficacy, and economic viability of the product contemplated. Whether it is produced by tissue cultures, by microorganisms or by transgenic animals, the product on the market will be regulated as a drug. Another possibility that may need safety consideration is the production of food substances using animal cell cultures. The recent regulatory issues relevant to transgenic animals and bioreactor cell lines are addressed in detail (Miao, 2013).

Food safety evaluation of transgenic animal is handled by national and international authorities. Theoretically, meat or milk from transgenic farm animals generated for nonfood purposes could also be consumed. For example, as the transgene in the “phosphorus-friendly” ENVIRON pig is expressed exclusively in the salivary gland; the meat contains only the transgene DNA not the transgene product. DNA is an essential part of nutrition and is not usually toxic upon uptake through the digestive tract. Large alimentary DNA fragments seem to survive gastrointestinal passage, enter the blood stream, but this does not lead to vertical gene transfer and there is no reason why a transgene should integrate into a consumer’s genome preferentially compared with nonrecombinant DNA. Nevertheless, debate is still ongoing. Large farm animals have little opportunity to escape and no chance of crossing with wild animals. More concern is warranted over possible escape of transgenic growth-enhanced fish from cage culture with access to free waters and subsequent spread of the transgene into the natural population. The FDA has completed the phase of public consultation before making a decision on allowing commercial production of AquaBounty’s genetically modified salmon and the fish going to market for human consumption in 2013. Physiochemical gene transfer methods and cloning by nuclear transfer do not have intrinsic biorisks, because the DNA is stably integrated into the host genome. Viral vectors are generally liable to the risk of recombination with wild-type viruses that in turn might create the means to spread the transgene.

All transferred DNA fragments should be characterized by sequencing, especially for large gene constructs, for example, artificial chromosomes. Transgenic animals designed for human consumption will be devoid of marker genes and other sequences not required for transgene function. Before using the transgenic animal for production, possible unwanted transgene effects are thoroughly checked by laboratory and veterinarian methods. Environmental issues and animal welfare are the most serious public concerns about animal biotechnology. Ethical assessments must openly address these uncertainties, with the precautionary principle providing a good criterion for responsible policies. Kochhar et al. (2005) described well the regulation and biosafety issues for transgenic animals, animal products, and feeds.

6.3.6 Detection methods of GM crops

The ever-increasing number of approvals granted spurred strong interest in developing methods for identifying GMOs in food. The availability of suitable identification procedures is necessary also for various food control activities, such as the observance of regulations on the labeling of GMOs and of regulations with respect to seed certification. The requirements on the specificity of detection methods will increase significantly with the number of distinct products available, the appearance of mixtures of distinct GMO products, and increased processing of such products or complex mixtures.

Traceability is defined in the EU General Food Law Regulation 178/2002/EC, inspired to the ISO standard, as the “ability to trace and follow food, feed, food producing animals and other substances intended to, or expected to, be incorporated into food or feed, through all stages of production, processing and distribution.” The detection method is a concept already widely applied to non-GE food/feed and it is not connected with their safety (Davison and Bertheau, 2007). It may include mandatory or voluntary labeling for the foods or feeds that contain or consist of GM crops or derived products. The European Union developed the most stringent regulatory framework for traceability of GE crops food/feed and derived products in the world. Their mandatory labeling for the unintentional presence of GE material in food or feed has the lowest threshold value at 0.9% based on the number of haploid genomes compared to other countries (Ramessar et al., 2008). Labeling requires the detection and quantification of the GE food/feed or derived product in the tested food/feed or seeds or any other product when applicable. After sampling and extraction of DNA, testing for GMOs is normally performed with qPCR (real-time quantitative polymerase chain reaction) – based methodologies that allow the detection and quantification of DNA at the same time. DNA-based qPCR testing utilizing GMO strain standards is used to determine the GMO levels from 0.01% to 5%. Other experimental strategies and analytical methods have been proposed (e.g., microarray, Luminex XMAP), but they require further evaluation. At the moment, in the European Union, mass fraction percentages are used for the certified reference material (CRM), or the copy number of transgenic DNA in relation to haploid genomes. For the detection of transgenes in mixtures composed by different ingredients, stacked transgenes and unauthorized events require specific approaches and strategies. As there is no uniform detection methods in different countries, a higher degree of international harmonization would be required in the future (Holst-Jensen et al., 2012).

Protein- and DNA-based methods employing western blots, ELISA, lateral-flow strips, Southern blots, qualitative-, quantitative-, real-time-, and limiting-dilution PCR methods, are some possible options for detection. Where information on modified gene sequences is not available, new approaches, such as near-infrared (NIR) spectrometry, might tackle the problem of detection of non-approved GM foods in Table 6.14.

GM products contain an additional trait encoded by an introduced gene(s), which generally produce an additional protein(s) that confers the trait of interest. Raw material (e.g., grains) and processed products (e.g., foods) derived from GM crops might thus be identified by testing for the presence of introduced DNA, or by detecting expressed novel proteins encoded by the genetic material. Both qualitative (i.e., those that give a yes/no answer) and quantitative methods are available.

6.3.6.1 Protein-based methods (i) In *protein-based testing methods*, immunoassay technologies with antibodies are sensitive qualitative and quantitative detection methods for many types of proteins in complex matrices when the target analyte is known. Both monoclonal (highly specific) and polyclonal (often more sensitive) antibodies can be

Table 6.14 Summary of the several detection for methods for GMO^a products

Parameter	Protein based			DNA based		
	Western blot	ELISA	Lateral-flow strip	Southern blot	Qualitative PCR ^b limiting dilution	QC-PCR and Real-time PCR
Ease of use	Difficult	Moderate	Simple	Difficult	Difficult	Difficult
Needs special equipment	Yes	Yes	No	Yes	Yes	Yes
Sensitivity	High	High	High	Moderate	Very high	High
Duration ^c	2 d	30–90 min	10 min	6 h ^d	1.5 d	2 d
Cost/sample (US\$)	150	5	2	150	250	350
Gives quantitative results	No	Yes [¶]	No	No	No	Yes
Suitable for field test	No	Yes [¶]	Yes	No	No	No
Employed mainly in	Academic laboratories	Test facility	Field testing	Academic laboratories	Test facility	Test facility

Abbreviations: ELISA, enzyme-linked immunosorbant assay; GM, genetically modified; QC-PCR, quantitative-competitive polymerase chain reaction; rDNA, recombinant deoxyribonucleic acid.

^aNear-infrared detects structural changes (not DNA or protein), is fast (<1 min) and inexpensive (~US\$1).

^bIncluding nested PCR and GMO Chip.

^cExcluding time allotted for sample preparation.

^dWhen nonradioactive probes are used; otherwise 30 h with ³²P-labeled probes.

^eAs in the antibody-coated tube format.

^fWith high precision.

^gSource: Ahmed (2002). Reproduced with permission of Elsevier.

used depending on the amounts needed and the specificity of the detection system (e.g., antibodies to whole protein or specific peptide sequences), depending on the particular application, time allotted for testing and cost. On the basis of typical concentrations of transgenic material in plant tissue ($>10\ \mu\text{g}$ per tissue), the detection limits of protein immunoassays can predict the presence of modified proteins in the range of 1% GMOs. Immunoassays with antibodies attached to a solid phase have been used in two formats: a competitive assay in which the detector and analyte compete to bind with capture antibodies, or a two-site (double antibody sandwich) assay in which the analyte is sandwiched between the capture antibody and the detector antibody; the latter assay is deemed preferable. Both western blot and ELISA techniques have been used for the analysis of protein products of Monsanto's transgenic Roundup Ready soybean (RRS), which is resistant to the herbicide glyphosate and contains the gene encoding *Agrobacterium* spp. strain CP4-derived EPSPS (Rogan et al., 1999). (ii) In *Western blot method* (i.e., the protein immunoblot) using gel electrophoresis to separate native proteins by 3D structure or denatured proteins by the length of the polypeptide, a sample containing the target protein below or above a predetermined threshold level is particularly useful for the analysis of insoluble protein. This method, however, is considered more suited to research applications than to routine testing and the detection limits of western blots vary between 0.25% for seeds and 1% for toasted meal. (iii) In a more sensitive antibody-based *ELISA* (ELISA), a sample solution containing a particular GMO protein is added to a multi-well solid plate on which GMO protein-specific antibody has been immobilized. If the GMO protein is present in the sample, it will bind to the immobilized capture antibody. After washing, a different antibody, also specific for the protein of interest and tagged with an enzyme, is added to the well. The enzyme-linked detection antibody will bind any GMO protein already immobilized to the well by the capture antibody. After another round of washing to remove any unbound antibody, the substrate for the enzyme is added which induces a color change in the solution. The degree of color change is directly proportional to the amount of GMO protein present in the well. Because there is no quantitative internal standard within the assay, no extra information can be obtained concerning the presence of GMOs at the ingredient level in food. (iv) In most common *lateral-flow strip*, which is a variation of ELISA, using strips rather than microtiter wells, led to the development of lateral-flow strip technology.

When the sample pad is submersed in a solution of homogenized test sample, the solution wicks up the nitrocellulose membrane on the strip, causing the fluid to pass over an area containing an excess of gold-labeled antibody specific to the GMO protein being tested. If that particular GMO protein is present in the sample then it will specifically bind to the gold-labeled antibody, and the antibody-protein complex will continue moving up the strip with the flow of fluid. The sample fluid then passes over two additional areas on the strip, a test line and a control line. The test line contains a second antibody, which is specific for the GMO protein being tested. When the gold-labeled antibodies with bound GMO proteins pass over the test line, the antibody-protein complex forms a sandwich with the immobilized second capture antibody. This results in the formation of a visible line on the strip indicating that that particular GMO protein is present in the sample.

In addition to microplate ELISA and lateral-flow devices, other immunoassay formats use magnetic particles as the solid support surface. The magnetic particles can be coated with the capture antibody and the reaction carried out in a test tube. The particles with bound reactants are separated from unbound reactants in solution by a magnet. Advantages of this format are superior kinetics because the particles are free to move in reaction solutions and increased precision owing to the uniformity of the particles.

Optimization and validation assays for ELISA (and also for DNA-based methods) are important aspects of standardizing this technology for GMO detection. Assay validation for food analysis is complex considering the large diversity of food matrices. Factors affecting optimization include: (i) selection of parameters (e.g. quality of kits, methods to test modified proteins and incubation times); (ii) selection of thresholds (e.g. limits for positive and negative tests); (iii) tracking of controls (in-house controls versus commercial kits); and (iv) the work environment (experience of the laboratory in performing tests, and potential contamination problems in the environment or by the individual(s) conducting the assays). Factors affecting validation include: (i) extraction efficiency, (ii) accuracy of results, (iii) precision and ability to distinguish between closely related values, (iv) sensitivity, limit of detection, (v) specificity, (vi) reproducibility, and (vii) consistency and reliability of detection. Given the complexity of food matrices, a practical approach to validate results is to use standard reference materials with known concentrations of GMOs in a matrix similar to that of the test sample. A collaborative validation study in Europe, involving 38 laboratories in 13 EU member states and Switzerland, was conducted to test accuracy and precision of ELISA with mAbs raised against CP4 EPSPS protein, and with a polyclonal antibody that conjugates with HRP for the detection of RRS in soybean flour (Lipp, 2000). The reference sample, which simulated an actual food matrix, contained 1.25% GMO. Results showed consistent detection on the basis of dry weight for samples containing approximately 0.35% GMOs.

6.3.6.2 DNA-based testing methods Whether or not a GMO is present in a sample can be tested by qPCR, but also by multiplex PCR, which uses multiple, unique primer sets within a single PCR reaction to produce amplicons of varying sizes specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes, that is, their base pair length, should be different enough to form distinct bands when visualized by gel electrophoresis. The DNA that has been engineered into a crop consists of several elements that govern its functioning, that are typically a promoter sequence, structural gene, and a stop sequence for the gene. Although several techniques are available, two are commonly used: Southern blot and particularly PCR analyses. Microarray-based technology for detecting gene expression is currently under development. (i) *Southern blot* involves fixing isolated sample DNA onto nitrocellulose or nylon membranes, probing with double-stranded (ds)-labeled nucleic acid probes specific to the GMO, and detecting hybridization radiographically, fluorometrically or by chemiluminescence. Although earlier probes were labeled with ^{32}P , nonradioactive fluorescein-labeled DNA digoxigenin-, or biotin-labeled DNA probes with sensitivity equal to ^{32}P probes, was recently used. These nonradioactive probes reduced detection to <1 h, as opposed to 24 h labeling required by ^{32}P . Because only one probe is used, and no amplification is carried out, this method is considered less sensitive than PCR, which employs DNA of two primers. Alternatively, the Southern blot technology has been attempted with fluorescent dyes (emitting at ~700 and 800 nm) coupled to a carbodiimide-reactive group and attached directly to DNA in a 5-min reaction. The signals for both dyes are detected simultaneously (limit in the low zeptomolar range) by two detectors of an infrared imager, something not yet possible with conventional radioactive or chemiluminescent detection techniques. (ii) In *Quantitative end-point PCR*, PCR was shown to be quantitative if an internal DNA standard was coamplified with target DNA. In systems such as the quantitative-competitive (QC)-PCR method, the presence of PCR inhibitors will be noticed immediately because

the amplification of both internal standard and target DNA will be simultaneously affected. qPCR consists of four steps: (1) coamplification of standard and target DNA in the same reaction tube; (2) separation of the products by an appropriate method, such as agarose gel electrophoresis and staining the gel by ethidium bromide; (3) analysis of the gel densitometrically; and (4) estimation of the relative amounts of target and standard DNA by regression analysis. (iii) In *Quantitative real-time PCR* some of the problems of conventional quantitative end-point PCR was circumvented. In theory, production of PCR products should proceed exponentially, but in practice it reaches a plateau between 30 and 40 cycles because certain reaction components become limiting. In conventional PCR, products of the reaction are measured at a single point in the reaction profile. Plotting the concentration of reactions shows that proportionality between DNA concentration (dynamic range) and PCR products occurs over a limited range of DNA concentrations. Real-time PCR also allows for detection of low copy. (iv) In *NIR spectroscopy*, which was used for nondestructive analysis of whole grains for the prediction of moisture, protein, oil, fiber, and starch, the technique has been used in attempts to distinguish RRS from conventional soybean. The advantages of this technique are: (1) it is fast (<1 min), (2) sample preparation is not necessary because it uses whole kernels (~300 g), and (3) it is therefore cheap. The major disadvantage is that it does not identify compounds, thereby necessitating a large set of samples to generate spectra. This calibration dataset is then used to predict the GMO event. Thus, this method cannot be more accurate than the reference method used to build the model. Moreover, a calibration needs to be developed for each GMO to be predicted. Furthermore, although NIR is sensitive to major organic compounds (e.g., vibration overtones of C–H, O–H, and N–H), its accuracy is limited. The several procedures for detecting GMOs in foods described here are presented in Table 6.6.

One example on a PCR detection system for 35S promoter region of cauliflower mosaic virus (Deisingh and Badrie, 2005) indicated the possibility of detecting GMO contents of foods and raw materials in the range of 0.01–0.1%. La Mura et al (2011) also applied QUIZ (quantification using informative zeros) to estimate the contents of RoundUp Ready™ soya and MON810 in processed food containing one or both GMs and found that the quantification in samples can be carried out without the certified reference materials using QUIZ. Other detection of RRS by loop-mediated isothermal amplification in conjunction with a lateral-flow dipstick has recently been reported (Xiumin et al., 2012).

Approval of GM crops for commercial production and for importation as food or feed follows extensive testing and is the responsibility of individual countries or cooperating countries (e.g., the European Union). The allergenicity assessment is an important one for individuals with specific food allergies. The primary concern is the potential transfer of a major allergen from a different species into a food crop as was the case when a Brazil nut 2S albumin was transferred into soybean to improve nutritional quality. Serum IgE testing, if the source of the gene is allergenic, and also amino acid sequence identity comparisons were also used to known allergens. If a significant sequence match is identified (e.g., >35% identity over 80 or more amino acids) using a well-curated allergen database (www.allergenonline.org), similar IgE testing would be required. The assessment also evaluates the stability of the protein in pepsin and abundance in food materials. Most countries have required evaluating endogenous allergens only for GM host plants (gene recipients) that are common causes of allergy (e.g., soybean), but not maize. Tests of herbicide-tolerant soybean event 40-3-2 (Monsanto Co., St. Louis, MO, USA) showed no differences in IgE binding to extracts of the parental line and two other commercial soybean varieties using 1D gel immunoblotting with a pool of five soybean-food-challenge-positive allergic subjects. Recently, the use of complex testing methods (e.g., proteomics) has been suggested by some regulatory agencies along with requests for testing of crops that rarely cause food allergy (e.g., corn).

For allergenicity tests, GM soybeans expressing the brasil nut 2S proteins, GM potatoes expressing cod protein genes, Bt toxin are known. Bawa and Anilakumar (2013) recently reviewed the detection methods and other safety, risks and public concerns on GM foods.

6.3.7 Detection methods of transgenic animals and fish

After transgenic animals and fish have successfully been created, the next major step is to identify the proper transgenic integration and safety consideration.

When the transgene produces a distinctive phenotypic effect, it can be noted easily, but often it does not result in such distinct effects, therefore other technique such as (i) Dots blot technique, and PCR technique must be employed. In Dots blot technique, the sample of DNA collected is fixed onto a support like nitrocellulose membrane; and then undergoes denaturation, that the DNA double helix separates. When treated with radioactively labeled probe of corresponding transgene, the sample DNA incorporated with transgene binds with the probe. After removing free probes by washing and analyzed by autoradiography, it reveals the presence of transgene, which can be detected by fluorescence produced by radioactive probes. The strength of radioactivity equals the strength of the radioactive probes, and the strength of radioactivity exhibited shows the strength of transgene integration. The PCR technique, which is the most widely used technique, can identify the transgenic animals similar to the GM crops described earlier. The analysis of transgenic proteins can be carried out by (i) identifying specific antibodies produced by transgenic proteins and (ii) studying the enzymatic properties of the concerned proteins. In the case of antibody identification, various immunologic assays using ELISA test are a classic example.

In order to analyze the transgene integrated, the isolated gene samples, which are detected with transgene integration, undergo restriction digestion with known restriction endonucleases. After the fragments are separated by agarose gel electrophoresis and these fragments are analyzed by southern blotting procedure, the integration of transgene can be confirmed by choosing the appropriate restriction enzyme. The presence of single or multiple integration of transgene is indicated by southern blotting. The mRNA produced from a transgene can also be detected as it is different from the native mRNA of the organisms. These can be purified and hybridized with a radioactive probe to detect mRNA production. Final detection of transgene integration is possible by protein expression of the transgene. The analysis of transgenic proteins can be done by two methods by identifying specific antibodies produced by transgenic proteins and by studying the enzymatic properties of the concerned proteins. Enzyme activities can be detected by using transgenic genes which produces different enzymatic activities or different pathway which is not shown by host enzymes. The main procedure followed is by transfer of scorable marker genes, which produce a definite phenotype. Main examples for these are *CAT* genes used mainly in fish or mammal cells, β -galactosidase, and luciferase gene used in fish.

In transgenic animals created by embryo microinjection, the site of integration of the transgene within the genome is a random event. Thus, when multiple embryos have been injected or infected with the same DNA, the integration site will be different in each founder animal. In the case of pronuclear injection, there is typically one insertion site, although multiple transgene copies are often found in a tandem array at that integration site. Lentivirus transgenesis is becoming an increasingly attractive alternative to pronuclear injection because it is more efficient in terms of successful transgene incorporation into the host genome, less invasive to the embryo, and technically less demanding to perform. Lentiviral delivery systems have been used successfully to generate transgenic mice, rats, pigs, and cattle. Independent of the method of transgene delivery, the insertion site can have profound effects on transgene expression. This can lead to phenotypic effects in

the transgenic animal that are not due to the transgene per se, but are a consequence of the integration site, a phenomenon referred to as *position effect*. To detect processed transgenic fish, PCR-based methods have been used. Detailed detection methods for transgenic animals and fish can be found in the book (Heller, 2006; Rehbein, 2006).

6.3.8 Containment: physical and biological

When designing DNA cloning experiments, any possible biohazards associated with the creation of recombinations of genes must be *contained*, to minimize the potential risks to humans and to the environment. Several agencies such as the WHO, the Medical Research Council of Canada, the US National Institutes of Health (NIH), and the Genetic Manipulation Advisory Committee of the United Kingdom have developed specific guidelines for the conduct of rDNA work. These guidelines are intended to prevent the inadvertent exposure and dispersal of recombinant molecules; to this end, they specify the implementation of stringent physical and biological containment (barrier) systems.

Physical containment is self-explanatory and encompasses several aspects of laboratory areas. *Biological containment* refers to measures genetically built in to a recombinant molecule or its propagating host for the purpose of limiting its chances of survival outside the laboratory. Both the physical and biological containment systems are divided into different levels. In the NIH system, which is generally recognized as the gold standard, levels of physical containment are designated P1 (the least) through P4 (the most stringent). An HV1 system provides a moderate level of containment while HV2 provides a high level of containment.

Persons in the United States and Canada wishing to conduct field trials of regulated substances in the environment must obtain a permit from the American Plant Health Inspection Service (APHIS) and USEPA or Environmental Canada. Any person, before undertaking a deliberate release of a *GMO* for the purpose of research and development or for any other purpose than placing on the market, must submit a notification to the competent authority of the state or province within whose territory the release is to take place. More than 30 species of fish including AquaBounty's GM salmon have been GE to combat diminishing wild fish stocks. In particular, biotechnological solutions are urgently required for containment to prevent interactions between transgenic fish and wild fish populations. Efficient physical and biological containment strategies remain as crucial approaches to ensure the safety of transgenic fish (Devlin et al., 2006).

6.3.9 Promises and limitations

Despite numerous food applications envisioned and the potential benefits of biotechnology in plant, animal, and food ingredients, many new products face technical obstacles that remain to be overcome before they can be launched into commercial production. There are several notable technical limitations: genetic maps (i.e., the identification of the location of desired genes on various chromosomes) have not been constructed for most of the industrially useful GRAS microorganisms, genetic systems for GRAS organisms (e.g., the availability of useful vectors) are at an early stage of development, and physiological pathways and the sequence of enzymatic steps leading from raw material to the desired product remain to be completely elucidated. The number of genes necessary is also a major limitation, since rDNA is most useful when only a single, easily identifiable gene is needed. It is more difficult to use rDNA technology when the genes have not yet been identified and several genes must be transferred. Additional problems regarding plasmid stability include

possible genetic transfer between plasmid and chromosome due to sequence homology or to transposons (“jumping genes,” i.e., sequences of DNA that can combine with foreign DNA by a mechanism that does not rely on sequence homology).

As more novel products are developed and are on the market, the emphasis will shift rapidly from the technical to the regulatory arena, where public perceptions of risks associated with the novel technology may threaten to hinder the achievement of its potential. Unless the public is convinced that the potential benefits exceed any potential risks, the technology cannot be used. Public acceptance of biotechnology is especially critical when novel technological methods are applied to the production of whole food. The public is more receptive to the application of biotechnology in developing human gene therapy and new drugs than in producing foods. One good example of the importance of public perception is the controversy around the marketing of BST, a hormone that stimulates increased milk production and can be produced by the rDNA process. Opponents of BST, mostly members of public interest groups and agricultural groups, have undertaken a nationwide campaign to prevent marketing of the recombinant hormone, and the controversy surrounding its safety and socioeconomic impact has created an inhospitable climate for foods and food ingredients produced by the new technologies. Thus, public perception seems to be much more important than scientific reality in determining the regulatory course.

Some of the unresolved issues on a national and an international basis include allergy considerations, labeling of biotechnology products, and social, moral, and ethical issues, as well as consumer acceptance. Calgene, developers of the GM Flavr Savr tomato, has withdrawn the products from the market after 1998, and many groups oppose the cultivation of Golden rice, GM salmon, and so on, claiming it will open the door to more widespread use of GMOs.

Among those without a basic understanding of scientific principles, however, there is a general fear of the unknown and a perception that scientists are tampering with Mother Nature or playing God. There is also a sector of the public who believes that human and animal genomes must not be treated simply as commodities available for commercial exploitation. If the primary beneficiaries of new technology are corporations and consumers believe that they will bear a disproportionate amount of risk, the technology will not be acceptable to the public. Some assurance will also be needed that regulatory agencies have anticipated, for the evaluation of long-term risks to humans, animals, and the environment. Despite negative consumer attitudes toward GE foods, food products derived from rDNA technology are already flooded. Educating the public about new biotechnology may be as critical as the technology and may have the greatest impact in promoting positive attitudes.

Summary

Genetic engineering has reached the stage that permits the relatively straightforward isolation and cloning of the gene for any well-characterized protein. GMMs and their derived products such as enzymes, vitamins, and other additives are well accepted with their purity and chemical authenticity. About 35 commercial (i) herbicide-resistant or (ii) insecticidal *Bt* (*B. thuringiensis*) protein containing crops including soy, maize/corn, canola, and alfalfa have been developed and many are already in the market, and many of their derived GM ingredients are on the grocery store shelves, that we eat. The future of biotech crops looks encouraging as commercialization of drought-tolerant maize is expected in 2013; Golden Rice in 2013/2014; and GM maize to be followed by Bt rice. Biotech crops could possibly contribute in accomplishing the 2015 Millennium Development goals, particularly in decreasing poverty by half, through maximizing crop productivity. Bananas that

produce human vaccines against infectious diseases such as Hepatitis B have also been developed. Yet, pest- and herbicide-resistant plants that will create superbugs and superweeds, respectively, are in the making. Resistance in that habitat always evolves whenever selective pressure is prevail, that could nullify the benefits of the transgenic crops in the long run. Some recombinant biotherapeutics using transgenic animals have gone into the market place; these include human insulin, human somatotropin, urokinase, blot clotting factor, livestock vaccine, interferon-2, and so on and more than 100 are under development. The drug, ATryn, an anticoagulant that can reduce blood clots during surgery or childbirth was extracted from the goat's milk. Consumers are more receptive on the recombinant biopharma products for human disease prevention. Although no GE food from farm animals has been made available commercially, AquaBounty's GM salmon and other fish will hit the market for human consumption soon. Farm animal and cow transgenics are undoubtedly important for biomedicine, but one main limitation has been the lack of public perception and acceptance of transgenics. Even if public perception moved in favor of transgenic food and if optimized gene transfer technology increased efficiency in the generation and exploitation of transgenics, there remain a number of issues such as safety testing, allergenicity, labeling, regulation, and policies. Any product or microorganism that had not previously been used as human food, or any process that had not previously been used to produce foods, could be considered to be novel, and hence subject to regulation. Consumer perception and ethical and moral issues will need public debate and input as well. Whether today's research projects will become tomorrow's products depends not only on making continued scientific progress but also on addressing concerns about environmental impacts and other risks, meeting regulatory requirements and dealing with marketplace realities.

People from all walks of life have given their opinions about GM foods, either in favor of or against them. People who speak in favor of GM foods find it to be a much cheaper, nutritious, and safe method, while people against them argue that pose many health concerns for human, environment, and ecology. The available data show GM foods are every bit as safe as conventional counterparts, yet critics of GM foods argue that current safety testing is not rigorous enough and fail to assess unintended consequences, especially health and environmental effects that arise over a longer period of time. The anti-GMO group overemphasizes the "right to know" aspect of GM labeling, while the pro-GMO and the biotech industry overemphasizes the cost aspect of GM labels. If consumers demand labeling, there is no option, but it is a very expensive process to screen them and it is impossible to 100% guaranteed. It is a complex issue and this subject requires a reasonable debate. We must look into the real important benefits of this technology that we cannot ignore.

Bibliography

- Acquaah, G. 2012. Clonal propagation and in vitro culture. *Principles of Plant Genetics and Breeding*, 2nd ed. John Wiley & Sons, Ltd, UK.
- Agrawal, V. and M. Bal, 2012. Strategies for rapid production of therapeutic proteins in mammalian cells. *BioProcess Intern.* 10: 32–48.
- Aguilera, J., A. R. Gomes, and I. Olaru. 2013. Principles for the risk assessment of genetically modified microorganisms and their food products in the European Union. *Int. J. Food Microbiol.* 167: 2–7.
- Ahmed, F. E. 2002. Detection of genetically modified organisms in foods. *Trends Biotechnol.* 20: 215–223.

- AMFEP. 2009. List of commercial enzymes October. Association of Manufacturers and Formulators of Enzyme Products. Accessed 21/07/04. Available from: <http://www.amfep.org/content/list-enzymes>.
- Amiri-Jami M., M. W. Griffiths. 2010. Recombinant production of omega-3 fatty acids in *Escherichia coli* using a gene cluster isolated from *Shewanella baltica* MAC1. *J. Appl. Microbiol.* 109: 1897–1905.
- Bairu, M. W., O. Adeyemi, and A. J. Van Staden. 2011. Somaclonal variation in plants: Causes and detection methods. *Plant Growth Regulation* 63: 147–173.
- Baldi, L., D. L. Hacker, M. Adam, F. M. Wurm. 2007. Recombinant protein production by large-scale transient gene expression in mammalian cells: state of the art and future perspectives. *Biotech. Lett.* 29: 677–684.
- Bawa, A. S. and K. R. Anilakumar. 2013. Genetically modified foods: Safety, risks and public concerns – a review. *J. Food Sci. Technol.* 50: 1035–1046.
- Berlin, J. and J. Bode. 1987. Plant and animal cell cultures. In *Fundamentals of Biotechnology*, P. Prauve, U. Faust, W. Sittig, and D. A. Sukatsch, Eds., pp. 134–177, Wiley-VCH Verlag GmbH, Weinheim, Germany.
- Bhojwani, S. S., and P. K. Dantu. 2013. Somaclonal variation. In: *Plant Tissue Culture: An Introductory Text*, pp. 141–154, Springer, New York.
- Birch, J. R. and A. J. Racher. 2006. Antibody production. *Adv. Drug Deliv. Rev.* 58: 671–685.
- Brown, D.C.W. and T.A. Thorpe. 1994. Crop improvement through tissue culture. *World J. Microbiol. Biotechnol.* 11: 409–415.
- Carvan, M. J., III, Dalton, T. P., Stuart, G. W., and D. W. Nebert. 2000. Transgenic zebrafish as sentinels for aquatic pollution. *Ann. N. Y. Acad. Sci.* 919: 133–147.
- Chen, C. M., T. K. W. Cheng, and H. L. Chen. 2009. Transgenic animals producing low-lactose milk and newly identified human small intestinal extracellular lactase-phlorizin hydrolase (ecLPH) gene. US patent 7501554 B2.
- Chen, H., J. Hu, J. Yang, Y. Wang, H. Xu, Q. Jiang, Y. Gong, Y. Gu, and H. Song. 2000. Generation of a fluorescent transgenic zebrafish for detection of environmental oestrogens. *Aquat. Toxicol.* 96: 53–61.
- Chicoine, L. G., C. L. Montgomery, W. G. Bremer, K. M. Shontz, D. A. Griffin, K. N. Heller, S. Lewis, V. Malik, W. E. Grose, C. J. Shilling, K. J. Campbell, T. J. Preston, B. D. Coley, P. T. Martin, C. M. Walker, K. R. Clark, Z. Sahenk, J. R. Mendell, and L. R. Rodino-Klapac. 2013. Plasmapheresis eliminates the negative impact of AAV antibodies on micro-dystrophin gene expression following vascular delivery. *Mol. Ther.* doi:10.1038/mt.2013.244.
- Clive J. 2012. ISAAA Report on Global Status of Biotech/GM Crop (<http://www.isaaa.org>)
- Cotton seed protein: Australian Consumers Association. 2004. Special report – Genetically modified foods. Accessed 21/07/04. Available from: <http://www.choice.com.au>.
- Curtin, M. E. 1983. Harvesting profitable products from plant tissue cultures. *Biotechnology* 1: 649–657.
- Davison J. and Y. Bertheau. 2007. EU regulations on the traceability and detection of GMOs: difficulties in interpretation, implementation and compliance. *Cab. Rev.* 2: 1–14.
- Day, A. G. and C. P. Lichtenstein. 1992. Plant genetic transformation. In *Plant Biotechnology*, M. W. Fowler, G. S. Warren, and M. Moo-Young, Eds., pp. 258–281, Pergamon Press, London.

- De Coster, S. D. and N. V. Larebeke. 2012. Endocrine-disrupting chemicals. Associated disorders and mechanisms of action. *J. Environ. Public Health* 2012: 1–52. DOI: 10.1155/2012/713696.
- Deisingh, A. K. and N. Badrie. 2005. Detection approaches for genetically modified organisms in foods. *Food Res. Int.* 38: 639–649.
- Devlin, R. H., L. F. Sundstrom, and W. M. Muir. 2006. Interface of biotechnology and ecology for environmental risk assessments of transgenic fish. *Trends Biotechnol.* 24: 89–97.
- Edgerton, M. D., J. Fridgen, J. R. Anderson Jr, J. Ahlgrim, M. Criswell, P. Dhungana, T. Gocken, Z. Li, S. Mariappan, C. D. Pilcher, A. Rosielle, and S. B. Stark. 2012. Transgenic insect resistance traits increase corn yield and yield stability. *Nat. Biotechnol.* 30: 493–496.
- Edwards, R. A. N. and G. H. Fleet. 1989. Food legislation and products of biotechnology. In *Biotechnology and the Food Industry*, P. L. Rogers and G. H. Fleet, Eds., pp. 279–289, Gordon and Breach Science Publishers, New York.
- Festing, S. and R. Wilkinson. 2007. The ethics of animal research. Talking point on the use of animals in scientific research. *EMBO Rep.* 8: 526–530.
- Fowler, M. W. 1987. Products from plant cells. In *Basic Biotechnology*, J. Bullock and B. Kristiansen, Eds., pp. 525–544, Academic Press, London.
- Francis G., Z. Kerem, H. P. Makkar, and K. Becker. 2002. The biological action of saponins in animal systems: a review. *British J. Nutr.* 88: 587–605.
- Franklin, S., A. Somanchi, J. Wee, G. Rudenko, J. L. Moseley, and W. Rakitsky. 2011. Tailored oils produced from recombinant heterotrophic microorganisms. US Patent 20110294174 A1.
- Gannon, F., R. Powell, T. Barry, T. G. McEvoy, and J. M. Screenan, 1990. Transgenic farm animals (minireview). *J. Biotechnol.* 16: 155–170.
- Gassmann, A. J. 2012. Field-evolved resistance to Bt maize by western corn rootworm: predictions from the laboratory and effects in the field. *J. Invert. Pathol.* 110: 287–293.
- Gillespie J., R. Nehring, C. Hallahan, C. Sandretto, and L. Tauer. 2010. Adoption of recombinant bovine somatotropin and farm profitability: Does farm size matter? *AgBioForum* 13: 251–262.
- Gong, Z., B. Ju, and T. J. Lam. 2000. Chimeric gene constructs for generation of fluorescent transgenic ornamental fish. WO 2000049150 A1.
- Griffin, T. J., G. Seth, H. Xie, S. Bandhakavi, and W. S. Hu. 2007. Advancing mammalian cell culture engineering using genome-scale technologies. *Trends Biotechnol.* 25: 401–408.
- Guardiola, J., J. L. Iborra, and M. Canovas. 1995. A model that links growth and secondary metabolites production in plant cell suspension cultures. *Biotechnol. Bioeng.* 34: 1331–1336.
- Hall, L., G. Tucker, J. Boniwell, J. Fletcher, J. Ray, C. Smith, C. Watson, W. Schuch, C. R. Bird, and D. Grierson, 1993. Antisense inhibition of pectinesterase gene expression in transgenic tomatoes. *Plant J.* 3: 121–129.
- Heller, K. J. 2006. *Genetically Engineered Food. Methods and Detection*, 2nd ed., Wiley-VCH Verlag GmbH Weinheim, Germany.
- Hernandez Gifford, J. A. and C. A. Gifford. 2013. Role of reproductive biotechnologies in enhancing food security and sustainability. *Animal Frontiers* 3: 14–19.

- Hill, J. E., A. R. Kapuscinski, and T. Pavlowich. 2011. Fluorescent transgenic zebrafish more vulnerable to predators than wild-type fish. *Transact. Am. Fish Soc.* 140: 1001–1005.
- Holst-Jensen, A., Y. Bertheau, M. de Loose, L. Grohmann, S. Hamels, L. Hougs, D. Morisset, S. Pecoraro, M. Pla, M. Van den Bulcke, D. Wulff. 2012. Detecting un-authorized genetically modified organisms (GMOs) and derived materials. *Biotechnol Adv.* 30: 1318–1335.
- Huang, T. K., and K. A. McDonald. 2012. Bioreactor systems for *in vitro* production of foreign proteins using plant cell cultures. *Biotechnol. Adv.* 30: 398–409.
- Jabed, A., J. Anower, S. Wagner, J. McCracken, D. N. Wells, and G. Laible. 2012. Targeted microRNA expression in dairy cattle directs production of beta-lactoglobulin-free, high-casein milk. *Proc. Natl. Acad. Sci. USA* 109: 16811–16816.
- James, C. 2012. Global Status of Commercialized Biotech/GM Crops: 2012. ISAAA Brief, No.44. ISAAA, Ithaca, NY.
- Jardin, B. A., C. B. Elias, and S. Prakash. 2012. Expression of a secreted protein in mammalian cells using baculovirus particles. *Methods Mol. Biol.* 801: 41–63.
- Jayapal, K. P., K. F. Wlaschin, W. S. Hu, and M. G. Yap. 2007. Recombinant protein therapeutics from CHO cells – 20 years and counting. *Chem. Eng. Prog.* 103: 40–47.
- Jesus, M. D., and F. M. Wurm. 2011. Manufacturing recombinant proteins in kg-ton quantities using animal cells in bioreactors. *Eur. J. Pharm. Biopharm.* 78: 184–188.
- Kang, J. X. and A. Leaf. 2007. Why the omega-3 piggy should go to market. *Nat. Biotechnol.* 25: 505–506.
- Karuppusamy, S. 2009. A review on trends in secondary metabolites from higher plants by *in vitro* tissue, organ and cell cultures. *J. Med. Plants Res.* 3: 1222–1239.
- Khan, K. H. 2013. Gene expression in mammalian cells and its applications. *Adv. Pharm. Bull.* 3: 257–263.
- Kim, J. Y., Y. G. Kim, and G. M. Lee. 2012. CHO cells in biotechnology for production of recombinant proteins: Current state and further potential. *Appl. Microbiol. Biotechnol.* 93: 917–930.
- Kochhar, H. P. S., G. A. Gifford, and S. Kahn. 2005. Regulation and biosafety issues in relation to transgenic animals in food and agriculture, feeds containing genetically modified organisms (GMO) and veterinary biologics. In *Applications of Gene-Based Technologies for Improving Animal Production and Health in Developing Countries*, H. P. S. Makkar and G. J. Viljoen, Eds., pp. 479–498, Springer- Netherlands.
- Kohler, G. and C. Milstein. 1975. Continuous cultures of fused cells producing antibodies of predefined specificity. *Nature* 256: 495–497.
- Koornneef, M. and D. Meinke. 2010. The development of *Arabidopsis* as a model plant. *Plant J.* 61: 909–921.
- Kwit, C., H. S. Moon, S. I. Warwick, and C. N. Stewart Jr. 2011. Transgene introgression in crop relatives: molecular evidence and mitigation strategies. *Trends Biotechnol.* 29: 284–293.
- Lai, L., J. X. Kang, R. Li, J. Wang, W. T. Witt, H. Y. Yong, Y. Hao, D. M. Wax, C. N. Murphy, A. Rieke, M. Samuel, M. L. Linville, S. W. Korte, R. W. Evans, T. E. Starzl, R. S. Prather, and Y. Dai. 2006. Generation of cloned transgenic pigs rich in omega-3 fatty acids. *Nat. Biotechnol.* 24: 435–436.
- Lankin, P. 2004. Somaclonal variation; origin and causes. In *Encyclopedia of Plant and Crop Science*, R. M. Goodman, pp. 1158–1161, Marcel Dekker, New York.

- Lazaris, A., S. Arcidiacono, Y. Huang, J. Zhou, F. Duguay, N. Chretien, E. Welsh, J. Soares, and C. Karatzas. 2002. Spider silk fibers spun from soluble recombinant silk produced in mammalian cells. *Science* 295(5554): 472–476.
- Ledford, H. 2013. Transgenic salmon nears approval. *Nature* 497 (7447): 17–18.
- Lee, O., A. Takesono, M. Tade, et al. 2012. Biosensor zebrafish provide new insights into potential health effects of environmental oestrogens. *Environ. Health Perspect.* 120: 990–996.
- Legler, J., J. L. M. Broekhof, A. Brouwer, P. H. Lanser, A. J. Murk, P. T. van der Saag, A. D. Vethaak, P. Wester, D. Zivkovic, and B. van Der. 2000. A novel *in vivo* bioassay for (xeno-) estrogens using transgenic zebrafish. *Environ. Sci. Technol.* 34: 4439–4444.
- Li, W., B. Hua, S. M. Saud, H. Lin, W. Hou, M. S. Matter, L. Jia, N. H. Colburn, and M. R. Young. 2014. Berberine regulates AMP-activated protein kinase signaling pathways and inhibits colon tumorigenesis in mice. *Mol. Carcinog.* doi: 10.1002/mc.22179.
- Lim, A. C., J. Washbrook, N. J. Titchener-Hooker, and S. S. Farid. 2006. A computer-aided approach to compare the production economics of fed-batch and perfusion culture under uncertainty. *Biotechnol. Bioeng.* 93: 687–697.
- Lipinski, C. and A. Hopkins. 2004. Navigating chemical space for biology and medicine. *Nature* 432 (7019), 855–861.
- Lipp, M. 2000. Validation of an immunoassay for the detection and quantification of Roundup-Ready[®] soybeans in food and food fractions by the use of reference material. *J. AOAC Intern.* 83: 99–127.
- Lloyd-Evans, L. P. M. 1994. Biotechnology-derived foods and the battleground of labelling. *Trends Food Sci. Technol.* 5: 363–367.
- Ma, B. L., R. E. Blackshaw, J. Roy, and T. He. 2011. Investigation on gene transfer from genetically modified corn (*Zea mays* L.) plants to soil bacteria. *J. Environ. Sci. Health B* 46: 590–599.
- Mallory-Smith, C. A. and E. Sanchez Olguin. 2011. Gene flow from herbicide-resistant crops: its not just for transgenes. *J. Agric. Food Chem.* 59: 5813–5818.
- Mandal, P. K., A. K. Biswas, K. Choi, and U. K. Pal. 2011. Methods for rapid detection of foodborne pathogens: An overview. *Am. J Food Technol.* 6: 87–102.
- Miao, X. 2013. Recent advances in the development of new transgenic animal technology. *Cell. Mol. Life. Sci.* 70: 815–823.
- Mercier, J. C. 1986. Exploiting new technologies. In *Animal Breeding*, C. Smith, J. W. B. King, and J. C. McKay, Eds., pp. 121–131, Oxford University Press, UK.
- Miller, P. D. and R. A. Morrison. 1991. Biotechnological applications in the development of new fruits and vegetables. In *Biotechnology and Food Ingredients*, I. Goldberg, and R. William, Eds., pp. 13–24, Van Nostrand Reinhold, New York.
- Miller, P. D., and J. A. Lauritis. 1989. DNA delivery by microprojectiles accelerated by a “vaccination gun”. *J. Cell. Biochem. Suppl.* 13D: M138.
- Muir, W. M. 2004. The threats and benefits of GM fish. *EMBO Rep.* 5: 654–659.
- La Mura, M., T. R. Allnut, A. Greenland, and L. D. Mackay. 2011. Application of QUIZ for GM quantification in food. *Food Chem.* 125: 1340–1344.
- Murai, N. 2013. Review: Plant binary vectors of Ti plasmid in *Agrobacterium tumefaciens* with a broad host-range replicon of pRK2, pRi, pSa or pVS1. *Am. J. Plant Sci.* 4: 932–939.
- Nam, Y. K., J. K. Noh, Y. S. Cho, H. J. Cho, K. N. Cho, C. G. Kim, and D. S. Kim. 2001. Dramatically accelerated growth and extraordinary gigantism of transgenic mud loach *Misgurnus mizolepis*. *Transgenic Res.* 10: 353–362.

- Nicolia, A., A. Manzo, F. Veronesi, and D. Rosellini. 2013. An overview of the last 10 years of genetically engineered crop safety research. *Crit. Rev. Biotechnol.* doi:10.3109/07388551.2013.823595.
- Noorden, R. V. 2010. Demand for malaria drug soars. *Nature* 466: 672–673.
- Nowak, R. 2002. Gene warfare to be waged on invasive fish. Accessed 21/07/04. Available from: www.newscientist.com.
- OECD. 2010. Safety Assessment of Transgenic Organisms: OECD Consensus Documents. OECD, Paris. Accessed 21/07/04. Available from: http://www.oecd.org/document/15/0,3746,en_2649_34385_37336335_1_1_1_1,00.html.
- Olempska-Beer, Z. S., R. I. Merker, M. D. Ditto, and M. J. DiNovi. 2006. Food-processing enzymes from recombinant microorganisms – a review. *Regul. Toxicol. Pharmacol.* 45: 144–158.
- Panda, R., H. Ariyaratna, P. Amnuaycheewa, A. Tetteh, S. N. Pramod, S. L. Taylor, B. K. Ballmer-Weber, and R. E. Goodman. 2013. Challenges in testing genetically modified crops for potential increases in endogenous allergen expression for safety. *Allergy* 68: 142–151.
- Peng, J., S. Shanshan X. Liguang, M. Wei, L. Liu, H. Kuang, and C. Xu. 2013. Development of a monoclonal antibody-based sandwich ELISA for peanut allergen ara h 1 in food. *J. Food Agr. Environ.* 11: 249–257.
- Peterson, D. G., E. A. Matitashvili, and D. E. Bauman. 2003. Diet-induced milk fat depression in dairy cows results in increased trans-10, cis-12 CLA in milk fat and coordinate suppression of mRNA abundance for mammary enzymes involved in milk fat synthesis. *J. Nutr.* 133: 3098–3102.
- Piecznik, G. 1999. Method and means for sorting and identifying biological information. US Patent 5866363.
- Primrose, S. B. 1987. *Modern Biotechnology*, pp. 137–159, Blackwell Scientific Publications, Oxford.
- Pullen, K. F., M. D. Jonson, A. W. Philips, G. D. Ball, and N. B. Finter. 1985. Very large scale suspension cultures of mammalian cells. *Dev. Biol. Stand.* 60: 175–177.
- Ramessar, K., T. Capell, R. M. Twyman, H. Quemada, and P. Christou. 2008. Trace and traceability – a call for regulatory harmony. *Nat. Biotechnol.* 26: 975–978.
- Rathore, K. S., S. Sundaram, G. Sunilkumari, L. M. Campbell, L. S. Puckhaber, S. Marcel, S. Palle, R. D. Stipanovic, and T. C. Wedegaertner. 2012. Ultra-low gossypol cottonseed: Generational stability of the seed-specific, RNAi mediated phenotype and resumption of terpenoid profile following seed germination. *Plant Biotechnol. J.* 10: 174–183.
- Rehbein, H. 2006. Genetic engineering of fish, and methods of detection. In *Genetically Engineered Food. Methods and Detection*, 2nd ed., K. J. Heller, Ed., Wiley-VCH Verlag GmbH, Weinheim, Germany.
- Rogan, G. J., Y. A. Dudin, T. C. Lee, N. S. Bhakta, J. N. Leach, P. R. Sanders, and R. L. Fuchs. 1999. Immunodiagnostic methods for detection of 5-enolpyruvylshikimatephosphate synthase in Roundup-Ready soybeans. *Food Control* 10: 407–414.
- Schafer, M. G., A. A. Ross, J. P. Londo, C. A. Burdick, E. H. Lee, S. E. Travers, P. K. Van de Water, and C. L. Sagers. 2011. The establishment of genetically engineered canola populations in the U.S. *PLoS ONE*. doi:10.1371/journal.pone.0025736.
- Seidel, G. E. Jr. 2000. Reproductive biotechnology and “big” biological questions. *Theriogenology* 53: 187–194.

- Shaner, D. L., R. B. Lindenmeyer, and M. H. Ostlie. 2012. What have the mechanisms of resistance to glyphosate taught us? *Pest Manag. Sci.* 68: 3–9.
- Sharma, H. and R. Mutharasan. 2013. Review of biosensors for foodborne pathogens and toxins. *Sens. Actuators B* 183: 535–549.
- Smetanska, I. 2008. Production of secondary metabolites using plant cell cultures. *Adv. Biochem. Eng. Biotechnol.* 111: 187–228.
- Stewart, C. N., Jr. 2006. Go with the glow: fluorescent proteins to light transgenic organisms. *Trends Biotechnol.* 24: 155–162.
- Tang, G., J. Qin, G. G. Dolnikowski, R. M. Russell, and M. A. Grusak. 2009. Golden Rice is an effective source of vitamin A. *Am. J. Clin. Nutr.* 89: 1776–1783.
- Taura, K., S. Yamamoto, S. Hayashi, S. Furusaki, and S. Shioya. 2014. A new bioreactor for paclitaxel production based on foam separation. *Asia-Pacific J. Chem Eng.* 9: 75–80.
- Thornton, C. R. and O. E. Wills. 2013. Immunodetection of fungal and oomycete pathogens: Established and emerging threats to human health, animal welfare and global food security. *Crit. Rev. Microbiol.* 39: 1–25.
- Walsh, G. 2010. Biopharmaceutical benchmarks. *Nat. Biotechnol.* 28: 917–924.
- Weathers, P. J., M. J. Towler, and J. Xu. 2010. Bench to batch: advances in plant cell culture for producing useful products. *Appl. Microbiol. Biotechnol.* 85: 1339–1351.
- Wei P. L., C. C. Tu, C. H. Chen, Y. S. Ho, C. T. Wu, H. Y. Su, W. Y. Chen, J. J. Liu, and Y. J. Chang. 2013. Shikonin suppresses the migratory ability of hepatocellular carcinoma cells. *J. Agric. Food Chem.* 61: 8191–8197.
- Wiersema, J. H. and B. León. 2013. *World Economic Plants: A Standard Reference*, 2nd ed., CRC Press, UK, pp. 1336.
- Willadsen, S. M. 1989. Cloning of sheep and cow embryos. *Genome* 31: 956–962.
- Xiumin, W., T. Da, G. Qingfeng, T. Fang, and W. Jianhua. 2012. Detection of Roundup Ready soybean by loop-mediated isothermal amplification combined with a lateral-flow dipstick. *Food Control* 29: 213–220.
- Yamamoto, H., P. Zhao, K. Yajaki and K. Inoue. 2002. Regulation of lithospermic acid B and shikonin production in *Lithospermum erythrorhizon* cell suspension cultures. *Chem. Pharm. Bull.* 50: 1086–1090.
- Yan L., K. Yan, W. Kun, L. Xu, Q. Ma, Y. Tang, W. Jiao, G. Gu, Y. Fang, and Z. Xu. 2012. Berberine inhibits the migration and invasion of T24 bladder cancer cells via reducing the expression of heparanase. *Tumour Biol.* 34: 215–221.
- Ye, X., S. Al-Babili, A. Klöti, J. Zhang, P. Lucca, P. Beyer, and I. Potrykus. 2000. Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* 287 (5451): 303–305.
- Zapiola, M. L. and C. A. Mallory-Smith. 2012. Crossing the divide: Gene flow produces intergeneric hybrid in feral transgenic creeping bentgrass population. *Mol. Ecol.* 21: 4672–4680.

Part III

Questions and Answers

1. What is the difference between traditional breeding and recombinant DNA technology (genetic engineering)?

Answer: Traditional breeding techniques involve mixing thousands of genes between only related species (crop to crop), which in addition to providing the beneficial trait, also result in the loss of other traits of that crop considered to be valuable. Significant time and effort, that may take longer than 10 years, is required to restore the other valued traits of that crop while maintaining the desired trait using traditional breeding techniques. Recombinant genetic modification permits food developers to add or enhance useful traits more specifically and precisely or remove undesirable traits from any organism (human gene to bacteria, etc) within very short time.

2. What are genetically modified (GM) organisms and GM foods?

Answer: Genetically modified organisms (GMOs) can be defined as organisms in which the genetic material (DNA) has been altered in a way that does not occur naturally. The technology is often called “modern biotechnology” or “gene technology”, sometimes also “recombinant DNA technology” or “genetic engineering”. It allows selected individual genes to be transferred from one organism into another, also between non-related species. Such methods are used to create GM plants – which are then used to grow GM food crops.

3. Why are GM foods produced and what are their benefits?

Answer: GM foods are developed and marketed because of some perceived benefits either to the producer or to the consumer of these foods with a lower price, greater benefit (in terms of durability or nutritional value), or both. The GM crops currently on the market are mainly aimed at an increased level of crop protection through the introduction of resistance against plant diseases caused by insects or viruses or through increased tolerance toward herbicides. Insect resistance is achieved by incorporating into the food plant the gene for toxin production from the bacterium *Bacillus thuringiensis* (Bt). This toxin is currently used as a conventional insecticide in agriculture and is safe for human consumption. GM crops that permanently produce this toxin have been shown to require lower quantities of insecticides in specific situations, for example, where pest pressure is high. Virus resistance is achieved through the introduction of a gene from certain viruses which cause disease in plants.

Virus resistance makes plants less susceptible to diseases caused by such viruses, resulting in higher crop yields. Herbicide tolerance is achieved through the introduction of a gene from a bacterium conveying resistance to some herbicides. In situations where weed pressure is high, the use of such crops has resulted in a reduction in the quantity of the herbicides used.

4. Are GM foods assessed differently from traditional foods?

Answer: When new foods are developed by natural methods, some of the existing characteristics of foods can be altered, either in a positive or in a negative way, but new plants developed through traditional breeding techniques may not be evaluated rigorously using risk-assessment techniques. Specific systems have been set up for the rigorous evaluation of GM organisms and GM foods relative to both human health and the environment. Hence, there is a significant difference in the evaluation process before the marketing process begins for these two groups of food.

5. How are the potential risks to human health determined?

Answer: The safety assessment of GM foods generally investigates: (i) direct health effects (toxicity); (ii) tendencies to provoke allergic reaction (allergenicity); (iii) specific components thought to have nutritional or toxic properties; (iv) the stability of the inserted gene; (v) nutritional effects associated with genetic modification; and (vi) any unintended effects which could result from the gene insertion.

6. What are the main issues of concern for human health?

Answer: The three main issues debated are tendencies to provoke allergic reaction (allergenicity), gene transfer, and outcrossing.

Allergenicity: While traditionally developed foods are not generally tested for allergenicity, protocols for tests for GM foods have been evaluated by the Food and Agriculture Organization of the United Nations (FAO) and WHO. No allergic effects have been found relative to GM foods currently on the market.

Gene transfer: Gene transfer from GM foods to cells of the body or to bacteria in the gastrointestinal tract would cause concern if the transferred genetic material adversely affects human health. This would be particularly relevant if antibiotic resistance genes, used in creating GMOs, were to be transferred. Although the probability of transfer is low, technology, which is called homologous integration, without antibiotic resistance genes has been used.

Outcrossing: The spread of genes from GM plants into conventional crops or related species in the wild as well as the mixing of crops derived from conventional seeds with those grown using GM crops may have an indirect effect on food safety and food security. Several countries have adopted strategies to reduce mixing, including a clear separation of the fields within which GM crops and conventional crops are grown.

Feasibility and methods for post-marketing monitoring of GM food products, for the continued surveillance of the safety of GM food products, are under discussion.

7. How is a risk assessment for the environment performed?

Answer: Environmental risk assessments cover both the GMO concerned and the potential receiving environment. The assessment process includes evaluation of the characteristics of the GMO and its effect and stability in the environment, combined with ecological characteristics of the environment in which the introduction

will take place. The assessment also includes studying unintended effects that could result from the insertion of the new gene.

8. What are some issues of concern for the environment?

Answer: The capability of the GMO to escape and potentially introduce the engineered genes into wild populations; the persistence of the gene after the GMO has been harvested; the susceptibility of non-target organisms (e.g., insects which are not pests) to the gene product; the stability of the gene; the reduction in the spectrum of other plants including loss of biodiversity; and increased use of chemicals in agriculture. The environmental safety aspects of GM crops vary considerably according to local conditions. Current investigations focus on: the potentially detrimental effect on beneficial insects or a faster induction of resistant insects; the potential generation of new plant pathogens; the potential detrimental consequences for plant biodiversity and wildlife, and a decreased use of the important practice of crop rotation in certain local situations; and the movement of herbicide resistance genes to other plants.

9. Are GM foods safe?

Answer: Individual GM foods and their safety should be assessed on a case-by-case basis and it is not possible to make general statements on the safety of all GM foods. GM foods currently available on the international market have passed risk assessments and are not likely to present risks for human health. In addition, no effects on human health have been shown as a result of the consumption of such foods by the general population in the countries where they have been approved. Continuous use of risk assessments based on the Codex principles and, where appropriate, including post market monitoring, should form the basis for evaluating the safety of GM foods.

10. How are GM foods regulated nationally?

Answer: In some countries, GM foods are not yet regulated. Countries that have legislation in place focus primarily on assessment of risks for consumer health. Countries that have provisions for GM foods usually also regulate GMOs in general, taking into account health and environmental risks, as well as control- and trade-related issues (such as potential testing and labelling regimes). In view of the dynamics of the debate on GM foods, legislation is likely to continue to evolve.

11. What kind of GM foods are on the market internationally?

Answer: All GM crops available on the international market today have been designed using one of the three basic traits: resistance to insect damage; resistance to viral infections; and tolerance toward certain herbicides. All the genes used to modify crops are derived from microorganisms.

12. What happens when GM foods are traded internationally?

Answer: No specific international regulatory systems are currently in place. However, several international organizations are involved in developing protocols for GMOs. The Codex Alimentarius Commission (Codex) is the joint FAO/WHO body responsible for compiling the standards, codes of practice, guidelines and recommendations that constitute the Codex Alimentarius: the international food code. Codex is developing principles for the human health risk analysis of GM foods. The Cartagena Protocol on Biosafety (CPB), an environmental treaty legally binding for its

Parties, regulates transboundary movements of living modified organisms (LMOs). Each country has its own regulatory systems.

13. Have GM products on the international market passed a risk assessment?

Answer: The GM products that are currently on the international market have all passed risk assessments conducted by national authorities. These different assessments in general follow the same basic principles, including an assessment of environmental and human health risk. These assessments are thorough and they have not indicated any risk to human health.

14. Why has there been concern about GM foods among some politicians, public interest groups and consumers, especially in Europe?

Answer: Consumers were generally not very aware of the potential of this new technology. In the case of food, consumers started to wonder about safety because they perceive that modern biotechnology is leading to the creation of new species. Where medicines are concerned, many consumers more readily accept biotechnology as beneficial for their health (e.g., medicines with improved treatment potential). In the case of the GM foods that we consume, the products were of no apparent direct benefit to consumers (not cheaper, no increased shelf life, no better taste). Consumers have questioned the validity of risk assessments, both with regard to consumer health and environmental risks, focusing in particular on long-term effects. Other topics for debate by consumer organizations have included allergenicity and antimicrobial resistance. Consumer concerns have triggered a discussion on the desirability of labelling GM foods, allowing an informed choice. At the same time, it has proved difficult to detect traces of GMOs in foods, as very low concentrations often cannot be detected by the PCR technique.

15. What is termination technology of GM seeds?

Answer: Terminator technology is the genetic modification of plants to make them produce sterile seeds. They are also known as suicide seeds. Terminator's official name – used by the United Nations and scientists – is Genetic Use Restriction Technologies (GURTs). Although Terminator seeds are not yet being sold, in 2007 biotech companies with the support of the US Department for Agriculture were conducting greenhouse tests for future commercialization.

This technology is a cause of concern for peasant farmers throughout the developing world because they would no longer be able to save seeds to reuse from one harvest to the next. Many poor farmers cannot afford to buy seeds each year. Instead, they save, swap, and share seeds that have been developed over generations. If farmers have no choice but to buy new seeds every year, the companies are guaranteed large profits at the expense of poor farmers' food security. This technology was developed so that sterile seeds cannot spread into the environment, and therefore biosafety would be ensured, but the GM terminator genes would themselves contaminate the non-GM crops, meaning that these non-GM crops would produce sterile seeds and would no longer be GM-free. Terminator technology could spell the end of locally adapted agriculture that could diminish the range of local and native seeds that are fundamental to local food systems. Having fewer and fewer companies in charge of the global seed supply has serious implications for global food security. The world's top four seed companies – Monsanto, DuPont, Syngenta, and Group Limagrain accounted for half of the global seed market. The seed corporations realized that the enforcement of intellectual property rights on seeds was costly and difficult.

Having a self-enforcing biological way to do this would be a much more effective way for seed corporations to protect and gain from their intellectual property.

16. Do you support genetically modified food or advocate against it? Give reasons for your choice.

Answer: Class discussion agenda.

Index

- a-crystallin domain (ACD), 91–2
accessory gland proteins (Acps), 106
acesulfame-K, 329, 335–9
acetate, 23–7, 56–71, 241–62, 290–6,
342–51, 467–83
acetic acid, xiv, 40–50, 265–7, 281–7,
342, 366–72
Acetobacter, 282–7, 298–306, 353–6, 366
acetoin, 243, 251–2, 342–51
acetolactate decarboxylase xiv,
acetyl-CoA, 32–6, 248–9
acetylcholinesterase (AChE), 114, 116,
458–64
N-acetylglucosaminyltransferases, 179
Achromobacter genus, 322–9
Acinetobacter genus, 289–96
Actinobacteria genus, 241, 266–70,
370–1
Actinomyces genus, 171, 241, 288–96,
386
active yeasts, 207–8, 234–7
acylhomoserine lactones
(AHLs), 119–20
adenosine diphosphate (ADP), 79
adenosine triphosphate (ATP), 10,
15–19, 50–53, 66, 77–9,
89–93, 116, 148–91, 249–62,
376–83
adenoviruses, 175–9
adsorption, 43–50, 63–4, 65–71, 84,
95–6, 109–16, 184–90
Aequorea victoria, 255–62
Aerobacter genus, 13, 302–6
Aeromonas genus, 274–80
Agaricus bisporus, 360–3
agarose, 95–6, 169–79, 184–90, 204
agricultural biotechnology, xvii, 37–8,
100, 105–6, 108, 109–10, 120,
121–2, 133–4, 135–9, 202,
255–62, 288–96, 323–9,
433–48, 451–2, 464–83
Agrobacterium genus, 171–2, 298–306,
439–48, 477–83
agrochemicals, 135–9
AIDS, 11, 138
air inlets, fermentor systems, 39–50,
72–84
air-lift bubble-column reactors, 41, 197,
455–8
alanine, 51–3, 294–6, 348–51, 355–6
alarm systems, computer
applications, 79, 81, 84, 197
albumin, 131–9, 178–9
Alcaligenes, 273–80, 288–96, 298–306
alcoholic beverages, xiii, xiv, 20–1, 37,
40–50, 195, 207, 212–37,
314–15, 319–29, 380
aldehydes, 24–7, 44–5, 245–62, 282–7,
344–51
alfalfa, 443–8
algae, 6, 82–3, 194, 211, 287–306, 352–4,
356–9, 467–83
alginate, 45, 63, 127–9, 131–9, 296–9,
300–6
alitame, 335–9
alkaline phosphatase, 46–50, 173
alkanes, 23–7, 56–71, 125–9, 288–96
alkylating agents, 25–7

- allantoin, 293–4
alleles, 27–30
allergens, 106, 112–13, 137–9, 388–97,
446–8, 452–3, 463–4, 470–2,
479–83
allergic rhinitis, 389, 393–4
aluminium, 370–1
Alzheimer's disease, 89, 278, 341
amines, 222–3, 247–9, 265–7, 269–70
amino acids, xiii, xiv, 7–21, 22–7, 37–93,
96–100, 105–8, 110–16,
153–61, 173–9, 196–204,
209–37, 247–9, 275–80,
326–9, 339, 348–51, 384,
468–83
aminoacylase, 46–50, 158–61
aminobutyric acid, 279, 374–83
aminoglycoside antibiotic
(Kanamycin), 29
aminopenicillanic acid, 13, 48–50
aminopeptidase, 255–62
aminopurine, 25–7
ammonia, 48–50, 282, 301–6
amperometric transducer
sensors, 111–16, 200–1
ampicillin, 165, 167–8, 172–9, 187,
188–90
amplification, 99–100, 182, 185, 190
amylases, 18–19, 23–7, 46–50, 129,
216–37, 243–62, 313–29,
330–9, 395–7
amyloids, 92–3, 199, 278, 341–51
anaerobic bacteria, 12–13, 18–19,
241–62, 288–96, 386–97
anaerobic vat reactors, 40–50, 54–5, 78,
197
anchovies, 268, 270
anemia, 89, 452–3
animal biotechnology, xii, 3–6, 8–11,
39–50, 82–3, 103, 171–2,
176–9, 184–90, 193–4,
255–62, 340–51, 380–3,
449–83
animal cells, 8–11, 39–50, 82–3, 103,
171–2, 176–9, 184–90,
193–4, 255–62, 315–29,
340–51, 449–64, 473–83
animal feeds, 290–6, 314–29, 349–51,
356–9, 380–3, 396–7, 449–64,
468–83
anthocyanidins, 385
anthracycline, 23–7
anthrax, 114, 179
anti-inflammatories, 121–2, 447–8
antibiotic resistant bacteria, 108,
118–20, 165–8, 172–9, 182,
189–90, 199, 203–4, 253–62,
372–3, 378, 397, 468–83
antibiotics, xiii, xiv, xvii, 13–19, 20–1, 27,
28, 29, 31–36, 57–8, 62–71,
72–84, 108, 110–20, 124–9,
137–9, 165–8, 199, 243,
253–62, 326, 381–3, 468–83
antibodies, xiv, xvii, 3–6, 36–7, 82–4, 96,
109–16, 134–9, 162–91,
193–4, 200, 442–3, 447–8,
457–64, 477–83
anticancer agents, 3, 4, 5–6, 32, 121–2,
131–9, 178–9, 193–4, 222–4,
242–3, 275–6, 360–1, 370–2,
385–97, 434–5, 442–3, 447–8,
457–64
anticoagulant antithrombin, 4–5, 193–4,
278, 302–3, 325, 451–2, 454,
457–8, 483
antifoam agents, 52–3, 77–84
antifreeze proteins, 255–62
antifungals, 124–9, 135–9
antigens, 109–16, 179, 457–64
antilisterian peptides, 375–83
antimalarial drugs, 32, 34–6, 196–7
antimicrobials, 108, 121–2, 129, 254–62,
374–83, 397
antioxidants, 121–2, 129, 222–4, 235–7,
275–80, 352–9, 360–3,
369–72, 384–97, 464–5
antisense RNA technology, 256–62,
440–8
aquaculture industry, 4, 359, 380–3,
452–3, 474–83
aqueous–alcoholic mediums, 248–9
aqueous–aqueous
concentration/purification
methods, 62–4, 83–4

- aqueous-organic
 concentration/purification
 methods, 62-4, 83-82-3
- arabinose, 219-22, 229-32
- Archaea organism domain, 6-11, 91-2,
 154, 194, 373, 392
- archaeosomes, 131-9
- arginine, 23-7, 222-4, 258-62, 294-6
- Arrhenius law, 17-18, 50-53, 68-8, 88
- Artemisia annua*, 34-6, 196
- artemisinin antimalarial, 34-6, 196-7
- Arthrobacter* genus, 13, 23-7, 241,
 303-6, 327-9, 332-9, 353-6
- artichokes, 226-32, 385
- Ascomycetes*, 19-21, 354-6, 370
- Ashbya* genus, 20-1, 354-6
- asparaginase, 44-5, 325-9, 349-51,
 468-83
- asparagine, 51-3, 325-9
- aspartame, 329, 334-9
- aspartase, 64-71
- aspartate, 22-7, 348-51
- aspartic acid, 48-50, 294-6, 334-9,
 348-51
- aspartokinase, 24-7, 196
- aspartyl phosphate, 24-7
- Aspergillus* genus, 20-1, 28-30, 48-50,
 177-9, 211-12, 223-4, 249,
 271-80, 315-29, 367-9, 468
- Aspergillus niger*, 48-50, 70-71, 178-9,
 186-90, 218, 281-7, 290-6,
 319-29, 330-9, 341-51
- Aspergillus oryzae*, 177-9, 186-90,
 210-11, 218, 275-80, 285-7,
 320-9, 346-51
- aspirin, 121-2
- asthma, 389, 393-4
- autolysis techniques, 62, 209-11, 222,
 256-62, 351
- autonomous replicating sequence
 (ARS), 167-79
- avian flu, 116
- Azotobacter* genus, 13, 19, 43-4,
 298-306, 352-4
- B-cells, 4, 179, 394
- baby hamster kidney cells (BHK), 3-6,
 178-9, 453-64
- Bacillus* genus, 9-11, 13, 18-19, 23-7,
 29-30, 64, 167-8, 223-4,
 250-62, 269-70, 271-80,
 298-306, 315-29, 353-9,
 442-8
- Bacillus subtilis*, 23-7, 117, 155-6,
 158-61, 165-8, 171-2, 174-9,
 255-62, 271-80, 327-9,
 330-9, 347-51, 355-6,
 381-3, 468
- backmixing, 40-50
- bacterial artificial chromosomes
 (BACs), 167-8
- bacterial families, 11-13
- bacteriocins, xi, xii, 243-62, 266-7,
 372-83
- bacteriophages, 129, 162-79, 199,
 243-4, 254-8, 259-62, 350,
 372-3, 382-3, 386-97
- baculoviruses, 175-9
- baker's yeast, xiii, xiv, 20-1, 62, 207-12,
 232-7, 304, 350-1
- baking processes, xiii, xiv, 235-7, 287,
 301, 328
- Basidiomycetes* genus, 19-21, 357-9
- batch cultures, 14-19, 39, 40-50, 54-71,
 72-84, 190, 194-5, 230-7,
 301, 355, 457-8
- batch sterilization, 68-8
- batch-stirred immobilized-enzyme/cell
 reactors, 45-50, 457
- benzaldehyde, 344-51
- benzene, 96-100
- benzoylarginine ethyl ester, 49-50
- beta carotene, 20-1, 131-9, 356-7,
 385-97, 445, 473
- Bifidobacterium* genus, 13, 129, 204,
 241-62, 266-70, 356, 381-3,
 384-97
- bile salt hydrolase (BSH), 127-9, 386,
 391-7
- binary fission replication, 7-11, 12-19,
 20-1, 147-61
- biocatalysts, 14-19, 20-1, 42-50, 100,
 121-39, 313-29
- biochemical-oxygen-demand
 (BOD), 83-4, 290-6, 369
- biochips, 134-9, 200-1

- biodiesels, 32
biofilms, 116–20
bioflavors, 339–51
biofuels, 31, 85, 315, 323–9
biogas, 37–8
biogenic amines, 269
biogums, 11, 254–62
bioinformatics, xi, 35–6, 100–108, 196, 199–200
biolistic method, 441–8
biological containment
 techniques, 481–3
biological measurements, scale-up
 processes, 78–84
biological value (BV), 293–6
biological-element component of
 biosensors, 109–16, 200
bioluminometers, 79–84
biomarkers, 106–8
biomass, 40–50, 55–71, 78–84, 85, 195, 197, 211, 226–32, 281, 287–96, 324, 468–83
biopharmaceuticals, 3–6, 10–11, 32–6, 64–71, 82–4, 85–93, 94–6, 100, 105–6, 109–16, 120–39, 193–204, 212, 254–62, 314–97, 434–64
biopolymers, 37–8, 58–71, 122, 296–306
bioreactors, 3–6, 39–50, 53–84, 197, 231–2, 455–64
biosensors, xi, xvii, 5–6, 11, 49–50, 77–84, 109–16, 134–9, 200–2, 453–4
bioseparations, 58–71, 78–84, 93–6, 98–100, 122, 123–9, 162–91, 197, 209–37, 327–9
BioSteel, 4, 193, 452
biosynthetic pathways, 22–30, 79, 82–4, 106, 196, 340–51, 355–6, 377–8
biotechnological engineering *see*
 bioengineering...
biotechnology concepts, xiii, xiv, xv, 3–145, 147–91, 193–204, 207–37, 241–306, 313–97, 411–29, 433–83, 491–5
bioterrorism, 110, 135, 136, 179
biotin (vitamin H), 23–7, 110, 169–79, 346–51, 352, 355–6, 473, 478–83
biotransformation processes, 340–51, 447–8
Blakeslea, 20–1, 356–9
blood biosensors, 109–16
blood pressure, 278–80, 370–2
blood product substitutes, 4–5
blotting and hybridization DNA
 manipulations, 103–4, 173, 185–90
blue cheeses, 211–12, 242, 340–1
bottom-fermenting yeasts, 217–18
botulins, 13–19, 113
bound immobilized-enzyme/cell
 reactors, 42–3
bovine chymosin (rennet) cloning and
 expression, 188–90, 203, 261–2
bovine mastitis, 381
bovine pancreatic trypsin inhibitor
 (BPTI), 91–2
bovine somatotropin (BST), xiv, 451–2, 473–83
bovine spongiform encephalopathy
 (BSE), 89, 178–9
bowl centrifugation, 61–2
breast cancer, 275, 370, 385, 434–5, 447
Brevibacterium genus, 23–7, 29–30, 246–62, 290–6, 349–51, 353–9
brewing processes, xiii, xiv, 20–1, 40–50, 63, 207, 212–37, 319–29, 467–8
brewing yeast, xiv, 20–1, 126–7, 207, 212–37, 467–8
Brochothrix genus, 379–83
bromelain, 125–9, 315–29
bubble-column reactors, 40, 41–50, 197, 455–6
budding cell division, 10–12, 20–1
buds, plant biotechnology, 435–48
bulk chemical products, 37–85
butanediol (BDO), 82–3
butanol, xiv, 13–19, 37, 82–3
buttermilk, 244–62, 341–51
butyl acetate, 50–53

- butyric acid, 13–19, 279, 343–4, 374–83
by-products, 38–9
- C-terminus of the protein, 88–9,
253–62, 374–83
- cake yeasts, 208, 234–7
- calcium, 18–19, 29–30, 43–50, 278,
282–7, 385–97
- callus, 435–48
- Camembert cheese, 211–12, 242, 247–9,
250–2
- cancer, 5–6, 24, 32, 103–6, 116, 121–2,
131–9, 178–9, 193–4, 222–4,
242–3, 275–6, 280, 333–4,
342, 360–1, 370–2, 385–97,
434–5, 442–3, 447–8, 452–3,
456–64
- cancer detection kits, 5–6, 116, 134–5,
193–4, 442–3
- Candida* genus, 21, 100, 207, 209,
288–96, 321–9, 352–63,
42–51
- canola/rapeseed, 136–7, 443–8, 469–72
- carbon nanotubes (CNTs), 114–16,
133–9
- carboxyl, 7–8, 44–5, 159–61, 246–62
- carboxymethylcellulose, 122–39,
297–306
- Carnobacterium* genus, 380–3
- carotenoids, 211, 356–7, 385–97, 469–83
- carrageenan, 45, 296–306
- carrier-mediated membrane
transport, 8–11
- catabolite activator protein
(CAP), 153–61
- catechins, 131–9, 370–2, 385–97
- cDNA, 97–8, 102–8, 161–79, 189–90,
203–4, 261–2, 451
- cell cultures, xvii, 3–6, 13–21, 31–6,
80–84, 194–5, 434–48,
453–64, 472–4
- cell walls, 10–11, 171–2, 194, 211,
260–2, 360–3, 376–83, 393–7,
435–48, 455–6
- cellulases, 23–7, 29–30, 229–32, 313–29
- Cellulomonas* genus, 13, 288–96,
298–306
- cellulose, 43–50, 60–71, 122–39,
186–90, 226–32, 264–7,
289–96, 297–306, 313–29
- cellulose cell walls, 10–11, 171–2, 194,
435–48
- centrifugal extrusion, 123, 126–9,
169–79
- centrifugation separation
methods, 58–71, 123, 126–9,
169–79, 184–90, 215–18,
220–2
- Cephalosporium* genus, 21, 28–30,
288–96
- Chaetomium cellulyticum*, 288–96
- chain termination (Sanger
sequencing), 180–1, 200
- chaperons, 87–92, 198–9
- charcoal, 224–5
- Cheddar cheese, 242, 245–9, 256–62,
343–4
- cheese, xiii, xviii, 13, 21, 71, 124–5, 129,
188–90, 194, 210–12, 227–8,
241–62, 267, 288–96, 301–6,
319–29, 340–1, 343–51, 385,
467–83, 318–29, 340–1,
343–4, 348
- chewing gum, 122, 343–4
- chinese hamster ovary cells
(CHO), 3–6, 177–9, 456–64
- chitin, 20–1, 184–90, 194, 360–3
- chitosan, 116, 122–39, 298–306
- Chlamydomonas*, 357–9
- Chlorella*, 290–6, 352–4, 357–9
- chlorophyll, 20, 469–83
- chloroplasts, 9–11, 90–93, 150–61
- chocolate, 385
- cholesterol, 8–11, 110–16, 222–4,
242–3, 255–62, 278, 370–2,
384–9, 391–7, 446–8
- chromatin, 10–11, 156–61
- chromatography, 65–6, 67–71, 84, 85,
92, 169–79, 189–90, 198–9,
201, 296, 462–4
- chromosomes, concepts, 6–11, 20–1,
25–7, 34–6, 147–91, 254–62
- chymosin, xviii, 5–6, 70–71, 188–90,
203, 252, 261–2
- chymotrypsin, 44–5

- citrate, 249–62, 342
 citric acid, xiv, 20–1, 37, 283–7
Citrobacter, 353–6
 clarification, 46–50, 210–11, 220–3, 296
 class I/II/III/IV bacteriocins, 373–83
 classification systems, 6–13, 18–21,
 89–93, 241–2, 373–5
Clavispora, 229–32
 cloned livestock, 4–5, 193–4
 cloned pets, 4, 193–4
Clostridium genus, 13, 19, 229–32,
 259–62, 274–80, 350–1,
 379–83
 coacervation techniques, 131–9
 coagulation, cheese-making
 processes, 245–9, 260–2
 cocoa, 21, 364–6, 385, 448
 coffee, 21, 63, 364, 367–9
 colicin-like bacteriocins, 373–83
 collagen, 8–11, 131–9, 264–7, 326
 colon cancer, 103–5, 342, 385, 447–8
 Colorado beetle, 443
 colorants, 20–1, 128–9, 135–9, 211,
 264–7, 352, 356–9, 446–8,
 464–83
 computer applications, 75, 79–84,
 96–100, 102, 106–8, 197, 199,
 327–9, 451
 conjugation mechanism in
 bacteria, 28–30, 116–20,
 148–61, 253–62
 Consultative Group on International
 Agricultural Research
 (CGIAR), 433
 containment techniques, 481–3
 contaminants, 3–6, 11, 16–17, 68–71,
 81–4, 92–3, 109–16, 118–20,
 136–9, 194–5, 199, 229–32,
 259–62, 266–70
 continuous cultures, 15–19, 39, 40–50,
 54–71, 79–84, 194–5, 457–8
 continuous stirred-tank reactors
 (CSTRs), 45–50, 231–2,
 457–8
 convection cooling, 236–7
 cooked sausages, 264–7
 cooling, scale-up processes, 75–84,
 235–6
 copper, 13, 138
 corals, 8–9
 core enzymes, 154–61
 corn syrups, 329–30, 331–9
Corynebacterium genus, 13, 22–7, 196,
 344–51, 353–6, 175–9, 196,
 344–51
 Costa Rica, 443–4
 cottage cheese, 242, 249–52
 cream yeasts, 208, 234–5
 creatinine, 48–50
 Creutzfeldt–Jakob disease (CJD), 11,
 89, 178–9
 cRNA, 103–8
 Crohn's disease, 179, 392
 crop improvements, 105–6, 108, 147,
 442–8, 469–72, 475–83
 crystallization step, 93–6, 92, 93–6,
 340–51
 cucumbers, 272–80, 348, 443–8
 curcumin, 131–9
 curdlan, 296–8, 303–6
 curds, cheese-making processes, 245–9
 cyanide poisoning, 326
 cyclic adenosine monophosphate
 (cAMP), 157–61, 346–51,
 376
 cyclonic reactors, 45–50
 cysteine, 210–11, 348–51
 cystic fibrosis, 119, 452–3
 cytokines, 4–5, 193–4, 360–3, 393–7
 cytoplasm, 6–11, 17–19, 20–1, 89–93,
 150–90, 376–83, 438–9
Dacrymyces deliquescens, 357–9
 daidzein, 275–80
 dairy products, xiii, xviii, 13, 71, 124–5,
 127–9, 186, 193–4, 200–1,
 210–11, 241–62, 300,
 319–29, 340–51, 380–3,
 389–97, 451–64, 467–83
 databases, 35–6, 98–100, 102–8,
 162–91, 199, 451
 dehydration, 245–9, 263
 dehydrogenases, 44–5, 64–71, 282–7,
 342–51
 dental health, 338, 382, 392–3
 deodorization, 111, 128–9

- deoxyadenosine triphosphate
(dATP), 148–61, 168–79
- deoxycytidine triphosphate
(dCTP), 148–61, 168–79
- deoxyguanosine triphosphate
(dGTP), 148–61, 168–79
- deoxynucleotide triphosphates
(dNTP), 169–79, 180–90,
204
- deoxythymidine triphosphate
(dTTP), 148–61, 168–79
- detergents, 89, 100, 184–90, 314–29
- dextrans, 63–71, 243–62, 298–9, 301–6
- dextrinization, 46–50
- dextrins, 215–37
- dextrose, 329–39
- DHA omega-3 fatty acids, 82, 131–9,
385–97, 452–3
- diabetes, 5, 10–11, 110, 135–6, 194, 200,
325, 333–9, 452–3
- diacetate dehydrogenase, 64–71, 217
- diagnostics applications, 5–6, 32–6,
64–71, 106, 109–16, 457–64
- diesel fuels, 137, 138, 225–32
- dietary supplements, 32, 383–97, 464–83
- digestion, 21, 83–4, 97–100, 184–90,
210–37, 293–6, 321–9, 342,
370–2, 384–97, 451–64
- digital set point computer controls
(DSC), 79–81
- diglycerides, 248–9
- diploid cells, 9–11, 12, 17, 20–1, 27,
28–30, 456–64
- direct digital computer controls
(DDCs), 77, 79–84
- direct vat inoculation (DVI), 257–62,
267
- direct vat set (DVS), 257–62
- direct-charge transfers (DCTs), 114, 116
- directed evolution protein
engineering, 97–100, 326–9
- dissolved oxygen (DO), 18–19, 75–84,
127–9
- distillation separation methods, 61–2,
230–2, 340–51
- DNA, xi, xii, xiv, xv, xvii, xviii, 6–11,
12–19, 24–30, 33–5, 77–84,
87–93, 96–100, 101–8,
112–13, 136–9, 147–91,
195–204, 253–62, 314, 326–9,
437–48, 449–64, 467–83
- DNA cleavage, 161–79, 180–90, 203–4
- DNA damage, nanoparticles, 138
- DNA gyrase, 148–91
- DNA helicase, 148–91
- DNA ligases, 148–60, 161–90, 203–4
- DNA microarrays, 101–8, 200
- DNA Plant Technology
Corporation, 441
- DNA polymerases, 148–91, 199, 204,
261–2, 326–9
- DNA replication, 147–61, 164–8
- DNA-based testing methods, 478–83
- DNA–RNA hybrids, 156–61, 169–79
- DNA-sequencing techniques, 34–6,
101–8, 136, 147–90,
199–200, 203–4, 255–62,
376–8, 478–83
- Dolly the sheep, 449–50
- downstream (product recovery)
processes, 37–9, 50–53,
57–71, 83–4, 85, 93–6, 175,
189–90, 196–7, 223–5,
256–62, 301–2, 318–29
327–9
- Drosophila melanogaster*, 177–9
- drug delivery systems, 120–39
- drying finishing operations, 60–61–2,
68–71, 78–84, 93–6, 254–62,
301, 318–29
- dual polarization interferometry, 102–8
- duodenal ulcers, 392
- dyes, 26–7, 102–8, 122–9, 185–6, 193,
434–5, 448
- eczema, 389, 393–4
- egg white, 255–62, 299–300
- eggplant, 469–70
- electrochemical biosensors, 111–16
- electrodes, 47–50, 77–84
- electrodialysis separation
methods, 61–2
- electrofusion of protoplast cells, 29–30
- electronic component of
biosensors, 109–16, 200

- electronic nose devices (e-nose),
 biosensors, 111–16, 136–9,
 201–2
- electrophoresis, 107–8, 169–79, 180–90
- electroporation, 30, 171–9, 254, 450–64,
 467–83
- Embden–Meyerhof pathway
 (EM), 212–13
- embryos, 3–6, 171–2, 449–64
- emulsification techniques, 131–9,
 208–37, 301–6
- endo/exonucleases, 46–50, 148, 161–91,
 346–51
- endoglucanase, 255–62
- Endomyces vernalis*, 289–96
- endopeptidases, 243, 250–2
- endoplasmic reticulum (ER), 9–11,
 20–1, 90–93
- enrichment techniques, strain
 improvement and
 tools, 22–7, 169–79, 459–64
- Enterobacter* genus, 271–80, 304–6,
 345–51
- entrapment techniques,
 biosensors, 109–16
- entrapped immobilized-enzyme/cell
 reactors, 42–3
- entropy, second law of
 thermodynamics, 85–93,
 128–9
- environmental bacterial growth
 factors, 16–19, 39, 40–50,
 72–84, 195, 265–7
- environmental health and safety
 (EHS), 137–9
- Enviropig, 4, 193–4, 452–3, 474–5
- enzyme-linked immunosorbant assay
 (ELISA), 109–10, 112–13,
 186–90, 463–4, 475–83
- enzymes, xi, xiii, xiv, xv, xvii, xviii, 3–19,
 20–1, 31–85, 96–100, 105–8,
 109–16, 120, 121–2, 124–9,
 148–91, 193–204, 209–37,
 243–62, 268–70, 298–306,
 313–29, 340–51, 352–9,
 375–83, 446–8, 457–64,
 467–83
- EPA omega-3 fatty acids, 385–97, 452–3
- epigallocatechin gallate
 (EGCG), 131–9, 370–2
- Erwinia* genus, 353–4, 367–9
- erythropoietin commercial hormone, 5,
 178–9, 193–4, 442–3
- Escherichia coli*, xiv, 3–6, 14–19, 22–7,
 32–6, 43–50, 64, 67, 69–71,
 81–4, 90–91, 98–100, 110,
 112–13, 114–16, 150, 152–9,
 161, 163–79, 183, 186–90,
 193–6, 204, 211, 229–32,
 253–62, 295, 320–29, 336,
 341, 349–51, 355, 358–9,
 380–3, 447–8, 463–4, 468
- esterases, 243–62, 319–29
- esters, 243–62, 319–29, 343–4, 447–8
- estrogen, 275–80, 453
- ethanol, xiv, 21, 37–8, 42–50, 55–71,
 212–37, 265–7, 281–7,
 288–96, 314–29, 342–51,
 366–72, 467–83
- ethics, xviii, 4, 483
- ethidium bromide, 96–100, 204
- ethyl, 25–7, 184–90, 209–37, 343–4,
 351
- ethylcellulose, 122–39
- ethylene, 49–50, 226–32
- ethylenediamine tetraacetic acid
 (EDTA), 455
- eukaryotes, 6–11, 20–1, 28–30, 32–6,
 57–71, 89–93, 103, 148–79,
 187–90, 194, 204, 371–83
- European Bioinformatics Institute
 (EBI), 108
- European Food Safety Authority
 (EFSA), 473–4
- European Medicines Agency
 (EMA), 95
- European Union, 374–5, 397, 444–5,
 451, 465, 466, 468–9, 471,
 473–5, 479
- evaporation uses, 54–71, 123–9, 132–9,
 209–37
- exopolysaccharides (EPSs), 253–62,
 296–306
- extracellular metabolites, 57–71
- extraction, 62–71, 162–90, 340–51
- extremophiles, 6–11, 18–19, 194

- fast protein liquid chromatography (FPLC), 65–6
- fat-soluble vitamins, 352–9
- fatty acids, 46–50, 82–3, 127–9, 134–9, 246–9, 252, 257–62, 340–1, 347–51, 382–3, 385–97, 452–3
- fed-batch cultures, 15–19, 81–4, 190, 194–5, 196, 232–7, 284–7, 355–6, 457–8
- feedstock, 37–9, 226–9, 231–2, 290–6, 305–6, 314–29, 349–51, 356–9, 380–3, 396–7, 449–64, 468–83
- fermentation, xiii, xiv, xv, xvii, xviii, 5–6, 11–21, 22–30, 37–84, 162–91, 193–204, 207–37, 241–306, 313–97, 455–64, 467–83
- fermentor systems, 38, 39–71, 81–4, 455–64
- fertilizers, 230–2
- fibre optics, 110–16, 201–2
- filtration separation methods, 58–71, 84, 93–6, 189–90, 209–37, 259–62, 291–6
- fish, 4–5, 132–9, 193–4, 211, 255, 263, 267–70, 291, 295–6, 303, 359, 381–3, 385–97, 452–65, 467–83
- flatulence problems with legumes, 323
Flavobacterium, 271–80, 289–96
- flavones, 385–97, 446–8
- flavonoids, 370–2, 385–97, 446–8
- flavors, xiii, 20–1, 46–50, 62, 63, 122–39, 193–4, 201, 207–37, 241–62, 264–7, 269–70, 281–7, 313–29, 339–51, 446–8, 468–83
- Flavr Savr tomatoes, xiv, xviii, 256, 440
- flow cytometry measurements, 78–84
- flowmeters, 77–84
- fluid bed coatings, 123, 126–9
- fluidized bed reactors, 40, 41–50, 126–9, 197
- fluorescent methods, 75–6, 78–84, 101–8, 109–20, 135–9, 185–90, 204, 255–62, 453
- fluoride, 60–71, 370–2
- fluorometer measurements, 78–84
- folding, protein folding and stability, 85–93, 100, 198–9
- folic acid, 110, 209, 352, 356, 360–3, 394
- food biotechnology concepts, xi, xii, xvii, xviii, 3–145, 193, 207–37, 241–306, 313–97, 433–83
- food nanotechnology, 114–16, 121–2, 130–39
- food packaging, 114–16, 122, 130–39, 215–18, 352–9
- food preservatives, 46–50, 121–2, 129, 130–39, 243–62, 263–70, 280–7, 363, 379–83, 464–83
- Food Safety Modernization Act (FSMA), 466–7
- Food for Specific Health Use (FOSHU), 384
- food spoilage, 118–20, 121–2, 136–9, 202, 245–70, 274–80, 379–83
- foot-and-mouth disease (FMD), 454–9
- free fatty acids (FFAs), 246–9, 252, 257–62, 347–51
- free radicals, 222–4, 385–97
- freeze-drying separation methods, 61–2, 68, 93–6, 254–62
- Frings reactor, 282
- fructo-oligosaccharides (FOSs), 337–9
- fructose, 46–50, 211, 218–37, 241–62, 289–96, 298–306, 329–39
- fruit, 212–37, 285–6, 290–6, 319, 322–9, 342–51, 385–97
- functional foods, xi, xii, 5, 13, 121–2, 130–39, 242–62, 383–97, 464
- functional genomics, 101–8, 199, 200
- fungi, 5–6, 19–21, 28–30, 40–50, 53, 175–9, 184–90, 207–37, 246–9, 260–2, 270–80, 287–96, 320–9, 356–9, 360–3, 467–83
- galacto-oligosaccharides (GOSs), 186–90, 395–7
- galactose, 211, 234–7, 249–52, 289–96, 353–4

- galactosidase, 46–50, 165–79, 187, 186–90, 204, 211, 255–62, 323–9, 390–7, 440, 451
- gamma rays, 26–7, 96–100
- gastrointestinal tract, 136, 138–9, 179, 267, 384–9, 392–7, 451–64
- gel electrophoresis, 107–8, 169–79, 204
- gel filtration chromatography, 65–71
- gelatin, 122–39, 220–2, 297–306
- gene cloning, xi, xvii, xviii, 4–6, 12, 69–71, 97–9, 100–108, 161–91, 193–4, 203–4, 229–32, 254–62, 327–9, 334–5, 349–51, 439–48, 449–64
- gene expression, xi, 3–6, 33–6, 80, 100–108, 116, 118–20, 153–61, 173–9, 186–90, 200, 203–4, 255–62, 460–4
- gene therapy, 450–1, 452–3
- gene transfer processes, 3–6, 449–64, 472–3
- generally regarded as safe (GRAS), 44, 177–9, 315, 334, 374–5, 380–3, 387–8, 466–7, 481–3
- genetic engineering concepts, xiii, xiv, xv, xvii, 3–6, 24–5, 27–30, 32–3, 71, 83–4, 147, 161–90, 193–4, 203–4, 212, 252–62, 265–7, 280, 329, 434–5, 441–8, 449–83
- genetic use restriction technology (GURT), 471–2
- genetical modification (GM), 4–6, 24–5, 31–2, 69–71, 111, 122, 137, 139, 147, 188, 219–22, 252–62, 265–7, 317, 434–48, 449–83
- genipin cross-linked alginate-chitosan (GCAC), 127–9
- genomics, xi, 25, 31–6, 100–108, 116, 196, 199, 370–1, 376–8
- germination, 433–48
- glucan, 211, 360–3, 385
- glucoamylase, 40–50, 129, 172–9, 218–37, 243–62, 318–29
- glucolactone, 281–7
- gluconic acid, 37, 111–16, 281
- glucosamine, 298–306
- glucose, 14–19, 23–7, 40–50, 51–3, 56–71, 81–4, 110–16, 200, 211, 218–37, 241–62, 281–7, 289–96, 298–306, 329–39, 455–64
- glucose isomerase, 46–50, 64–71, 129, 320–29
- glucose oxidase (GOX), 49–50, 110–16, 325
- glutamate, 23–7, 209–37, 347–51, 467–83
- glutamic acid (monosodium glutamate (MSG)), xiv, 37, 209–11, 294–6, 336–7, 347–51
- glycolipids (sphingolipids), 7–11
- glycolysis, 241–2, 246–9
- glycoproteins, 18–19, 178–9
- glycosides, 29, 384, 446–8
- glycosylation, 70–71, 105, 171–9
- goats, 4–5, 193–4, 257, 451–64, 483
- Golden Rice, 356–7, 435, 443, 445, 482–3
- Golgi complex, 9–11
- Gram-negative bacteria, 12–13, 62, 119–20, 158–9, 171–2, 304, 374–83
- Gram-positive bacteria, 12–13, 17–19, 30, 62, 119–20, 158–9, 171–2, 241–62, 374–83, 386–97
- granulocyte-colony stimulating factor (GCSF), 5–6, 193–4
- granulocyte-macrophage colony stimulating factor (GMCSF), 5–6, 193–4
- grapes, 212–14, 218–37, 447–8
- green teas, 369–72
- growth mediums, 4–6, 11–21, 38–53, 74–84, 195, 196–7, 298–306, 456–64
- guanosine, 23–7, 152–61, 209–37, 347–51
- guanosine monophosphate (GMP), 209–37, 345–51
- guar gum, 296–9, 300
- gum arabic, 123–4, 128–9
- Haematococcus pluvialis*, 211, 358–9
- Haemophilus aegyptus*, 163–4, 163–4

- halocin bacteriocins, 373–83
Hansela anomala, 343–51
Hansenula polymorpha, 176–7, 273
haploid cells, 9–11, 12, 17, 20–1, 27,
28–30, 218, 224–5
hard cheeses, 242–62, 322–9
head cancer, 434
heart disease, 222–3, 326, 370–2, 385–9,
391–7, 448, 452–3
heat transfers, 39, 53–71, 72–84, 197
Helicobacter pylori, 389, 392
hemophilia, 11
hepatitis B, xiv, 5, 114, 193–4, 212,
442–3, 483
hepatitis C, 11
herbicides, 110, 442–8, 472–4, 482–3
histamine, 269–70
histidine, 23–7, 269–70, 294–6, 327–9,
348–51
HIV, 114, 139
homoserine, 22–7, 117–20, 196
hops, 209, 213–37
horizontal gene transfer (HGT), 472–3
human growth hormone, xiv, 5–6, 11,
193–4, 457
human insulin, xiv, 5–6, 10–11, 70–71,
175, 193–4, 203, 212, 333–4,
483
human milk, 395–6, 453–4
human serum albumin (HSA), 179
humans, xiv, 5–6, 8–9, 10–11, 70–71,
103, 106–8, 175–9, 442–3
hybridization, 102–8, 156–61, 162–91,
200, 203–4, 437, 438–9,
461–4
hybridomas, xiv, 4–6, 84, 179, 455–64
hydrocarbons, 13–19, 55–71, 287–8,
290–6, 298–306
hydrogen peroxide (H₂O₂), 241–2,
265–7, 322–9
hydrolyzed vegetable protein
(HVP), 209–11
hydrophobic interaction
chromatography
(HIC), 65–6, 67–71, 95–6,
198–9
hydrophobic surfaces on proteins,
protein folding and
stability, 87–93
hydroxisocaproate
dehydrogenase, 64–71
hydroxyl ethylcellulose, 122–39
hydroxylamine, 26–7, 96–100, 327–9
hyphae cells, 19–21, 28–30, 184–90
Hyphomicrobium, 289–96
hypoxanthine–aminopterin–thymidine
(HAT), 4–6, 456–64
ice cream, 186, 211, 243, 300, 301–6,
343–4
immobilization processes,
biosensors, 109–16, 200–1
immobilized-enzyme/cell reactors, 40,
42–50, 84, 197
immortalized tumor cell lines, 4–6
immunobiologicals, 3–6, 32, 178–9,
193–4, 242–62, 360–3,
388–97, 447–8, 457–64,
468–83
immunoglobulin E (IgE), 479–80
immunoglobulin G (IgG), 96, 393–4
immunosensors, 109–16
impellers, 55–71, 72–84
inactive yeasts, 207–8, 234–7
inclusion bodies (IBs), 57–71, 89–93,
175–9, 190
industrial alcohols, 225–32
industrial biotechnology concepts, 33–6,
96–100, 147–91, 194, 196–8,
207–37, 241–306, 313–97,
433–83
inflammatory conditions, 5, 121–2, 325,
335, 392–7, 447–8
influenza, 116, 163–4, 179, 454
insect cells, 3, 178–9, 255
insecticides, 114, 193–4, 442–8, 469–72,
482–3
insertion methods, vectors, 29–30,
170–9, 187–90, 203, 261–2
interferon, 5–6, 193–4, 212, 442–3,
457–64, 483
interleukins, xiv, 3–6, 100, 178–9,
193–4, 393–4, 442–3, 458–64
introns, 160–1, 169–79

- ions, 8–11, 17–19, 43–50, 61–2, 63–71,
89, 107–8, 139, 180–1, 198–9,
284, 376–83
- iron, 13, 136–9, 278, 360–3
- irritable bowel syndrome (IBS), 242–3,
335, 388–9, 392–7
- isoaccepting tRNAs, 150–61
- isoleucine, 23–7, 294–6
- isomalt, 329, 337–9
- isomerization, 86–93, 333, 447–8
- jellies, 322, 363
- jellyfish, 4–5, 8–9, 453
- Kaposi's sarcoma, 434
- kappa-casein, 188, 245–9
- Kefir, 242–3, 356
- kilning processes, 215–18
- Klebsiella*, 64, 345
- Klenow fragments, 172–3, 180–1, 183
- Kluyveromyces* genus, 48–50, 70–71,
176–9, 186–90, 203, 207, 209,
290–6, 319–29, 176–9,
186–90, 203, 207, 227–8,
261–2, 320–9, 383
- koji, 21, 195, 224–5, 268, 269–71,
276–80, 283–7
- Kreb's cycle, 248–9
- Kunitz inhibitor, 66
- labeling needs, 139, 463–4, 465, 469–83
- lactitol, 329, 337–9, 396–7
- lactalbumin, 131–9
- lactamase, 255–62, 326
- lactase, 46–10, 71, 129, 186–90, 211,
243–62, 319–29, 451–2
- lactate, 110, 200–1, 241–62, 467–83
- lactic acid, xiv, 13, 19, 29–30, 37, 48–50,
200–1, 210–11, 216, 220–5,
231–2, 237, 241–70, 281,
284–5
- lactic acid bacteria (LAB), xiv, 13, 19,
29–30, 37, 48–50, 200–1,
210–11, 216, 220–5, 231–2,
237, 241–62, 264–70, 271–80,
284–5, 338, 366–72, 373–83,
384–97, 468–83
- Lactobacillus acidophilus*, 234–7,
242–62, 277–8, 356, 381–3,
386–97
- Lactobacillus brevis*, 234–7, 259–62,
272–80, 367–9
- Lactobacillus bulgaricus*, 242–62,
284–5, 328–9, 356, 387, 393–7
- Lactobacillus casei*, 64, 129, 234–7,
242–62, 266–70, 278, 386–7,
393–7
- Lactobacillus delbrueckii*, 222, 223–5,
234–7, 284–5, 328–9, 387,
393–7
- Lactobacillus* genus, 13, 29–30, 48–50,
64, 129, 222, 241–62, 265–70,
284–5, 287–8, 298–306,
343–51, 380–3, 386–97
- Lactobacillus plantarum*, 127, 234–7,
254–62, 266–70, 271–80
- Lactobacillus rhamnosus*, 258–62,
278–80, 392–7
- Lactococcus* genus, 129, 175, 241–62,
266–70, 343–51, 386–97
- lactones, 117–20, 342–51, 355–6
- lactose, 15–19, 46–50, 51–3, 129, 177–9,
186–90, 200–1, 211, 249–62,
288–96, 298–306, 389–97,
454
- lager, 217–18
- lasers, 102–8, 113–16
- LDL, 222–4, 385
- leading strands, 149–61
- leathers, enzymes, 324–5, 328, 446–8
- leavening processes, 232–7, 467
- lecithins, 66, 124–9, 277–80
- Legionella pneumophila*, 112–13
- legumes, 323–9, 446–8
- Lentinus edoides* (Japanese shiitake
mushroom), 360, 362–3
- leucine, 23–7, 46–50, 51–3, 64–71,
224–5, 258–62, 294–6,
349–51
- Leuconostoc* genus, 13, 220–2, 241–62,
298–306, 380–3, 386–97
- leukaemia, 325
- libraries, 98–100, 102–8, 162–91, 204,
451
- linoleic acids, 348, 385–97

- lipases, 46–50, 203, 248–9, 255–62, 313–29, 340–51
- lipids, 7–11, 21, 32–6, 110–16, 122, 184–90, 252–62, 347–51, 376–83, 389–97, 446–8
- lipolysis, 245–9
- lipolytic enzymes, 257–62, 340–51
- liposomes, 29–30, 45, 258–62
- liquefying, 318–29
- Listeria monocytogenes*, 112–13, 125–9, 257, 379–83
- Lithospermum erythrorhizon* (shikonin), 434–5, 446–8
- liver disease, 136
- locust ben gum (carob), 297
- low calorie sweeteners, 329, 337–9
- low fat cheeses, 258–62
- lung diseases, 341–2, 381–3, 434–5
- lycopene, 20–1, 128–9, 131–9, 356–9, 385–97
- lymphoblastoid cells, 456–64
- lymphocytes, 4–6, 179, 394, 447–8, 455–64, 468–83
- lymphokines, 3–6, 178–9, 193–4
- lympholactoids, 455–64
- lysine, 13–19, 22–7, 83–4, 127–9, 196, 294–6, 348–51, 360–3
- lysis, 17–19, 29–30, 68, 129, 178–9, 184–90, 209–11, 376–83
- lysozyme, 29–30, 44–5, 173, 184–90, 204, 255–62, 326–9, 473–4
- macromolecules, 6–11, 60–62, 89–93, 147–61, 190
- macrophages, 394
- macular degeneration, 385
- magic-bullet medicines, 82–3–2
- malaria, 34–6, 179, 196–7
- malic acid, 48–50, 220–2, 281, 285–7
- maltidol, 329, 337–9
- mammalian cells, 3–6, 39–50, 82–3, 103, 137, 176–9, 184–90, 449–64
- marketing, 37, 162, 212–14
- mass spectrometry (MS), 102–8, 112–16
- mass transfers, 39, 50–54, 57–71, 72–84, 197, 457–8
- matrix microcapsules, 122–9
- Maxam–Gilbert DNA-sequencing technique, 180–1, 190
- meiosis processes, 9–11, 148–61
- melamine formaldehyde (MF), 123–9
- membrane reactors, 45–50
- membrane structures, 6–11, 43–4
- menthol, 343, 448
- metabolic control analysis (MCA), metabolic engineering, 33, 35–6
- metabolic engineering (ME), xi, 31–6, 79, 196–7, 211, 229–32, 349–51, 358–9
- metabolic flux analysis (MFA), metabolic engineering, 33, 35–6
- metabolic pathway analysis, 33–6, 196, 249–62
- metabolites, xi, 8–11, 21, 22–30, 36–86, 199, 209–37, 313–29, 334–9, 467–83
- metabolomics, 101–8, 199
- methane, 12–19, 37–8, 53, 55–71, 78–84, 226–32, 288–96, 389–97
- Methanobacterium* genus, 13
- Methanobrevibacter* genus, 392
- Methanococcus* genus, 13
- methanol, 55–71, 226–32, 288–96, 298–306, 317–29
- Methylococcus* genus, 13, 19
- Methylomonas* genus, 13, 288–96
- methylquanosine, 152–61
- Michaelis–Menten constant of enzyme kinetics, 14–15
- microarrays, 101–8
- microbial biomass protein (MPB), 287–96
- microbial factors affecting performance/economics, 37–9, 80–81
- microbial fuel cells (MFCs), 111–12
- microbial physiology, scale-up/scale-down processes, 72–81, 95, 197–8
- microcapsules, 120–39, 202–3
- Micrococcus* genus, 13, 18–19, 44–5, 241, 265–70, 353–6

- microencapsulation
immobilized-enzyme/cell
reactors, 45–50
- microencapsulations, xi, 45–50, 120–39,
202–3
- microfiltration separation
methods, 60–62
- microfluidization techniques, 132–9
- microinjections, 171–2, 439–50, 467–83
- microorganisms, xiii, xiv, xv, 3–6, 10–11,
20–1, 193–4, 315, 466–83
- milk, 4–6, 46–50, 124–5, 186, 193–4,
200–1, 241–62, 301–2,
319–29, 347–51, 380–3,
395–6, 451–64
- miscoded protein structures, 86–93
- misfolded protein structures, 86–93
- mitochondria, 9–11, 20–1, 89–93,
158–61
- molasses, 57, 223–5, 226–32, 283–7,
288–96, 329
- molds, 19–21, 28–30, 39, 53, 70, 211–37,
242, 246–9, 275–80, 340–1
- molecular thermodynamics, xi, 85–96,
198–9
- monoclonal antibodies, xiii, xiv, xvii,
3–6, 37, 82–4, 161, 178–9,
193–4, 442–3, 458–64
- moss plants, 3–6, 448
- mRNA, 97–100, 101–8, 147–91, 199,
256–62, 440, 480–3
- Mucor* genus, 21, 29–30, 211–12, 260–2,
316–29
- multilamellar vesicles (MLVs), 124–9,
130–39
- multiwall carbon nanotubes
(MWCNTs), 114, 116
- muscular dystrophy, 452–3
- mushrooms, xiv, 360–3
- mutagenic agents (mutagens), xiii, xv,
16, 22–3, 24–7, 71, 96–100,
154–61, 185, 204, 316, 326–9,
375–83
- mutation techniques, xiii, xv, 18, 22–7,
96–100, 195–6, 327–9
- mycelium, 20–1, 28–30, 292–6
- Mycobacterium* genus, 13, 30
- mycotoxins, 112–13, 211–12
- myeloma tumor cells, 4–6, 179, 456–64
- nano electronic nose (e-nose), 136–9,
201–2
- nano-capsules, 130–39
- nanobiosensors, 113–16, 134–9, 200–2
- nanobiotechnology, xi, 11, 109–16,
129–39, 200–2, 397
- nanoclays, 133–9
- natural selection and mutation, strain
improvement and
tools, 22–7
- neurological maladies, 5, 105–6, 136–9,
178–9, 199
- Newtonian fluids, 58–60, 73–4, 197
- next-generation
DNA-sequencing, 106–8,
180–90
- niacin (vitamin B3), 356, 360–3
- nicotinamide (vitamin P), 342, 352, 356
- nisin, 243–62, 373–83, 473
- nitrocellulose, 186–90, 204
- nitrogen, 13–19, 164–90, 208–37,
263–70, 294–6, 301–6,
434–48
- nitroso-hemochrome, 265–7
- nitrosoguanidine, 25–7, 96–100, 344–51
- nitrosylation, 105
- nitrous acid (HNO₂), 26–7
- nitrous oxide (N₂O), 125–9–8
- non-Newtonian fluids, 58–60, 74–5, 77,
196–7
- non-stirred reactors, 40
- nonrecombinant DNA techniques, plant
biotechnology, 437–9, 470–2
- Nocardia* genus, 13
- Northern blotting technique, 173,
186–90
- nuclear fusion (karyogamy), 28–30,
148–61
- nuclear magnetic resonance
(NMR), 79–84
- nuclear transplantation, 449–64
- nucleic acids, 20–1, 57–71, 81–4, 102–8,
109–16, 147–91, 200–4,
209–37, 246–62, 292–6,
446–8
- nucleoids, concepts, 6–11

- nucleolus, cell concepts, 9–11
nucleotides, 13–19, 22–7, 37–85,
96–100, 102–8, 109–16,
148–90, 195–204, 209–37,
326–9, 334–9, 351, 362–3,
446–8
nucleus, cell concepts, 6–11, 20–1,
28–30, 147–91, 194, 449–64
nutraceuticals, xi, xii, 5, 82–3, 130–39,
242–62, 338, 383–97
oats, 137, 385–97, 446–8
obesity, 329, 333
odors, 111, 128–9, 136–9, 201, 241–62,
269–70, 275–80, 313–29,
339–51, 446–8
oligonucleotides, 102–8, 161, 162–90,
204, 326–9
oligopeptides, 119–20
oligosaccharides, 8–11, 122, 186–90,
211, 230–2, 323–39, 384,
385–91, 395–7
olives, 270, 273–80, 305
omega-3 fatty acids (PUFA), 127–8,
134–9, 384, 385–97, 452–4,
468
online sensors, 79–84, 112, 116
opiates, 446–8
optical biosensors, 111–16
oral health, 338, 382, 392–7
organelles, concepts, 6–11, 20–1, 42–53,
109–16
organic acids, xiv, 20–1, 37–8, 47–8,
50–53, 57–71, 281–7, 446–8
Organization for Economic Cooperation
and Development
(OECD), 466–7
ornamental fish, 4–5, 193–4, 453–4
ornamental plants, 433–48
osmoregulation, solutes, 17–19
osteoporosis, 243, 278, 283, 370–1
ovarian cancer, 434, 447–8
ovaries, 3–6, 171–2, 177–9, 449–64
oxidation, 105, 121–9, 246–62, 333–9,
352–9, 369–72, 447–8
oxygen, environmental bacterial growth
factors, 18–19, 20–1, 40–53,
57–71, 72–84,, 215–37, 299
oxygen transfer rates (OTRs), 52–3,
57–71, 74–84, 196–7
Paecilomyces varioti, 289–96
palladium-coated semiconductor
enzyme thermistors, 47–50
pancreas, 110, 321–9
pantothenic acid (vitamin B5), 110, 352,
355–6, 359, 360–3
papain, 46–50, 315–29
papaya, 443–8
papillomavirus, 179
parasexual recombinations in
eukaryotes, 28–30, 147–61
parasites, 20–1, 34–6, 451–64
Parkinson's disease, 89, 452–3
Parmesan cheese, 248, 257–8, 260–2
pasteurization, xiv, 210–12, 217–18,
224–5, 232–3, 244–62, 267,
277–80, 313–14, 320–9,
361–2
patents, 36–7
pathogens, 106, 109–20, 136–9, 201–3,
242–3, 245–70, 288, 379–83,
384–97, 451–64
pectin, 8–11, 21, 46–50, 219–22,
297–306, 322–9, 367–72
pediocins, 255–62, 375–83
Pediococcus genus, 13, 17–19, 222–4,
241–62, 266–70, 386–97
penicillin, xiv, 13, 24–5, 40–50, 64–71,
96, 114, 116, 325–9
Penicillium chrysogenum, 28–30, 48–50,
211–12, 354–6
Penicillium genus, 20–1, 28–30, 48–50,
53, 211–12, 246–52, 316–29,
356–9, 367–9
Penicillium roqueforti, 211–12, 246–9,
340–1
pentoses, 289–96
pepsin, 260–2, 315–29
peptic ulcers, 121–2, 389, 392
peptides, 85–93, 119–20, 122–9, 134–9,
184–90, 243–62, 275–80,
373–83, 384, 446–8
peptidyl-prolyl cis–trans isomerases
(PPI), 86–93, 159–61

- pesticides, 110, 111–13, 114–16, 122–9, 135–9
- petrochemical products, 288–96
- Phaffia* genus, 358–9
- phage inhibitory media (PIM), 259–62
- phage-tail like bacteriocins, 373–83
- phages, 99–100, 102, 129, 162–79, 188–90, 252–62, 462–4
- phagocytes, 393–7
- pharmaceuticals, xvii, 3–6, 10–11, 13–19, 32–7, 40–50, 64–71, 82–4, 85–93, 94–6, 100, 105–6, 109–16, 120–39, 147, 161–2, 168–79, 212, 254–62, 314–97, 434–64
- pharmacodynamics, 459
- pharmacokinetics, 459
- phenolic off-flavor (POF), 218
- phenolics, 218, 385–97, 446–8, 469–83
- phenotypes, 27, 35–6, 101–8
- phenotypic expression of mutations, 27, 35–6
- phenylalanine, 23–7, 82–4, 258–62, 294–6, 334–9, 349–51
- phosphoenolpyruvate (PEP), 249–62
- phosphoenolpyruvate–lactose phosphotransferase (PEP-PTS), 249–62
- phosphoketolase, 241–62
- phospholipids, 7–11, 18–19, 110–16, 130–39, 258–62
- phosphorylase, 64–71, 105–8, 345–51
- photo-bioreactors, 3–6, 45
- photometers, 47–8, 78–84, 113–16
- photosynthesis, 10, 46–50, 356–9, 381
- Phycomyces*, 356–9
- phytoestrogens, 275–80, 385
- Pichia pastoris*, 176–9, 336, 342–51, 355–6, 383
- piezoelectric sensors, 111–16
- pigments, 20–1, 122–9, 352, 356–9, 446–8
- plant biotechnology, xii, 3, 4–6, 11, 25, 34–6, 39–50, 171–2, 179, 189–90, 193–4, 255–62, 300–1, 315–29, 340–51, 356–9, 433–48, 464–83
- plant cells concepts, 8–11, 25, 34, 39–50, 161, 164, 171–2, 179, 189–90, 193–4, 255–62, 300–1, 315–29, 340–51, 356–9, 433–48, 469–83
- Plant Pest Act, 464–5
- plant sterols, 385–97
- plants, xii, xiv, 3, 4–11, 25, 34, 39–50, 161, 164, 171–2, 179, 189–90, 193–4, 255–62, 300–1, 315–29, 356–9, 433–48, 464–83
- plasmid vector per unit cell (copy number), 173–9
- plasmids, 6–11, 28–30, 134–9, 161–91, 203–4, 252–62, 280, 376–8, 440, 450–64
- Plasmodium falciparum* malaria parasite, 34–6, 196
- plasmolysis techniques, 17–19, 62, 209–37
- plastids, 90–93, 438–48
- Pleurotus ostreatus* (oyster mushroom), 360, 362–3
- polyacrylamide, 45, 107–8, 180–90
- polyadenylated tails, 153–61
- polyalkylcyanoacrylate, 134–9
- polyanhydride polybis p-carboxyphenoxy-sebacic acid (PCPP-SA), 133–8
- polyethylene glycol (PEG), 29–30, 439
- polymerase chain reaction (PCR), 99–100, 103–8, 112–16, 169–79, 180–90, 203–4, 463–4, 475–6, 478–83
- polymerases, 99–100, 103–8, 112–16, 148–61, 169–79, 180–90, 199, 204, 261–2
- polymerization, 123–9, 148–90
- polymorphism, 94–6, 101–8
- polypeptides, 86–93, 124–9, 137–9, 153–61, 175–9, 186–90, 457–64
- polyphenols, 131–9, 222–4, 313–29, 348, 372
- polysaccharides, xiii, xiv, 13–19, 60–62, 63–71, 211, 212–37, 243–62,

- 280–306, 360–3, 446–8, 467–83
- polysulfones, 60–71
- polyunsaturated fatty acids, 348, 384, 452–4
- postmenopausal symptoms, 384–5
- potentiometric sensors, 111–16
- poultry, 4, 260–2, 266, 359, 380–3, 464–5, 474
- poverty, 483
- power consumed per unit volume (P/V), 74–84, 196–7
- power number (Newton's number), 73–5
- prebiotics, 186–90, 242–3, 302, 384–97
- precipitation methods, 63, 93–6, 318–29
- preservatives, 46–50, 121–2, 129, 130–39, 243–62, 263–70, 280–7, 363, 379–83, 464–83
- probiotics, 121–2, 131–9, 242–3, 244–62, 263–70, 373–4, 381–3, 384–97, 467–83
- prochymosin, 70–71, 189–90, 255–62
- proflavine, 26–7
- prokaryotes, 6–11, 12, 32–6, 57–71, 89–93, 103, 147–63, 188–90, 194, 204
- Propionibacteria* genus, 13, 23–7, 48–50, 242–62, 355–6, 393–7
- propionic acid (PPA), 13, 48–50, 246–9, 287
- prostate cancer, 279, 370, 385, 447–8
- proteases, 46–50, 178–9, 187–90, 209–37, 255–62, 313–29, 375–83
- protein cloning route (shotgun cloning), 161–2, 168–79
- protein disulfide isomerases (PDI), 91–2
- protein efficiency ratio (PER), 293–6
- protein and enzyme engineering, xi, xiii, xvii, 3–6, 33–6, 96–100, 161, 252–62, 326–9–9
- protein folding and stability, 85–93, 100, 198–9
- protein microarrays, 101–8
- protein synthesis, 147–91
- protein-based detection methods, 475–83
- protein–protein interactions, 101–8
- proteinases, 243–62
- proteolysis, 173–9, 184, 245–62, 268–70, 340–51
- proteomics (protein expression), xi, 33–6, 100–108, 116, 150–61, 186–90, 196, 199
- protists, 6–11, 194
- protoplasts, 29–30, 171–9, 253–62, 437, 438–9, 467–83
- protozoa, 6, 103, 392
- Pseudomonas* genus, 13, 18–19, 117, 164–5, 171–2, 250–2, 273–80, 289–96, 298–306, 321–9, 343–51, 358–9
- pullulan, 13–19, 64–71, 218–37, 285–7, 296–300, 303–6, 319–29
- pulp/paper industry, 209, 287–8, 297, 324–5, 328
- purification, 57–60, 62–3, 64–71, 78, 83–4, 93–6, 98–100, 122, 161–79, 183, 183–90, 197, 198–9, 318–29, 340–51, 462–4
- pyridoxine (vitamin B6), 278, 352, 355–6, 359
- pyruvate, 200–1, 250–2, 342–51
- quantum dots (QD), 114–16
- quorum quenching (QQ), xi, 116, 118–20, 202
- quorum sensing (QS), xi, 116–20, 202, 374–83
- radiation sickness, 277–8
- radioactive nucleotides, 172–3, 203–4
- raffinose hydrolysate, 46–50, 234, 323–9, 395–6
- random-energy model of Bernard Derrida, 88
- (RM), 35, 96–100, 327–9
- rapid expansion of supercritical solution (RESS), 125–9
- Rauth Crusher–Stemmer, 219–22
- real-time sensors, 79–84, 112, 116

- recombinant bovine somatotropin (rBST), 451–2
- recombinant DNA technology (rDNA), xi, xii, xiv, xv, xvii, xviii, 3–6, 27–30, 33–6, 37–8, 62, 69–71, 80–84, 85–6, 96–100, 147, 161–91, 193–204, 211–12, 220–2, 229–32, 253–62, 280, 282–3, 295–6, 316, 327–9, 334–5, 349–51, 353–4, 358–9, 411–29, 434–5, 439–48, 449–83, 491–5
- regulations, xvii, xviii, 4, 5–6, 82–4, 95, 118–20, 136–9, 177–9, 202–3, 222–4, 233–4, 293–4, 374–5, 382–3, 396–7, 451–2, 463–83
- renewable sources, biomass, 289–91, 323–4
- rennet, xiv, xviii, 5–6, 13, 46–50, 187–90, 203, 212, 245–9, 260–2, 315–29
- reproduction, xiii, 9–13, 17, 20–1, 28–30, 99–100, 106, 116–20, 147–61, 435–48, 449–64
- restriction endonucleases (restriction enzymes), 162–79, 187–90, 203–4, 256, 261–2
- restriction fragment length polymorphism (RFLP), 185–90
- retinol (vitamin A), 132–9, 352, 356–7, 443, 445
- retroviruses, 169–70, 175–9
- reverse transcriptase (RT), 104–8, 168–91, 261–2
- Rhizobium* genus, 164–5
- Rhizomucor miehei*, 190, 320–29
- Rhizopus* genus, 21, 211–12, 278–80, 286–7, 319–29, 330–9, 468
- rho independent terminators, 155–61
- Rhodococcus maris*, 358–9
- Rhodopseudomonas* genus, 290–6, 354–6
- Rhodotorula* genus, 21, 355–9
- rhodoxanthin, 356–9
- riboflavin (vitamin B2), 13, 20–1, 278–80, 352, 354, 355–6, 359, 360–3, 473
- ribonucleotides, 24–7, 209–37, 326, 345–51, 362–3
- ribosomes, 6–11, 108, 147–9, 150–90
- ripening, 245–9, 251–2, 254–62, 319–29, 340–1, 348–51
- RNA, 6–11, 36, 77–8, 87–93, 97–100, 101–8, 115–16, 147–91, 203–4, 210–11, 256–62, 293–4, 314, 345–51, 362–3, 440–8, 452–3
- rotary disk atomization, 123, 126–9
- rotary drum vacuum filters, 60–62, 292–6
- rRNA, 6–11, 108, 147–91, 199–200, 294
- saccharification, 46–50, 223–31, 286, 319–29, 330–2
- Saccharomyces cerevisiae*, 20–1, 28–36, 48–50, 64, 81–4, 103, 165–8, 171–2, 176–9, 189–90, 196–7, 208–9, 211, 212–32, 234–7, 288–96, 319–39, 346–51, 367–9, 381–3, 388
- Saccharomycopsis*, 20–1 –30
- Sacharomyces*, 28–30
- safety issues, xii, xvii, xviii, 4–6, 36–6, 44, 82–4, 95–6, 115–16, 122, 135–9, 177–9, 202–3, 222–4, 254–62, 280, 293–6, 315–29, 333–51, 374–5, 386–8, 396–7, 434–5, 448, 449–83
- SAGE (serial analysis of gene expression), 101–8
- Sake*, 21, 195, 224–5
- Salmonella* genus, 112–13, 114, 116, 257, 373–4, 380–3, 463–4
- salt stock pickles, 272–80
- salted fish, 270
- Sanger DNA-sequencing technique, 180–1, 200
- saponins, 279–80, 446–8
- saprophytic characteristics of fungi, 19–21
- sauerkraut, 270–2, 274–80, 347–51, 467

- sausages, 21, 211–12, 263–7, 347–51, 379–83
- scale-down processes, 71–84, 197–8
- scale-up processes, xi, xvii, xviii, 36–85, 189–90, 196–7
- Scenedesmus* genus, 290–6
- Schizosaccharomyces pombe*, 20–1, 176–9, 223–5
- Schwannionmyces castelli*, 218, 218
- Scytalidium acidophilum*, 291–6
- second law of thermodynamics, 85–93
- secondary metabolites, 313–29, 445–8
- selection step, directed evolution protein engineering, 99–100
- selective separation of racemic mixtures of amino acids, 40–50
- sequencing by ligation (SOLiD sequencing), 180–1
- sequencing by synthesis (Illuminia), 180–1, 199–200
- serine, 105–8, 294–6
- sexual reproduction, 20–1, 28–30, 99–100, 106, 116–20, 148–61, 449–64
- shikokin, 193, 434–5
- Shine–Delgarno sequence (SD), 158–61, 174–9
- sickle cell anemia, 452–3
- silicas, 32–6, 44–5, 46–50, 66, 109–16, 200
- simian eadenovirus SV, 40 150, 459–60
- single cell protein (SCP), 13–19, 20–1, 37–8, 40–50, 207–37, 287–96, 467–83
- single-primer method, 99–100, 169–70, 180
- site-directed mutagenesis (SDM), 96–100, 327–9
- sodium alginate, 122–39, 297–306
- sodium dodecyl sulfate (SDS), 62, 173, 184–90
- solid–solid separation recovery operations, 58–71
- solid-state fermentation (SSF), 21, 40–50, 195, 229–32
- somatropin, xiv, 451–2, 473–83
- sorbitol, 184–90, 337–9, 352–4, 360–3
- sorghum, 213–14, 228–32, 362
- sour breads, 234–7
- sour cream, 242–62, 341–51
- sourdough, 232–7
- Southern blotting technique, 103–4, 173, 185, 186–90, 204, 478–83
- spider's web protein, 4, 193–4, 452–4
- Spirulina maxima*, 288–96
- Spongiooccus* genus, 357–8
- spontaneous mutations, 24–7
- Sporotrichum pulverulentum*, 290–6
- spray drying/chilling, 123, 126–9, 132–9, 210–11, 301, 318–29
- ssDNA, 98–9
- Staphylococcus* genus, 112–13, 117, 119, 265–7, 321–9, 381–3
- starch, 46–50, 122, 129, 131–9, 215–37, 289–306, 314–29
- starter bacteria, 245–9, 254–62, 263–7, 280
- sterilization, xiv, 68–71, 72–84, 197, 267, 321–9, 435–48
- steroids, xiii, xiv, 13–19, 37–8, 40–50, 446–8
- stevioside, 329, 336–7
- stirred reactors, 40–50, 72–84, 197, 231–2, 457
- stonewashed jeans, 324
- storage finishing operations, 217–18, 318–29
- straight-dough process, 235–7
- strain improvement and tools, 22–36, 96–100, 355–6, 459–64
- Streptococcus (Lactococcus)* genus, 13, 29–30, 117, 241–62, 298–306, 393–7
- Streptomyces* genus, 29–30, 53, 64, 171–2, 241, 266–70, 327–9, 332–9, 357–9, 386–97
- streptomycin, xiv, 27, 64
- submerged nozzle processes, 123, 126–9, 195, 282, 315–29
- substrates, 13–19, 31–6, 45–53, 55–71, 72–84, 98–100, 105–8, 109–16, 148–91, 224–5, 288–96, 313–29, 347–51
- sucralose, 335–9
- sufu, 21, 279–80

- sugar cane, 223–5, 226–32, 323–8,
329–39, 438, 443–8
- sulfhydryl oxidase, 46–50
- sulfite, 207, 209–37, 288–96
- sulfolobin bacteriocins, 373–83
- superoxide dismutases, 19, 243–62,
279–80
- supersaturation, crystallization
dynamics, 93–6
- supervisory computer controls
(SCC), 79–81
- supervisory control and data acquisition
(SCADA), 80–81, 197
- surface koji (solid state) culture
reactors, 40–50
- surface plasmon resonance
(SPR), 109–16
- surfactants, 82–4, 379–83
- sweeteners, 329–39, 348–9, 446–8
- symbiotic relationships, 116–17
- synbiotics, 396–7
- synthetic biology, xi, 31–6, 82–3, 161–2,
168–79, 196–7, 340–51
- synthetic DNA cloning route, 161–2,
168–79, 203–4
- synthetic hormones, 3, 4–6, 178–9
- systems ME, 35–6
- systems/synthetic biology, xi, 31–6, 196,
198
- T-DNA, 440
- tannins, 217–18, 220–2, 385–97, 446–8
- tautomeric DNA bases, 25–7
- tea, 364, 369–72, 385
- tempeh, xiii, 271, 278–80
- tetracycline, xiv, 165–8, 172–9
- tetramethylpyrazine (TMP), 344–51
- textiles, enzymes, 324–5, 328
- texturizing effects, 211, 241–62, 275–80
- thaumatin, 255–62, 329, 336–7
- Thermoanaerobacterium*
saccharolyticum, 229–32
- thermocouple controls, 76–84
- thermodynamics, xi, 85–96, 198–9
- Thermus aquaticus*, 18–19, 183
- thiamine (vitamin B1), 23–7, 210–11,
352, 355–6, 359, 360–3,
386–7
- thickeners, 211, 297–306
- threonine, 22–7, 105–8, 196, 294–6,
348–51
- thymidine kinase (TK), 461–4
- tocopherol (vitamin E), 131–9, 278, 352,
356
- tofu, 279–80
- toluenesulfonamide, 333–4
- tomatoes, xiv, xviii, 256, 348, 357–9,
385–97, 438, 440, 443–8
- Torulopsis* genus, 21, 228–9, 234–7,
273–80, 321–9
- totipotent cells, 434–48
- toxins, 34–6, 71, 81–4, 88–9, 92–3,
109–20, 133, 134–9, 176–9,
185–6, 194–5, 199, 211–12,
218, 222, 242–3, 269–70, 280,
293–6, 334, 341, 379–83,
442–8, 458–64, 467–83
- traceability issues, 475–83
- transcription concepts, 33–6, 101–8,
118–20, 147–52, 153–61,
168–91, 199, 261–2, 460–4
- transcriptomics (gene
expression), 33–6, 101–8,
118–20, 153–61, 196, 460–4
- transducer component of
biosensors, 109–16, 200
- transduction mechanism in
bacteria, 28–30, 118–20,
147–61, 253–62
- transformation, 28–30, 54–71, 147–68,
170–91, 203–4, 253–62,
340–51, 447–8, 450–64
- transgenic biotechnology *see* genetic
engineering,
- transit gene expressions (TGE), 3–6, 80,
460–4
- transition single base changes, 25–7
- translation concepts, 105–8, 150–61,
171–9
- translocation concepts, 160–1
- transport mechanisms, 8–11, 16–19
- transposon DNA sequences, 25–7,
29–30, 255–62, 376–8
- tricarboxylic acid (TCA), 283–7, 347–51
- Trichoderma* sp., 23–7, 289–96, 342–51

- triglycerides, 48–50, 110–16, 131–9, 341–51
- tRNA concepts, 147–9, 150–61, 174–9
- trypsin, 44–5, 46–50, 66–71, 91–2, 325–6, 442–3, 455–64
- tryptophan, xiv, 14–19, 23–7, 157–61, 294–6, 334–5, 348–51, 360–3
- tumor necrosis factor cells (TNF), 179, 394
- turmeric, 131
- tyrosine, 23–7, 105–8, 294–6, 349–51
- U-Loop technology, 291–2
- ubiquitin, 105–8
- ulcerative colitis, 392
- ultra-formaldehyde (UF), 123–9
- ultrafiltration separation
methods, 47–50, 58–9, 60–62, 70–71, 189–90
- ultrasonication techniques, 132–9
- ultraviolet (UV), 19, 25–7, 69–70, 96–100, 185–90, 204
- umami effect, 209–11, 269–70
- untranslated regions (UTRs), 153–61
- unwinding proteins, 148–91
- urethane (EC), 222–4, 280
- uric acid, 293–4
- urokinase, 458–64, 483
- US Department of Agriculture
(USDA), 82–3, 443–8, 451–2, 464–5, 471–2
- US Environmental Protection Agency
(USEPA), 82–3, 481
- US Food and Drug Administration
(USFDA), 44, 80–81, 82–3, 95–6, 136–7, 177–9, 202–3, 233, 293–6, 334–9, 355, 358–9, 370–1, 374–5, 382–3, 396–7, 451–2, 463–5, 466–8, 470–1
- vaccines, xiv, 3, 4–6, 37–8, 82–3, 124–9, 131–9, 179, 193–4, 256–62, 381–3, 442–3, 448, 449–64, 473–83
- vacuum cooling, 236–7
- vaginal microbiota, 382, 385–7, 393–7
- van der Waals (VDW), 43–4, 87–93, 128–9
- vanillin, 340, 344–51, 446–8
- vectors, 29–30, 170–9, 187–90, 203, 261–2, 161–2, 164–8, 173–9, 180, 186–90, 203–4, 254–62, 349–50, 440, 459–64, 467–83
- Vibrio anguillarum*, 381–3, 255–62, 381–3
- vinegar, xiv, 40–50, 281–7
- vitamin A (retinol), 132–9, 352, 356–7, 443, 445
- vitamin B complex, 243, 269, 352, 360–3
- vitamin B1 (thiamine), 23–7, 210–11, 352, 355–6, 359, 360–3, 386–7
- vitamin B2 (riboflavin), 13, 278, 352, 354, 355–6, 359, 360–3, 473
- vitamin B3 (niacin), 356, 360–3
- vitamin B5 (pantothenic acid), 110, 352, 355–6, 359, 360–3
- vitamin B6 (pyridoxine), 278, 352, 355–6, 359
- vitamin B12, 13, 110, 278–80, 352, 354–5, 360–3, 394, 473
- vitamin C (ascorbic acid), xiv, 125–9, 131–9, 235–7, 264–7, 352–4, 359, 360–3
- vitamin D, 132–9, 352, 356
- vitamin E (tocopherol), 131–9, 278, 352, 356
- vitamin K, 132–9, 278, 352, 356
- vitamins, xiii, xiv, 11, 13–19, 20–1, 22–7, 32, 57–71, 109–16, 124–9, 131–9, 210–11, 235–7, 264–7, 278, 352–60, 384, 397, 443, 445, 446–8, 467–83
- waste-disposal processes, 82–4, 227
- Western blotting technique, 173, 185–90
- whey, 46–50, 131–9, 186–7, 207, 227–8, 243–62, 282–7, 288–96, 395–6
- whisky, 223–5
- white wines, 219–22
- wine, xiii, xiv, 20–1, 207, 212–14, 218–37, 282–7, 329, 358–9, 380, 467–8

- World Health Organization
(WHO), 122, 137, 338,
464–5, 481
- wort, 215–18, 230–2
- X-rays, 26–7, 96–100, 173–9, 180
- xanthan gum, 63, 296–8, 299–306
- Xanthomonas campestris*, 299–306
18–19, 298–306
- xanthophylls, 356–9
- xenotransplantation commercial
opportunities, 4–6
- xylose, 229–32, 295–6, 338–9
- Yarrowia lipolytica*, 350–1
- yeast artificial chromosomes
(YACs), 167–8
- yeast two-hybrid screen (Y2H), 100–7
- yeasts, xiii, xiv, 3–6, 17, 19–21, 28–30,
34–6, 40–50, 51–3, 62,
70–71, 101–8, 126–7,
152–61, 166–79, 184–90,
193–4, 203–4, 207–39, 242,
249, 273–87, 288–96, 343,
345–54, 366–72, 467–83
- yogurt, 194, 242–3, 244–62, 301, 342–4,
384–97, 467–83
- zeaxanthin, 356–9
- zirconia, 46–50
- Zygomycetes (Phycomycetes)*, 19–21
- zygotes, 9–11, 20–1, 147–61
- Zymomonas mobilis*, 228–32,
298–306

GENERAL FOOD SCIENCE & TECHNOLOGY, ENGINEERING AND PROCESSING

Food Texture Design and Optimization	Dar	9780470672426
Nano- and Microencapsulation for Foods	Kwak	9781118292334
Extrusion Processing Technology: Food and Non-Food Biomaterials	Bouvier	9781444338119
Food Processing: Principles and Applications, 2nd Edition	Clark	9780470671146
The Extra-Virgin Olive Oil Handbook	Peri	9781118460450
Mathematical and Statistical Methods in Food Science and Technology	Granato	9781118433683
The Chemistry of Food	Velisek	9781118383841
Dates: Postharvest Science, Processing Technology and Health Benefits	Siddiq	9781118292372
Resistant Starch: Sources, Applications and Health Benefits	Shi	9780813809519
Statistical Methods for Food Science: Introductory 2nd Edition	Bower	9781118541647
Formulation Engineering of Foods	Norton	9780470672907
Practical Ethics for Food Professionals: Research, Education and the Workplace	Clark	9780470673430
Edible Oil Processing, 2nd Edition	Hamm	9781444336849
Bio-Nanotechnology: A Revolution in Food, Biomedical and Health Sciences	Bagchi	9780470670378
Dry Beans and Pulses: Production, Processing and Nutrition	Siddiq	9780813823874
Genetically Modified and non-Genetically Modified Food Supply Chains: Co-Existence and Traceability	Bertheau	9781444337785
Food Materials Science and Engineering	Bhandari	9781405199223
Handbook of Fruits and Fruit Processing, second edition	Sinha	9780813808949
Tropical and Subtropical Fruits: Postharvest Physiology, Processing and Packaging	Siddiq	9780813811420
Food Biochemistry and Food Processing, 2nd Edition	Simpson	9780813808741
Dense Phase Carbon Dioxide	Balaban	9780813806495
Nanotechnology Research Methods for Food and Bioproducts	Padua	9780813817316
Handbook of Food Process Design, 2 Volume Set	Ahmed	9781444330113
Ozone in Food Processing	O'Donnell	9781444334425
Food Oral Processing	Chen	9781444330120
Food Carbohydrate Chemistry	Wrolstad	9780813826653
Organic Production & Food Quality	Blair	9780813812175
Handbook of Vegetables and Vegetable Processing	Sinha	9780813815411

FUNCTIONAL FOODS, NUTRACEUTICALS & HEALTH

Antioxidants and Functional Components in Aquatic Foods	Kristinsson	9780813813677
Food Oligosaccharides: Production, Analysis and Bioactivity	Moreno-Fuentes	9781118426494
Novel Plant Bioresources: Applications in Food, Medicine and Cosmetics	Gurib-Fakim	9781118460610
Functional Foods and Dietary Supplements: Processing Effects and Health Benefits	Noomhorm	9781118227879
Food Allergen Testing: Molecular, Immunochemical and Chromatographic Techniques	Siragakis	9781118519202
Bioactive Compounds from Marine Foods: Plant and Animal Sources	Hernández-Ledesma	9781118412848
Bioactives in Fruit: Health Benefits and Functional Foods	Skinner	9780470674970
Marine Proteins and Peptides: Biological Activities and Applications	Kim	9781118375068
Dried Fruits: Phytochemicals and Health Effects	Alasalvar	9780813811734
Handbook of Plant Food Phytochemicals	Tiwari	9781444338102
Analysis of Antioxidant-Rich Phytochemicals	Xu	9780813823911
Phytonutrients	Salter	9781405131513
Coffee: Emerging Health Effects and Disease Prevention	Chu	9780470958780
Functional Foods, Nutraceuticals & Disease Prevention	Paliyath	9780813824536
Nondigestible Carbohydrates and Digestive Health	Paeschke	9780813817620
Bioactive Proteins and Peptides as Functional Foods and Nutraceuticals	Mine	9780813813110
Probiotics and Health Claims	Kneifel	9781405194914
Functional Food Product Development	Smith	9781405178761

INGREDIENTS

Fats in Food Technology, 2nd Edition	Rajah	9781405195423
Processing and Nutrition of Fats and Oils	Hernandez	9780813827674
Stevioside: Technology, Applications and Health	De	9781118350669
The Chemistry of Food Additives and Preservatives	Msagati	9781118274149
Sweeteners and Sugar Alternatives in Food Technology, 2nd Edition	O'Donnell	9780470659687
Hydrocolloids in Food Processing	Laaman	9780813820767
Natural Food Flavors and Colorants	Attokaran	9780813821108
Handbook of Vanilla Science and Technology	Havkin-Frenkel	9781405193252
Enzymes in Food Technology, 2nd edition	Whitehurst	9781405183666
Food Stabilisers, Thickeners and Gelling Agents	Imeson	9781405132671
Glucose Syrups - Technology and Applications	Hull	9781405175562
Dictionary of Flavors, 2nd edition	DeRovira	9780813821351

FOOD SAFETY, QUALITY AND MICROBIOLOGY

Practical Food Safety	Bhat	9781118474600
Food Chemical Hazard Detection	Wang	9781118488591
Food Safety for the 21st Century	Wallace	9781118897980
Guide to Foodborne Pathogens, 2nd Edition	Labbe	9780470671429
Improving Import Food Safety	Ellefson	9780813808772
Food Irradiation Research and Technology, 2nd Edition	Fan	9780813802091
Food Safety: The Science of Keeping Food Safe	Shaw	9781444337228

Food Science and Technology from Wiley Blackwell

Decontamination of Fresh and Minimally Processed Produce	Gomez-Lopez	9780813823843
Progress in Food Preservation	Bhat	9780470655856
Food Safety for the 21st Century: Managing HACCP and Food Safety throughout the Global Supply Chain	Wallace	9781405189118
The Microbiology of Safe Food, 2nd edition	Forsythe	9781405140058

SENSORY SCIENCE, CONSUMER RESEARCH & NEW PRODUCT DEVELOPMENT

Olive Oil Sensory Science	Monteleone	9781118332528
Quantitative Sensory Analysis: Psychophysics, Models and Intelligent Design	Lawless	9780470673461
Product Innovation Toolbox: A Field Guide to Consumer Understanding and Research	Beckley	9780813823973
Sensory and Consumer Research in Food Product Design and Dev, 2nd Ed	Moskowitz	9780813813660
Sensory Evaluation: A Practical Handbook	Kemp	9781405162104
Statistical Methods for Food Science	Bower	9781405167642
Concept Research in Food Product Design and Development	Moskowitz	9780813824246
Sensory and Consumer Research in Food Product Design and Development	Moskowitz	9780813816326

FOOD INDUSTRY SUSTAINABILITY & WASTE MANAGEMENT

Food and Agricultural Wastewater Utilization and Treatment, 2nd Edition	Liu	9781118353974
Sustainable Food Processing	Tiwari	9780470672235
Food and Industrial Bioproducts and Bioprocessing	Dunford	9780813821054
Handbook of Sustainability for the Food Sciences	Morawicki	9780813817354
Sustainability in the Food Industry	Baldwin	9780813808468
Lean Manufacturing in the Food Industry	Dudbridge	9780813810072

FOOD LAWS & REGULATIONS

Guide to US Food Laws and Regulations, 2nd Edition	Curtis	9781118227787
Food and Drink - Good Manufacturing Practice: A Guide to its Responsible Management (GMP6), 6th Edition	Manning	9781118318201
The BRC Global Standard for Food Safety: A Guide to a Successful Audit, 2nd Edition	Kill	9780470670651
Food Labeling Compliance Review, 4th edition	Summers	9780813821818

DAIRY FOODS

Lactic Acid Bacteria: Biodiversity and Taxonomy	Holzappel	9781444333831
From Milk By-Products to Milk Ingredients: Upgrading the Cycle	de Boer	9780470672228
Milk and Dairy Products as Functional Foods	Kanekian	9781444336832
Milk and Dairy Products in Human Nutrition: Production, Composition and Health	Park	9780470674185
Manufacturing Yogurt and Fermented Milks, 2nd Edition	Chandan	9781119967088
Sustainable Dairy Production	de Jong	9780470655849
Advances in Dairy Ingredients	Smithers	9780813823959
Membrane Processing: Dairy and Beverage Applications	Tamime	9781444333374
Analytical Methods for Food and Dairy Powders	Schuck	9780470655986
Dairy Ingredients for Food Processing	Chandan	9780813817460
Processed Cheeses and Analogues	Tamime	9781405186421
Technology of Cheesemaking, 2nd edition	Law	9781405182980

SEAFOOD, MEAT AND POULTRY

Seafood Processing: Technology, Quality and Safety	Bozaris	9781118346211
Should We Eat Meat? Evolution and Consequences of Modern Carnivory	Smil	9781118278727
Handbook of Meat, Poultry and Seafood Quality, second edition	Nollet	9780470958322
The Seafood Industry: Species, Products, Processing, and Safety, 2nd Edition	Granata	9780813802589
Organic Meat Production and Processing	Ricke	9780813821269
Handbook of Seafood Quality, Safety and Health Effects	Alasalvar	9781405180702

BAKERY & CEREALS

Oats Nutrition and Technology	Chu	9781118354117
Cereals and Pulses: Nutraceutical Properties and Health Benefits	Yu	9780813818399
Whole Grains and Health	Marquart	9780813807775
Gluten-Free Food Science and Technology	Gallagher	9781405159159
Baked Products - Science, Technology and Practice	Cauvain	9781405127028

BEVERAGES & FERMENTED FOODS/BEVERAGES

Encyclopedia of Brewing	Boulton	9781405167444
Sweet, Reinforced and Fortified Wines: Grape Biochemistry, Technology and Vinification	Mencarelli	9780470672242
Technology of Bottled Water, 3rd edition	Dege	9781405199322
Wine Flavour Chemistry, 2nd edition	Bakker	9781444330427
Wine Quality: Tasting and Selection	Grainger	9781405113663

PACKAGING

Handbook of Paper and Paperboard Packaging Technology, 2nd Edition	Kirwan	9780470670668
Food and Beverage Packaging Technology, 2nd edition	Coles	9780813812748
Food and Package Engineering	Morris	9780813814797
Modified Atmosphere Packaging for Fresh-Cut Fruits and Vegetables	Brody	9780813812748

For further details and ordering information, please visit www.wiley.com/go/food

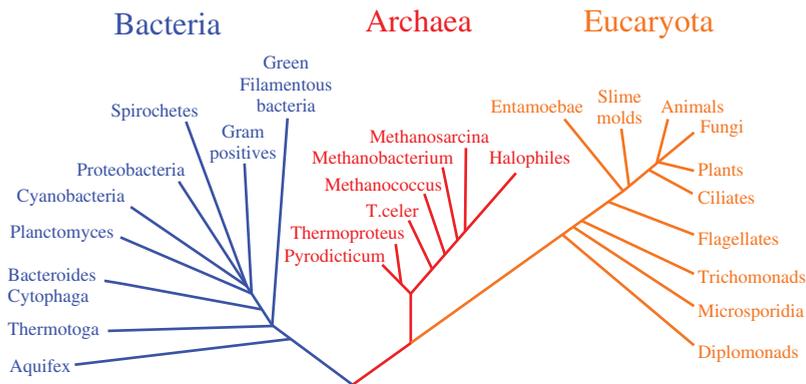


Figure 1.2 Concept of three life domains based on rRNA data, showing the separation of bacteria, archaea, and eukaryotes. *Source:* Wikipedia (June, 2007); http://en.wikipedia.org/wiki/Three-domain_system.

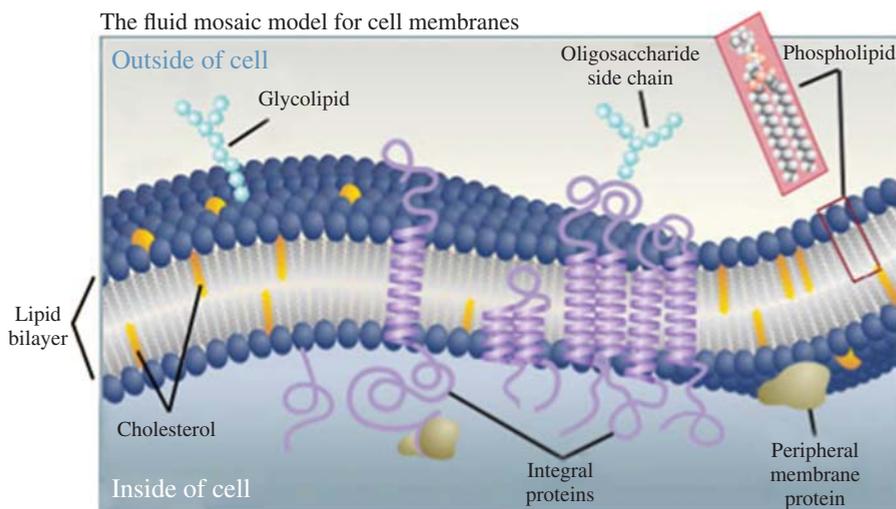


Figure 1.3 Fluid mosaic model of the structure of a membrane. *Source:* http://www.biology.arizona.edu/cell_bio/problem_sets/membranes/fluid_mosaic_model.html.

Some typical cells

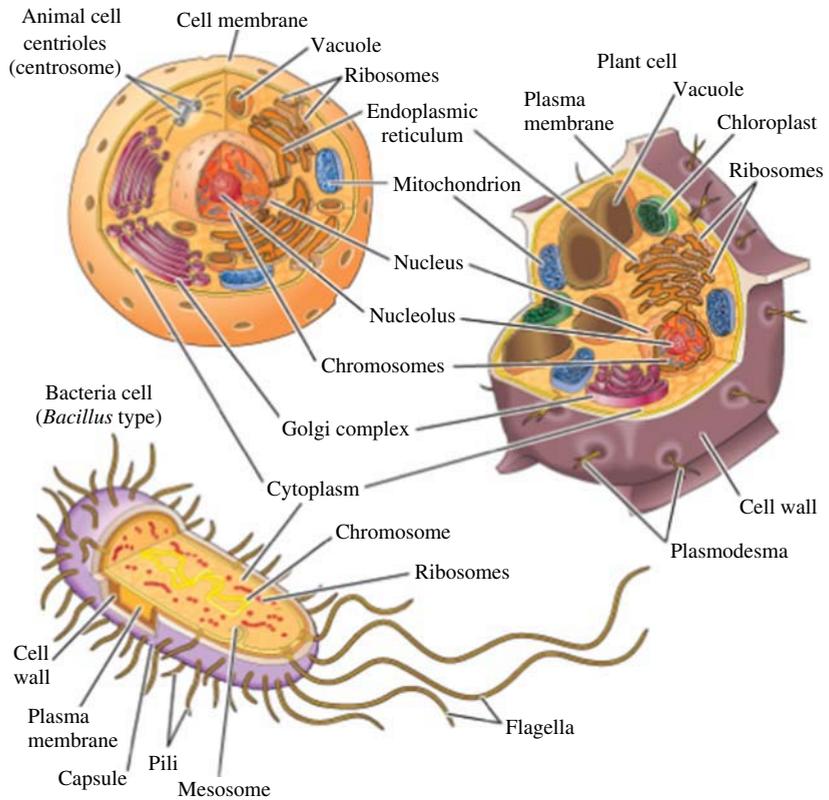


Figure 1.4 Anatomy differences of typical animal, plant, and bacterial cells. *Source:* Reprinted with permission from Encyclopædia Britannica, © 2010 by Encyclopædia Britannica, Inc.

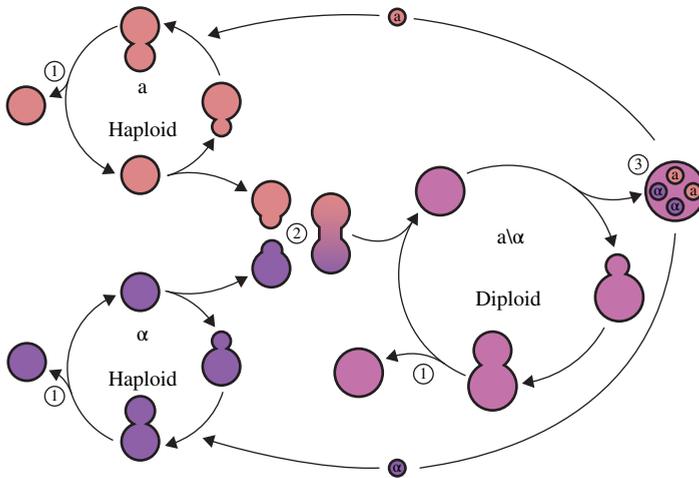


Figure 1.5 Sexual reproduction in the yeast life cycle. *Source:* http://en.wikipedia.org/wiki/File:Yeast_lifecycle.svg.

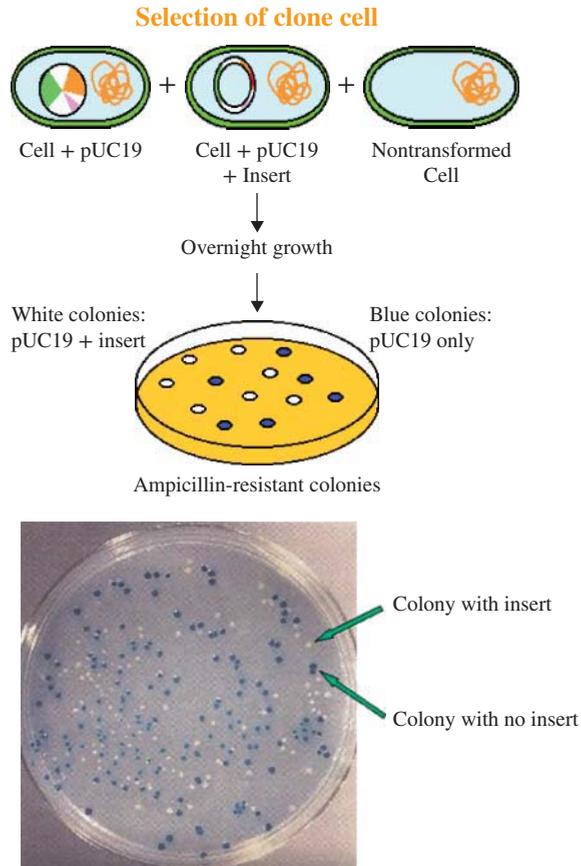


Figure 2.15 Selection of positive β -galactosidase clone which shows white colonies on agar plates containing ampicillin and X-gal. Animation on “Construction of a plasmid vector and cloning of β -galactosidase” and quiz (http://highered.mcgraw-hill.com/sites/0072556781/student_view0/chapter14/animation_quiz_2.html) can be viewed.

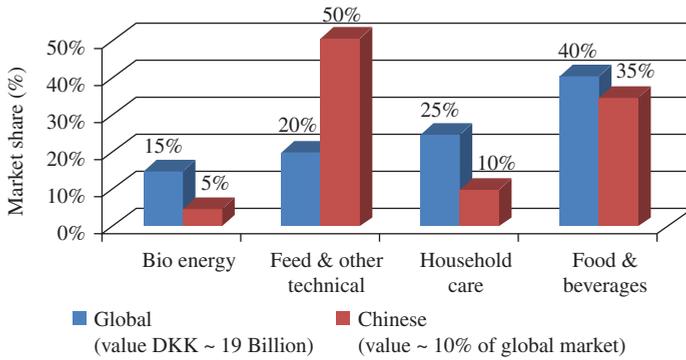


Figure 5.1 Global versus Chinese enzyme market in 2010. *Source:* Adapted from Li, S, et al. 2012. Technology prospecting on enzymes: application, marketing and engineering. *Comput. Struct. Biotechnol. J.* 2:1–11, e201209017, <http://dx.doi.org/10.5936/csbj.201209>.

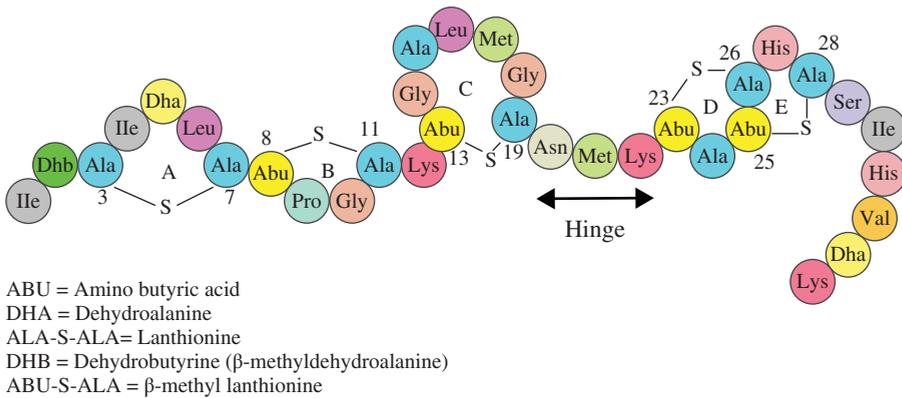


Figure 5.17 Structure of nisin. *Source:* <http://hu.wikipedia.org/wiki/Nizin>. Reproduced under a Creative Commons License.

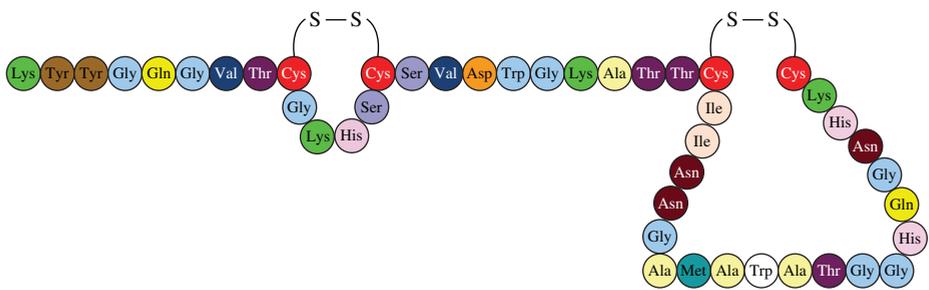


Figure 5.18 Structure of Pediocin PA-1. Source: Desriac et al., 2010. *Marine Drugs* 8:1153–1177.

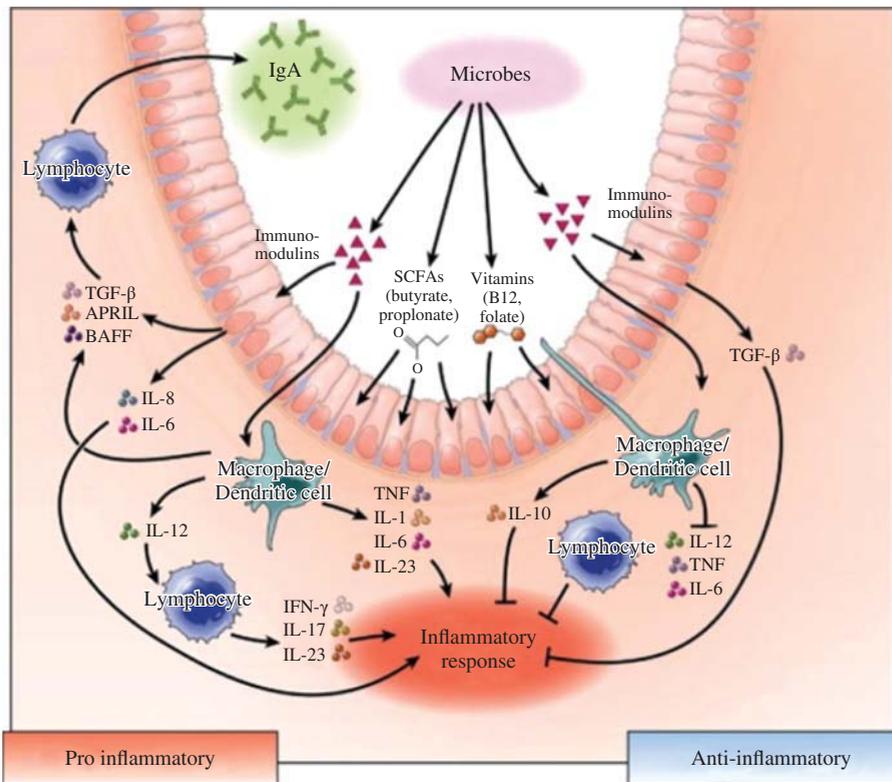


Figure 5.20 Mechanisms of immunomodulation by beneficial microbes. Probiotics can modulate the immune system in the intestine through the luminal conversion process. The bacteria produced secreted soluble factors and metabolites, such as short-chain fatty acids (SCFAs) and vitamins using substrates from the diet. These bioactive compounds can affect the function of intestinal epithelium and mucosal immune cells, resulting in production of cytokine and related factors such as a proliferation-inducing ligand (APRIL) and B-cell activating factor (BAFF). *Source:* Adapted from Preidis and Versalovic, 2009. *Gastroenterology* 136: 2015–2031. Reproduced with permission of Elsevier.

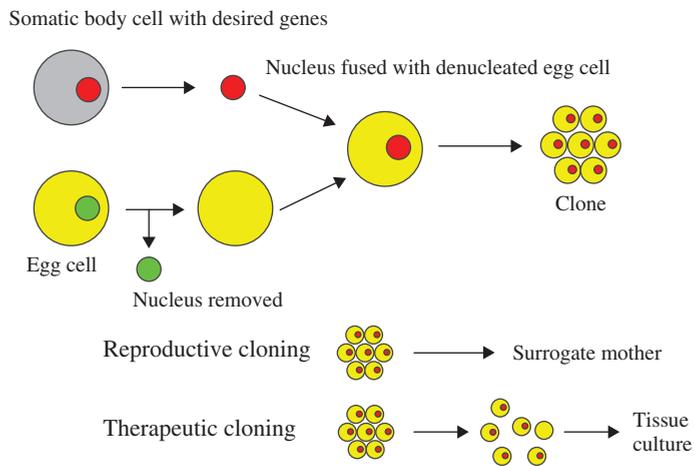


Figure 6.5 Somatic-cell nuclear transfer. *Source:* http://en.wikipedia.org/wiki/Somatic-cell_nuclear_transfer. Reproduced under a Creative Commons License.

WILEY END USER LICENSE AGREEMENT

Go to www.wiley.com/go/eula to access Wiley's ebook EULA.