WILEY-VCH

Edited by David B. Wilson, Hermann Sahm,
Klaus-Peter Stahmann, and Mattheos Koffas

Industrial Microbiology

Industrial Microbiology

Industrial Microbiology

Edited by
David B. Wilson, Hermann Sahm,
Klaus-Peter Stahmann, and Mattheos Koffas

WILEY-VCH

Editors

Prof. Dr. David B. Wilson (†)
Cornell University
Department of Molecular Biology and Genetics
Ithaca, New York 14850
United States of America

Prof. Dr. Hermann Sahm
University of Düsseldorf
Universitätsstr. 1
40225 Düsseldorf
Germany

Prof. Dr. Klaus-Peter Stahmann
Brandenburg University of Technology Cottbus-Senftenberg
Institute for Biotechnology
Universitätsplatz 1
01968 Senftenberg
Germany

Prof. Dr. Mattheos Koffas
Rensselaer Polytechnic Institute
Biological Sciences
110 8th Street
Troy, New York 12180
United States of America

Cover
Image credits: Fluorescence of riboflavin in fungal hyphae (Courtesy of Klaus-Peter Stahmann) and industrial fermenter (Courtesy of martinjoppen.de).

Translation of Chapters 1, 4, 6, 7, 9, and 13 from the German language edition:
Industrielle Mikrobiologie
edited by Hermann Sahm, Garabed Antranikian, Klaus-Peter Stahmann and Ralf Takors

Library of Congress Card No.:
applied for

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library.

Bibliographic information published by the Deutsche Nationalbibliothek
The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available on the Internet at <http://dnb.d-nb.de>.

Print ISBN: 978-3-527-34035-4
ePDF ISBN: 978-3-527-69729-8
ePub ISBN: 978-3-527-69731-1

Cover Design Adam-Design, Weinheim, Germany
Typesetting SPi Global, Chennai, India
Printing and Binding

Printed on acid-free paper

10 9 8 7 6 5 4 3 2 1

This book is dedicated to
Nancy, Allison, Ashley, and Laurie
Ursel, Matthias, and Andreas
Sandra, Samira, and Falk
Amalia, George, and Thomas.

Contents

Preface

"Nature is by far the best chemist and the best engineer. Nature also has the best engineering process: evolution."

Dr. Frances H. Arnold,
Linus Pauling Professor of Chemical Engineering at Caltech,
2018 Nobel Laureate in Chemistry.

In the endeavor of the chemical industry to reduce dependence on fossil raw materials, the application of microorganisms contributes to an increasing extent. Not only bacteria and fungi but also archaea are able to explore renewable resources efficiently and environmentally friendly and convert them into sustainable products. As an innovative cross-disciplinary field, the application of industrial microbiology will gain importance not only in the traditionally related areas of food and pharmaceutical industry but also increasingly in the chemical industry. Today, the global market for microbial products is in the order of 10^{11} US$. In many states, funding programs are running to replace significant proportions of chemical processes with biological ones.

The future potential of industrial microbiology lies in the fact that it bundles the know-how of biologists, chemists, engineers, and bioinformaticians. This leads to a quality that no specialist can achieve on their own. In recent decades, microbiology, especially by the successful approaches of molecular biologists, has developed fundamentally. The foundation of microbial strain development was and is still random mutagenesis and subsequent selection. However, the modern methods of genetic engineering lead to a targeted change in production strains, down to the position of a single base pair in the DNA, more quickly and accurately. This discipline called Metabolic Engineering is not only suitable to overproduce metabolites, but, in the form of the so-called Synthetic Microbiology, will also help to become independent from secondary metabolism of rare organisms such as plants, fungi, or unculturable bacteria. Not a single one of the dangers of genetic engineering feared in the 1980s become true. On the contrary, drugs produced by Genetically Modified Organisms (GMOs) fill the shelves in pharmacies and are safe and successful.

This textbook is an update of a German edition published by SPRINGER in 2013. Experienced scientists working at universities, research units, or in industry report selected aspects concerning successfully applied processes of industrial microbiology. Representative examples show which processes lead to recyclable

materials of special quality. In the first two chapters, a historical overview is given first (Chapter 1) followed by an introduction to process engineering (Chapter 2). Both chapters are of paramount importance. As food is the most important commodity to the reader as a human being, it will be discussed as the first product group (Chapter 3), allowing the students to rediscover it from a new perspective. In Chapter 4, "Technical Alcohols and Ketones" as well as in Chapter 5 "Organic Acids", it becomes clear that yields related to sugar as a substrate reach around 100%. In the production of L-enantiomers of amino acids (Chapter 6), the high selectivity of enzymes is most important. The importance of vitamins (Chapter 7) and antibiotics (Chapter 8) is well known. About 10^5 tons of vitamin C per year are produced with the help of bacteria. The penicillins excreted by fungi, cultivated in steel vessels as large as houses, exceed an annual market value of 10^{10} US$. In Chapter 9, the realization of the great promise of industrial microbiology becomes clear. With the help of genetic engineering pharmaceutical proteins, human-identical insulin and even analogues with improved active profiles can be produced by microorganisms on an industrial scale, so that the needs of more than 10^8 diabetics can be met. This not only means availability in principle but also affordability. Microbially produced enzymes (Chapter 10) are used in a wide range of applications. Today, everyone can use protease-containing detergents at home, e.g. for washing or in tiny amounts to clean contact lenses. Large companies in the United States apply bacterial amylases to hydrolyze more than 10^9 bushels (about 10^8 tons) of corn starch annually, which can then be used in other microbial processes, e.g. by brewer's yeast for the production of 10^{10} gallons (about 10^9 liters) of fuel alcohol. Microorganisms are also used in the production of polysaccharides (Chapter 11). Xanthan for example is added as a thickener to food products such as ketchup. In order to modify steroids for the production of cortisone or contraceptives, microorganisms are used for regioselective biotransformations in multistep processes (Chapter 12). As hydrometallurgy can be accelerated by iron- and sulfur-oxidizing bacteria, both, not only vessel-based but also open pit mining in kilometer scale is increasing to extract copper even from sources where classical techniques are inefficient (Chapter 13). In Chapter 14, highly developed waste water treatment plants are described, where microorganisms not only have a high potential for biosynthesis but also are suitable for degradation. In the future, we will be well advised to not only produce substances but also to consider during the design phase how microorganisms can quickly degrade them in order to prevent their accumulation in any environment. As microorganisms play key roles in nature's material cycle, they might become more important to close cycles urgently needed for human economy.

We are grateful to our colleagues who contributed to this textbook by writing their chapters. It was a pleasure for us to cooperate with internationally recognized scientists. Our colleagues in industry deserve special praise for sacrificing nights or weekends for their contributions. Sometimes, graphically presented relationships had to be simplified in their complexity without getting wrong. We thank Susanne Nieland, MSc, who did not give up until both discussion partners, authors and editors, were satisfied with a recognizable focus of a black and white or a rarely colored graphic. Furthermore, we are grateful to WILEY-VCH,

especially Dr. Frank Weinreich and Dr. Andreas Sendtko, for their help and patience because more than one round was needed to reach the wished quality.

Sadly, during the development of this book, our first editor, David, fell seriously ill and was not able to continue working with us any longer. The idea of an American–German coedition was born after an invited talk David gave at a special meeting of the German Association of General and Applied Microbiology in Senftenberg. David was a very generous and thoughtful colleague, researcher, and teacher who was a pioneer in the study of cellulases and was devoted to the goal of deriving clean fuels from plants. We are very pleased that his efforts helped to bring about this textbook and hope that we helped our authors explain the topics selected in a way that both undergraduate and graduate students can understand. As science and engineering develop at an increasingly rapid pace, causal explanations can only be given for selected topics. We strongly support efforts to discuss open issues in seminars as many outstanding questions remain, e.g. the reason for citrate overproduction of *Aspergillus niger*.

We also hope that this textbook will arise the interest of many students of natural sciences and engineering. We are convinced that industrial microbiology will continue to be a success and hope that our book will help both our teaching colleagues and very young people to make their own contributions, whether at a research or teaching institution or in an industry.

Summer 2019

Hermann Sahm
Klaus-Peter Stahmann
Mattheos Koffas

1

Historical Overview and Future Perspective

Bernhard Eikmanns[1], *Marcella Eikmanns*[2], *and Christopher J. Paddon*[3]

[1] *University of Ulm, Microbiology and Biotechnology, Albert-Einstein-Allee 11, 89081 Ulm, Germany*
[2] *Ochsengasse 34, 89077 Ulm, Germany*
[3] *Amyris Inc., 5885 Hollis St, Suite 100, Emeryville, CA 94608, USA*

CHAPTER MENU

1.1 Use of Fermentation Procedures Before the Discovery of Microorganisms (Neolithic Era = New Stone Age Until 1850)

The origins of industrial microbiology go back to prehistoric times, as human beings began to learn more about food spoilage, preservation, and storage. Based on their experiences, they developed diverse methods for preserving and refining foods. As we now know, many of these procedures are based on chemical changes brought about by microorganisms. These methods were progressively refined over time and applied in larger scales. Empirical knowledge was initially passed on verbally and later in a written form. Artisans made use of the various fermentation properties of microorganisms, being unaware of the microorganisms involved and the (micro-)biological and biochemical processes taking place. It was not possible to identify the microorganisms or explain the mechanisms of the chemical changes they caused until around 1850. Table 1.1 provides a historical summary of food manufacturing products and procedures as well as microbiological discoveries up to 1850.

Industrial Microbiology, First Edition.
Edited by David B. Wilson, Hermann Sahm, Klaus-Peter Stahmann, and Mattheos Koffas.

Table 1.1 Microbiological procedures and important discoveries from prehistoric times (Neolithic Era) to 1850.

Period/year	Procedures/products/discoveries
Prior to 4000 BC	Finds from Mesopotamia and from regions south of the Alps prove that flatbread was prepared from a grain pulp and then baked. It is likely that yeast dough is already unknowingly used, as it results in lighter, more flavorful bread.
From 4000 BC	The first sources show that the Sumerians in Mesopotamia and shortly thereafter the Egyptians, use grain pulp for beer production and sugary fruit juices for wine production.
From 3000 BC	In Mesopotamia and Egypt, sour dough bread and sour milk products (cheese) are produced. Vinegar is used as a preservative.
From 2000 BC	In Asia (China, Japan), soybeans are fermented with the help of fungi and bacteria (soy sauce) and rice wine is produced. In Egypt, beer brewing is "refined." Babylon's King Hammurabi (1728–1686 BC) issues strict beer laws in the "Code of Hammurabi."
From 1300	Saltpeter production: throughout Europe, excrements were converted to potassium nitrate needed for gunpowder. It was unknown that microorganisms were the catalysts.
Around 1680	Van Leeuwenhoek discovers and describes bacteria and yeasts by use of self-prepared simple microscopes.
1789	Lavoisier identifies the products of alcoholic fermentation.
1837/1838	Cagniard-Latour, Schwann, and Kützing attribute alcoholic fermentation to living yeasts, which divide themselves by means of budding.

The beginning of the New Stone Age (Neolithic Era, Neolithic Revolution) is marked by the transition from a nomadic lifestyle, centered on collecting wild plants and hunting wild game, to a farming lifestyle based on food production (agriculture and livestock breeding) and storage. In the Fertile Crescent (a crescent-shaped region in the Middle East, which includes parts of what is now Israel, Lebanon, Syria, Turkey, Iraq, and Iran), this radical change in subsistence occurred around 9000 BC. Goats, sheep, and cattle were domesticated, and barley, emmer wheat, and one-grain wheat were bred from wild grasses. The wine grape was also cultivated in this area. In other parts of the world, agriculture and livestock breeding practices were not established until later on, and they were often based on different kinds of livestock and crops.

It can be assumed that as soon as human beings adopted a settled lifestyle in the Fertile Crescent, they began to experiment with producing alcoholic beverages. Here, it is important to distinguish between the production of wine and the production of beer. Wine is made from sugary liquids (fruit juices, but also diluted honey). When fruit gets smashed or fruit juice is left standing, fermentation sets in quite quickly. This is due to the fact that sugar-consuming yeasts are naturally found on the skins of sweet fruits. With beer, on the other hand, the raw material is grain. Here, the fermentable sugar must first be released from the starch.

Grain was originally consumed in pulp form, prepared using water and crushed grains. Later, the pulp was formed into a flatbread and then baked. This advancement allowed the bread to be stored longer and made it easier to be transported. Microorganisms (yeast and lactic acid bacteria) were already being used in ancient Egypt (3000 BC – 395 AD) to prepare poriferous bread. The ancient Egyptians, also known in antiquity as "bread eaters," observed that bread became lighter and more easily digestible when the bread dough was left standing for some time before it was baked. The process of making beer by means of fermentation of either liquid bread dough or bread that has been baked and then soaked in water has been in practice since approximately 5000 BC. The first records of beer production are about 5500 years old and come from the Sumerians, who resided in Mesopotamia, which is now Iraq, between 5000 and about 1800 BC. Clay tablets were found that show how grain (barley and emmer wheat) was shucked and ground and how the flour was transformed into a flatbread, which was then baked and used to produce beer (called "kasch" or "bread beer"). Clay vessels were used for the fermentation of the flour cakes, which were baked and then kept moist. The success of the fermentation was dependent on the randomly incorporated microorganisms and the conditions at hand for each given attempt. Honey, cinnamon, and other spices were added to the beer; it was therefore generally sweet and could certainly not be stored for a very long period of time.

In Egypt, beer was also being produced out of bread dough by 2500 BC ("henket"; Figure 1.1). It is unclear, however, whether the Egyptians adopted the brewing trade from the Sumerians or developed it themselves. In 1990, a 3300-year-old brewery belonging to King Echnaton (reign: 1351–1334 BC) was excavated. The findings included intact clay vessels, instruments, and ingredients preserved by the dry heat (malt, grain, and dates). From these findings, researchers were able to deduce that the Egyptians had mastered malt preparation (germination and initiation of enzyme formation) and mashing (enzymatic conversion of starch into sugar under optimized conditions) and that they had used these techniques in making beer.

In 1902, a pillar more than 2 m high and consisting of green diorite was found in Susa, in present-day Iraq, which dates back to the Babylonian King Hammurabi (reign 1728 to 1686 BC). Today, the pillar is on display in the Louvre in Paris (see Figure 1.2). The stone stele depicts the King of Shamash, the Babylonian god of law and justice. The text engraved in the pillar, the so-called "Code of

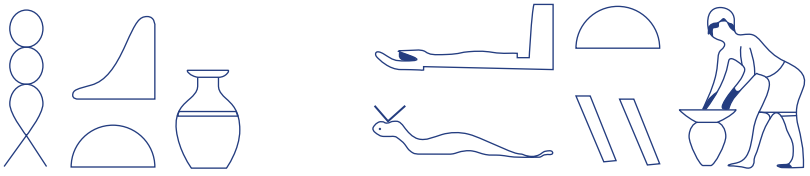


Figure 1.1 More than 3000 year-old Egyptian hieroglyphica, credentials of unconscious use of microorganisms for beer production at ancient times.

(a) (b)

Figure 1.2 Pillar with the Code of Hammurabi (a) and cutout of the engraved cuneiform scripts (b).

Hammurabi," represents the oldest set of laws in the world. These provisions address a variety of matters in both the public and private sector, including a series of exceedingly strict laws concerning the production and trading of beer. These laws stipulate quality requirements and establish the maximum prices allowed for about 20 varieties of beer. The Code also details the punishments to be administered to those who violate the laws of beer production. If a brewer was caught watering down his beer, for instance, he was to be drowned in his own beer barrels. The same punishment applied to innkeepers who allowed patrons to pay for their beer with silver rather than grain. Hammurabi's laws also dealt with the subject of wine. The Code of Hammurabi refers to wine as one of the earth's most valuable gifts to be handled with love, respect, and esteem. As was the case with beer, these provisions lay out fixed prices and punishments for violating the laws of wine production and trade.

Similar to beer production, the cultivation of grapevines and fermentation of grape juice to produce wine can also be traced back to the early cultures inhabiting the Fertile Crescent. The complicated production process for wine meant that it was considerably more expensive than beer. Wine therefore remained a privilege for the upper class of society until about 1000 BC. In ancient Egypt and Mesopotamia, wine was also processed into vinegar, which was then used as a seasoning or – in diluted form – a beverage. The first fermentation of soybeans dates back to this time period as well. Sake, or rice wine, also falls into the category of beer-like beverages as it is produced from a grain – rice in this case. Records from what is today China, and later from Japan, prove that sake was being produced in Asia by 2000 BC.

As human beings began adopting a settled lifestyle, a long period ensued in which food fermentation processes were refined, expanded, and passed on to other regions. Until far into the Middle Ages, however, there are no reports of new processes emerging for using microorganisms in daily life.

During the late Middle Ages (from roughly 1300), saltpeter manufacturing took root in Europe, delivering potassium nitrate (KNO_3, saltpeter) for gunpowder production. Nitrate is formed out of organically bound nitrogen in soil, with the help of nitrifying bacteria. To begin with, surfaces saturated with human and animal excrement served as the starting material for saltpeter production. Later, urine and blood were used as direct nitrogen sources in saltpeter huts and the nitrification was kept in progress in well-ventilated beds (Figure 1.3). Microbial production was abandoned in the nineteenth century, when large natural deposits of saltpeter were discovered in Chile.

A significant development in the empirically proven process of microbial production was the Orléans Process, which was established in the fourteenth century. With this method, vinegar was produced in large, open vessels in warm rooms. The large surface area of contact with the air provided the acetic acid bacteria, which were collecting on the surface, with ample amounts of oxygen. It was already evident to scientists at this time that adequate ventilation increased the effectiveness of the process. Since the nineteenth century, acetic acid has been produced either via the "round pump procedure" (a variation on rapid vinegar manufacturing in a trickle bed; Figure 1.4) or the submersion procedure, i.e. in a liquid culture, made from wine, brandy, and fermented fruit, with intensive ventilation.

With the commencement of the modern age (approximately 1500) and the breakthrough of the natural sciences in the seventeenth century, scientists began to examine natural phenomena systematically, by means of observation, experimentation, and measurement. Academies were established with the specific purpose of providing a forum to present and discuss the results of this natural research. The findings were also published in scientific journals. The emerging modern sciences of physics and chemistry provided the tools, along with

Figure 1.3 Saltpeter manufacture toward the end of the middle ages. Heaps of earth, soaked with animal and human excrements and blood were aerated with picks and rakes. The ammonium (and ammonia, respectively) released from the nitrogen-containing organic matter was oxidized by aerobic nitrifying bacteria to nitrate (NO_3^-). Nitrate then was leached out as potassium or sodium nitrate. The depleted soil was recovered and recycled. Source: Reproduced from "Berg- und Probirbuch" by Lazarus Erker (1574).

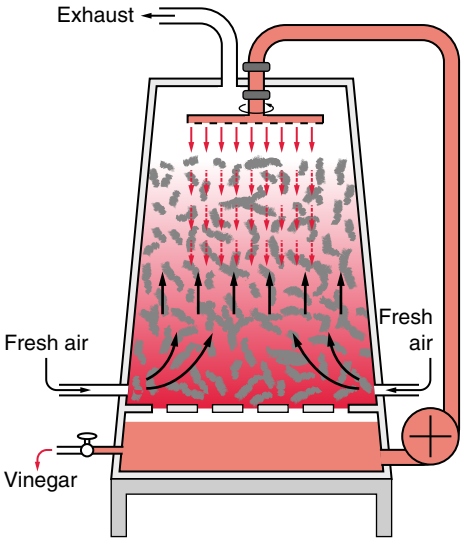


Figure 1.4 Acetic acid production from nineteenth century on. The vessel is filled with beechwood chips, grown over with acetic acid bacteria. 6 – 10% alcohol was trickled from the top of the vessel, at the outlet (bottom) 4 – 10% acetic acid was collected. Aeration was provided by air counterflow to supply the microorganisms with oxygen.

numerous technical inventions and innovations (e.g. lens grinding technology), with which the production processes for bread, wine, beer, and vinegar were scientifically analyzed in the eighteenth and nineteenth centuries. From today's perspective, the works of Antonie van Leeuwenhoek (1632–1723) mark the beginning of microbiology. He was the first to observe various microorganisms, including bacteria, with the aid of a microscope equipped with only one lens. He described the "animalcules," as he referred to them, in great detail. Although the process of fermentation had already been in practice for a long time, it was not yet recognized that these microorganisms played a significant role.

Because of its huge practical relevance, alcoholic fermentation became the subject of numerous studies. At the beginning of the nineteenth century, scientists were able to describe the process in terms of both phenomenology and quantity. Antoine de Lavoisier (1742–1794) focused on sugar fermentation, identifying two products: alcohol and carbon dioxide. Joseph Gay-Lussac (1778–1850) identified the quantity relation of sugar (two mol each of alcohol and CO_2 per mol of sugar). Charles Cagniard-Latour (1777–1859), Theodor Schwann (1810–1882), and Friedrich Kützing (1807–1893), all supporters of the vitalist philosophy of the 1830s, collected proof that fermentation is sustained by living microorganisms. Independently of each other, they all arrived at the conclusion that yeast was responsible for alcoholic fermentation. One question, however, remained under dispute: did these living microorganisms appear spontaneously by means of abiogenesis or was an inoculum of unknown nature necessary to initiate the process? In opposition to the ideas of the vitalists, chemists Jöns Berzelius (1779–1848), Friedrich Wöhler (1800–1882), and Justus von Liebig (1803–1873) were of the opinion that fermentation involved purely chemical processes of decomposition. It was not until the 1850s that Pasteur's findings were able to settle this debate.

1.2 Investigation of Microorganisms and Beginning of Industrial Microbiology (1850 Until 1940)

Louis Pasteur (1822–1895, Figure 1.5) proved through experimentation that the fermentation processes common during his time were invariably linked to the specific microorganisms present and that the observed chemical changes were based on the physiological abilities of these microorganisms. Between 1856 and 1875, Pasteur studied the life cycle of yeasts and compared how they processed sugar in the presence and absence of oxygen. He also looked at bacterial fermentation (lactic acid and butyric acid fermentation) and the microorganisms responsible for it. He demonstrated that "failed fermentations," i.e. those that did not result in the formation of the desired product, could be traced back to contamination with other microorganisms (Figure 1.6). With the introduction of sterilization techniques (pasteurization), Pasteur established the necessary conditions for breeding microbial pure cultures.

The founding of modern microbiology is accredited to both Robert Koch (1843–1910), who demonstrated that infectious diseases such as anthrax, typhus, and cholera were caused by bacterial pathogens, and Louis Pasteur. Industrial microbiology also has its roots in Pasteur's research from 1850. Industrial microbiology refers to the section of microbiology that relates to the microorganisms used by humans to modify and produce substances, as well as the industrial procedures developed for this purpose. Table 1.2 provides an overview of the microbiological procedures between 1850 and 1940, as well as the relevant scientific discoveries in the fields of microbiology and biochemistry from the same period.

Toward the end of the nineteenth century, numerous public and private research institutes sprouted up throughout Europe, focusing on the use of fermentation in food production, food processing, quality control, hygiene, and

Figure 1.5 Portrait of Louis Pasteur at around 1885 (Painting by Albert Edelfeldt 1854–1905).

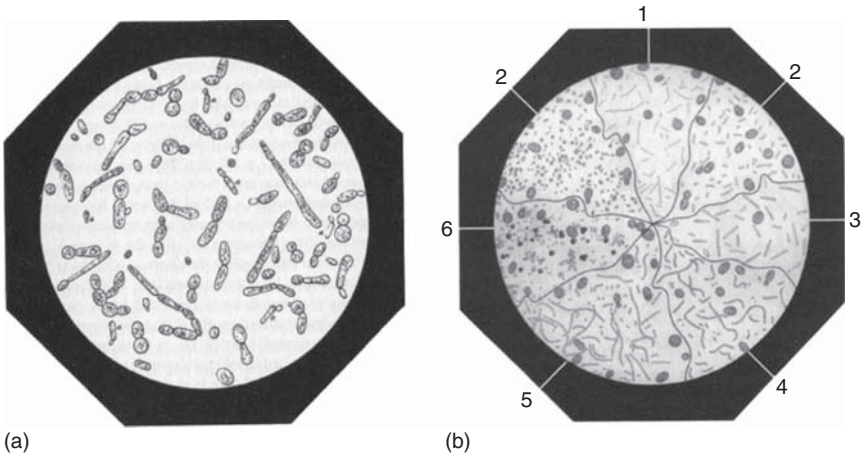


Figure 1.6 Yeast at the beginning of fermentation (a) and from "failed fermentations" (b). Drawing prepared by Pasteur (1876).

Table 1.2 Industrial applications of microbiological procedures and scientific discoveries from 1850 to 1940.

Period/year	Procedures/products/discoveries
1857–1877	Pasteur describes alcoholic fermentation, lactic acid, and butyric acid fermentation and explains the processes of wine and beer production. He introduces sterilization via "pasteurization" and other sterilization techniques for handling microorganisms.
1867	The "Vienna Process" is used for large-scale production of baker's yeast.
1870	Koch develops procedures for cultivating microorganisms and founds medical microbiology.
1877	Kühne introduces the term "enzyme" for temperature-sensitive, active ferments from living cells.
From 1881	Lactic acid is produced with the help of lactic acid bacteria.
1894	Fischer proves the specificity and stereoselectivity of enzymes.
1896	Buchner proves the existence of fermentation enzymes in yeast cell extract.
From approximately 1900	Municipal wastewater treatment plants are established in larger cities.
1915	Clostridia are used for large-scale production of acetone and butanol. Glycerin is produced with the help of yeasts from molasses.
From 1923	*Aspergillus niger* is used for large-scale production of citric acid.
1928	Fleming discovers penicillin and its effect on bacteria.
1939–1941	Penicillin is isolated and purified.

epidemic control. In the food industry, the growing understanding of microbial involvement triggered a technical innovation boom. Production was increasingly moved from craftsman's establishments to industrial facilities and there was a dramatic increase in the quantities being produced. In economic terms, the most significant impacts were observed in the areas of beer, wine, and alcohol production. In 1873, 3.6×10^9 l of beer was produced in Germany. In 1890, it was nearly double that amount. The wine production in Europe (mainly in France, Italy, and Spain) amounted to be roughly 11×10^9 l toward the end of the nineteenth century. The production of pure alcohol for consumption and industrial purposes went up to more than 5.9×10^8 l. This alcohol was mainly produced from molasses (a byproduct in the sugar industry), fruit, grains, and potatoes. Around two thirds of the alcohol produced was processed into high-proof alcoholic beverages.

The so-called Vienna Process became an established means of attaining baker's yeast in the second half of the nineteenth century. This process was particularly successful because it used bacteriologically pure cultures (inoculum) and followed a sterile proceeding. The yeast was bred in liquid culture (submersion culture). Rising CO_2 gas bubbles transported the yeast cells to the surface, where they were then extracted and rinsed with cold water. Using filter and threaded presses, most of the water was then removed from the yeast mass.

Lactic acid was produced via fermentation in 1881 with lactic acid bacteria. The company Boehringer Ingelheim took up industrial-scale production in 1895. Twelve years later, more than 20 other companies were also producing lactic acid out of sugar or sugar-containing substrates, primarily for the production of food, but also for applications in the emerging pharmaceutical industry. Increasing importance was placed on keeping substrates and culture vessels sterile before inoculation. Scientists also began to realize the importance of using pure cultures of the respective microorganism for the inoculations and monitoring the quality of the end product. New measures were taken to increase product yield: the culture conditions – for instance, the addition of oxygen – were regulated and the culture vessels were designed to comply with the requirements of the respective processes.

At the start of the twentieth century, efforts were made to scientifically explain the formation of butanol and acetone from carbohydrate-based substances with the help of microorganisms that were still unknown at that time. The aim was to enter into large-scale production with these substances. Butadiene, a basic compound used for the production of synthetic rubber, can be chemically synthesized from butanol. The increasing demand for rubber, which was amplified following the start of World War I, resulted in a shortage of natural rubber and an increase in price on the world market. Therefore, there was a tremendous economical interest in finding alternative methods of producing rubber. Acetone was needed as a solvent in the production of cordite, an explosive compound out of which ammunition fuses were produced. Chaim Weizmann (1874–1952), a chemist at Manchester University, director of the Ammunition Lab of the Royal British Admiralty from 1916 to 1919 and later the first president of Israel, identified and isolated the bacterium *Clostridium acetobutylicum*. He held important patents for the solvent fermentation catalyzed by these bacteria. As

a result, a large-scale fermentation production process for butanol and acetone was developed within a short time. Scientists from various fields (biologists, chemists, and process engineers) collaborated to develop this process, which used pure *C. acetobutylicum* cultures instead of undefined bacterial mixed cultures. For the most part, cornstarch was used as the starting material. The production facilities were primarily located in Great Britain, and then later in the United States and Canada. The historical details of the development, research, and use of the solvent fermentation process are discussed in Chapter 4.

In Germany, the start of World War I meant that industrial grease became scarce. The glycerol contained in industrial grease was required in vast quantities for producing explosives (nitroglycerin). A fermentation process was thus developed to produce glycerol with the help of yeast in production facilities specifically designed for this purpose. Molasses was used as a substrate. Adding sulfite considerably increased the yield of glycerol. Sulfite binds with the acetaldehyde, which is produced while the substance is being converted into alcohol, and thereby reduces the formation of ethanol. In 1916, more than 1000 tons of glycerol was produced with this method each month. In order to combat the shortage of feed imports in Germany during World War I, about 10 000 tons of feed yeast (*Candida utilis*) was produced as well, and here again, molasses served as a substrate.

Citric acid was produced in Europe for the first time in 1920. Large-scale production began in 1923, using the fungus *Aspergillus niger*. This fungus was cultivated by means of a surface procedure, i.e. in flat basins with a large area of contact surface between the culture and the air. This process was initially unsuccessful because of the fact that it was difficult to maintain sterile conditions in the flat culture vessels. The ultimate success was dependent on several changes: the selection of rapid growth strains, the optimization of the culture medium to accommodate the nutritional needs of the fungus, and the introduction of acid conditions (pH 3.5) for conducting the procedure right from the start. Shortly thereafter, starting in 1928, *A. niger* and *Penicillium* strains were then used for producing gluconic acid. Since roughly 1940, citric acid and gluconic acid have been primarily produced in submersion procedures, i.e. in a liquid culture, on a large scale and with air being piped in.

Since the end of the nineteenth century, wastewater treatment plants have been purifying municipal and industrial wastewater. In this field, microorganisms are responsible for the breakdown of organic compounds. The introduction of microbial wastewater processing reduced the risk of infection in increasingly populated areas and prevented the unpleasant smells that developed during natural decomposition in creeks, rivers, and especially in standing water. The first wastewater treatment plants originated in Europe and initially included only one aerobic level of decomposition, in which the wastewater was channeled over a substrate covered with microorganisms (trickling filters or aerobic fixed-bed reactor process). Starting in 1914, this oxidative decomposition step was the activated sludge process, in which flaky aggregated microorganisms (activated sludge) were added to wastewater and the mixture was then ventilated via stirring units. Digestion towers were also introduced for anaerobic conversion of the obtained sludge (secondary sludge). As early as the 1920s, the gas produced in the digestion tower was

being used for heating and lighting purposes. Wastewater processing is different from most microbial processes, in which scientists generally strive to use pure cultures. Even today, sewage treatment facilities deliberately use complex, naturally established microbial communities, which only fulfill their function as a stabile system because of their diversity.

Although Pasteur's work proved that microorganisms were responsible for the chemical changes in fermentation processes, it remained unclear into the 1890s exactly what was happening in the cells of the microorganisms and which biological structures were involved. Scientists were aware of "unformed, unorganized ferments" from plants, fruits, and the pancreas. As early as 1887, Wilhelm Kühne (1837–1900), a physiologist from Heidelberg, referred to these soluble, heat-sensitive structures as "enzymes." The "lock and key principle," however, which explained the specificity and stereoselectivity of enzymes, was not formulated until 1894 by Emil Fischer (1852–1919). The conversion of sugar into alcohol via cell-free yeast extracts (cell-free fermentation) was not proven until 1896 by Eduard Buchner (1860–1917). Buchner traced the activity to a soluble substance containing protein, which he called Zymase. As was later discovered, it actually involved the entire enzyme system of glycolysis and ethanol production from pyruvate. Ultimately, these studies were largely responsible for the subsequent rapid developments in biochemical and physiological research. These developments led to significant advancements in the first half of the twentieth century, including the identification of cell components (e.g. proteins and nucleic acids) and metabolic intermediates (e.g. ATP and coenzymes), as well as the elucidation of central metabolic pathways of glycolysis (Embden–Meyerhof–Parnas pathway) and the citric acid cycle (Krebs cycle). Although these studies were only partially conducted on microorganisms, their universally applicable results provided the basis for an understanding of metabolism in all living creatures – including microorganisms that are used in industrial production processes. Consequently, various techniques were developed for directly or indirectly influencing microbial metabolism in order to optimize production processes and yield. The new field of microbial enzyme technology emerged, thanks to biochemical research and the resulting understanding of protein structure and function as well as enzyme catalysis and kinetics. Microbial enzyme technology concerns the production of enzymes and their manifold uses – both in everyday life and for scientific applications.

1.3 Development of New Products and Procedures: Antibiotics and Other Biomolecules (From 1940)

The early 1940s mark the beginning of a chapter of expansion in the history of industrial microbiology, characterized by an abrupt widening in the spectrum of substances produced with the aid of microorganisms. An overview of the new products and microbial procedures can be found in Table 1.3. A prominent new class of substances emerging in the 1940s was antibiotics, i.e. compounds that are

Table 1.3 Microbial procedures with industrial application from approximately 1940.

Period/year	Procedures/products
From 1942	Large-scale production of penicillin with *Penicillium chrysogenum*
1946	Industrial production of streptomycin
From 1950	Further large-scale fermentation production of antibiotics, L-amino acids, vitamins, organic acids, and other compounds with the help of bacteria and fungi
	Large-scale establishment of microbial transformation in steroids
From 1960	Production of microbial enzymes (hydrolases), which are added to detergents
From 1965	Microbial production of rennet for cheese manufacturing
From 1966	Extraction of copper and uranium in the United States and especially in South America (Chile) with the help of microorganisms (bioleaching)
1977/1978	Recombinant *E. coli* strains developed for producing somatropin (human growth hormone) and insulin
1982	Introduction of insulin as first recombinant drug on the market
From 1985	Production of a multitude of recombinant proteins for the pharmaceutical industry as well as the detergent, paper, and textile industries and for scientific and diagnostic applications
From 1995	Use of recombinant microorganisms in the production of L-amino acids, vitamins, and industrial chemicals (e.g. polymer precursors)

formed by microorganisms and inhibit the growth of bacteria. Starting in 1950, a multitude of new compounds emerged with origins in microbial metabolism, such as L-amino acids, organic acids, and vitamins. Large-scale production of microbial enzymes started in 1960.

As had been the case with World War I, World War II also made a significant impact on the development of new products, as clearly demonstrated by the introduction of penicillin production in Great Britain. The springboard for the discovery was the search for new antibacterial methods for treating soldiers wounded in war. It was a well-known fact that bacterial infections could, in theory, be treated, as demonstrated in Germany in the 1930s, by the antibacterial effect of several compounds from the sulfonamide substance class. Outside of Germany, however, these chemically manufactured compounds were only available to a limited extent. Penicillin, the first known antibiotic, was discovered by Alexander Fleming (1881–1955) in England in 1928. In 1921, he had already proven the existence of the enzyme lysozyme in egg whites, which also has antibacterial effects. Fleming observed that the mold *Penicillium chrysogenum* excreted a substance that inhibited the growth of staphylococci (Figure 1.7). He was able to demonstrate that the compound he referred to as penicillin was effective against a range of other human pathogens as well. Although Fleming himself realized the potential of his discovery, his findings remained virtually unexploited until 1939. This was due to the difficulties involved in isolating and

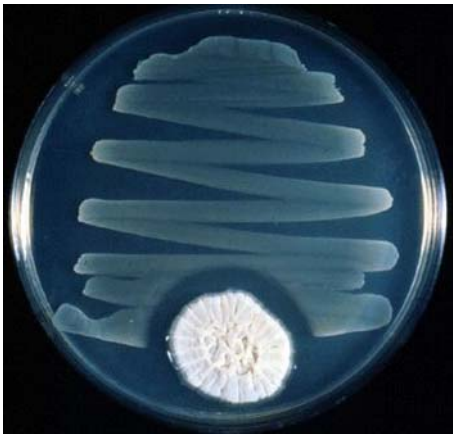


Figure 1.7 Reproduction of an agar plate (solid medium) contaminated with *Penicillium notatum*, randomly observed by Sir Alexander Fleming. *Staphylococcus aureus* do not grow in the neighborhood of the fungus as the penicillin concentration is above the minimal inhibitory concentration (with kind permission of Christine L. Case).

purifying penicillin because of its extremely low concentration in the culture supernatant.

During the war, Howard W. Florey, Ernst B. Chain, Norman Heatley, and their staff at Oxford University again addressed the issue of purifying penicillin in 1939. In 1941, they were successful in isolating a large amount of penicillin and establishing an activity test. Penicillin's effectiveness in treating bacterial infections was demonstrated in a small-scale clinical study. In order to convert penicillin production from a laboratory procedure to an industrial-scale process, the biochemists from Oxford sought out help in 1941 from other scientific institutions, government agencies, and industrial partners in the United States. A cooperative effort began, which brought together microbiologists, biochemists, chemists, physicians, pharmacologists, and process engineers from England and the United States. The coordinators were academic and industrial managers as well as government agencies. Penicillin exploitation and concentration increased immensely (from 3 to 1500 units/ml, Figure 1.8) with the isolation of new strains and further development of familiar strains. Other important factors in these advancements were the optimization of the culture medium composition (e.g. the use of corn steep liquor and the addition of lactose) and the optimization of the culture conditions. Pilot and industrial facilities were constructed for the surface cultivation and continuous submersion cultivation of *P. chrysogenum,* and suitable technical process management was developed for each type of cultivation. The biggest practical problems were encountered in attempting to keep the fermentation facilities free of contamination. Several factors increased the risk of contamination: the large scale, long incubation time, intensive ventilation, and numerous fittings, valves, pipes, and instruments in and on the facilities. It also became necessary to optimize the processing of the relatively sensitive penicillin (downstream processing: isolation and purification), the product analysis (identification of yield, purity, and activity), and the product formulation (conversion to a product that could be stored and transported). These aspects then needed to be converted for industrial-scale applications as well. As a result of these efforts, several companies began large-scale production of penicillin in

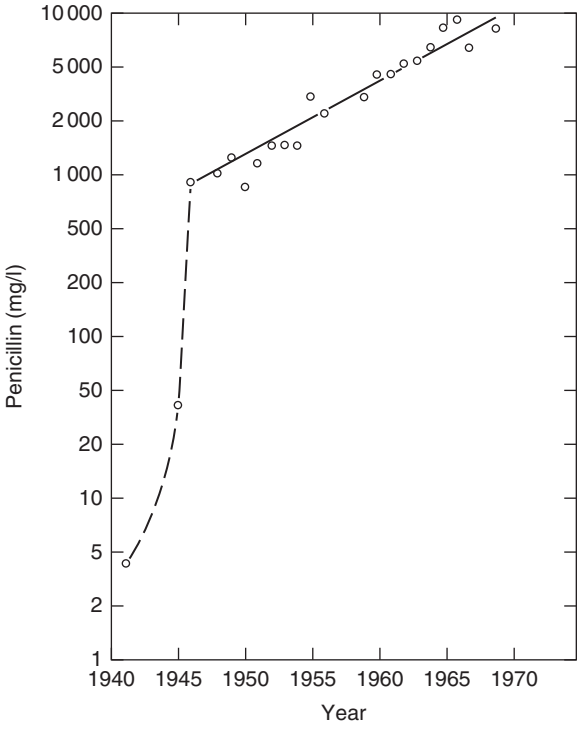


Figure 1.8 Progression of penicillin concentrations in fermentations from 1941 to 1960. Modified from: A.L. Demain (1971). Overproduction of microbial metabolites and enzymes due to alteration of regulation. Advances in Biochemical Engineering/Biotechnology, 1 : 113–142 (with kind permission of Springer Nature).

1943 (e.g. Merck, Squibb, and Pfizer). It was initially only available to the allied soldiers and later became available to the civilian population as a drug for treating bacterial infections.

The success of industrial penicillin production marks the beginning of the "antibiotic era." A large number of researchers devoted themselves to searching for new antibiotics, developing further previously identified antibiotics, and marketing them professionally. The results included the discovery and/or development of penicillin derivatives with a broader spectrum of efficacy and improved pharmacokinetic properties. The American microbiologist Selman A. Waksman (1888–1973), to whom the term "antibiotic" is attributed, discovered Actinomycin A in 1940. With the help of his assistant Albert Schatz, Waksman also discovered the aminoglycoside streptomycin in 1943, the first antibiotic to be successfully used in treating tuberculosis (*Mycobacterium tuberculosis*). Guiseppe Brotzu (1895–1976) isolated the mold fungus *Acremonium chrysogenum* (formerly known as *Cephalosporium acremonium*) in Italy in 1945. Cephalosporin C, a β-lactam antibiotic like penicillin, was isolated from this fungus and tested for its antibiotic effect in Florey's lab in Oxford.

Over the years, as researchers gained collective experience in constructing facilities and developing scientific procedures, they also added to the ever-increasing understanding of the primary and secondary metabolism of many microorganisms. Thanks to this collective wealth of knowledge and experience, a multitude of further compounds originating from microbial metabolism

were synthetically produced with microbial procedures and developed to manufacturing maturity starting in 1950. These compounds included amino acids (e.g. L-glutamic acid and L-lysine), special organic acids (e.g. itaconic acid), steroids, and vitamins (e.g. vitamin C). In addition to the large amounts of base chemicals being produced, such as alcohol and solvents, increasing amounts of so-called "fine chemicals" were being produced, with a comparatively smaller production quantity and higher level of chemical purity. Large-scale production of microbial enzymes began around this time as well. These enzymes were used in many facets of everyday life, e.g. in food processing (e.g. glucose isomerization and starch liquefaction), animal feed production (e.g. phytase), detergent production (e.g. proteases and lipases), in the pharmaceutical industry (e.g. hydrolysis of penicillin G for the synthesis of semisynthetic derivatives), and in the textile and paper industries (hydrolyses and oxidases). The subsequent chapters will discuss the microorganisms used for manufacturing various products, the relevant metabolic pathways and regulation, the development of suitable production strains, and traditional and modern production procedures that are still in use today.

Since about 1980, the term "biotechnology" has been used to refer to the use of organisms, cells, or cell components to manufacture or transform substances. The generalized field of biotechnology is divided into several subcategories. White biotechnology, also called *Industrial Biotechnology*, refers to all the aspects of industrial production. Depending on the definition, the range of products spans to include base and fine chemicals, food and food additives, precursors for the agricultural and pharmaceutical industries, and additives for the manufacturing industry. The terms red, green, brown, gray, and blue biotechnologies refer to biotechnical procedures with applications in the areas of medicine/pharmaceuticals, agriculture, environment, waste management, and marine organisms, respectively.

1.4 Genetic Engineering Is Introduced into Industrial Microbiology (From Roughly 1980)

The appearance of genetic engineering changed the scope of industrial microbiology. With its roots in the mid-1960s, genetic engineering is still under development today. These new methods allow scientists to make specifically defined changes (mutations) to the genetic material of microorganisms. The synthesis of individual enzymes/proteins in the cells can be switched off or intensified, and it also becomes possible to synthesize enzymes/proteins with modified properties. Strains that have been designed by means of genetic engineering are superior to those resulting from classical mutagenesis (radiation, mutagenic chemicals) in that they do not exhibit any other unspecific mutations with undesired effects. With genetic engineering methods, it is possible to influence the regulation of metabolic pathways in industrially used microorganisms, as well as the yield of products and side products. It is possible to produce all kinds of proteins, even those from higher eukaryotes, using techniques of reordering genetic material

(recombination) and introducing recombinant genetic material into microbial cells. Posttranslational modification (e.g. glycosylation) is the only area in which there are limitations.

The methods used in genetic engineering have their roots in the scientific discipline of molecular biology or molecular genetics, which started in the 1940s and concerns the structure and function of nucleic acids (DNA and RNA). The most important discoveries in this field are listed in Table 1.4. In 1944, Oswald Avery was able to demonstrate at the Rockefeller Institute in New York that genetic information must be contained in nucleic acids. He hereby disproved the popular theory that proteins were the carriers. The double-helix structure of DNA was discovered in 1953 by the American and British microbiologists James Watson and Francis Crick at Cambridge University's Cavendish Institute. The first step in deciphering the genetic code was made by German biochemist Heinrich Matthaei in Marshall W. Nirenberg's lab at the National Institute of Health (NIH) in Bethesda, when he identified the corresponding amino acid (L-phenylalanine) for the first codon (UUU). This first step paved the way for translating the DNA base sequences in the amino acid sequences in proteins. Five years later, all 64 base triplets had been successfully decoded. The French

Table 1.4 Molecular biology findings and genetic engineering methods.

Period/year	Discoveries/methods
1944	Avery identifies nucleic acids as carriers of genetic information.
1953	Watson and Crick decipher the structure of DNA.
1959	Jaques and Monrod establish the operon model and describe the regulation of allosteric enzymes.
1961–1966	The genetic code is deciphered.
1962	Stanier and van Niel differentiate and define prokaryotes and eukaryotes.
1962–1968	Various bacterial restriction and modification systems are discovered.
1965	Khorana and Kornberg establish the *in vitro* oligonucleotide synthesis.
1973	Cohen and Boyer create recombinant DNA (cloning of restriction fragments).
1976	Scientists are successful in chemically synthesizing a gene.
1977	Maxam, Gilbert, and Sanger establish methods for DNA sequencing.
1979	Smith establishes methods for site-directed mutagenesis.
1983	Mullis describes the polymerase chain reaction (PCR) for the amplification of DNA.
From 1990	The so-called "omics" techniques are developed (genomics, transcriptomics, proteomics, metabolomics, and fluxomics), as is the field of "metabolic engineering."
From 1995	Genomes of bacteria and fungi are sequenced.
From 2000	Metagenomic projects capture the entirety of genetic information for a habitat.
2010	Venter and staff replace a bacterial genome with DNA produced *in vitro*, thus successfully engineering a bacterium with a synthetic genome.

scientists François Jacob and Jacques Monod from the Pasteur Institute studied the organization of genes in bacterial chromosomes and developed the operon model for coordinated expression of bacterial genes in 1959. Between 1962 and 1968, various restriction and modification systems in bacteria were discovered. Their function is to break down foreign DNA that has penetrated the cells or to prevent destruction of the cell's own DNA. The restriction enzymes also play an important role in numerous genetic engineering methods, making it possible to cut DNA segments and then reassemble them in the desired order.

Microbiology research findings have resulted in a multitude of important genetic engineering methods (Table 1.4), without which it would not have been possible to develop new products and product strains for industrial manufacturing processes. These include *in vitro* oligonucleotides synthesis (Kornberg et al. 1964), the first cloning of restriction fragments (Cohen et al. 1973), the construction of cloning and expression plasmids, the classical methods of DNA sequencing (Maxam and Gilbert 1977; Sanger et al. 1977), site-directed mutagenesis (Gillam and Smith 1979), polymerase chain reaction (PCR; Mullis and Faloona 1987), and the cloning of synthetic genes (Saiki et al. 1988). With the help of automated DNA sequencing, the first bacterial genome was decoded in 1995 and the baker's yeast genome shortly thereafter, in 1997. New technologies toward the end of the last century included DNA chip technology (or DNA microarray technology) for genome-wide expression analysis (transcriptomics) as well as methods for identifying the entirety of available proteins in a cell or organism (proteomics). The field of bioinformatics emerged in order to accommodate the vast amounts of data processing required for these methods. The high-throughput sequencing of genomes has been possible with the technologies of pyrosequencing since 2001 and "Illumina/Solexa" since 2005. Thanks to these technologies, more than 4000 prokaryotes and 450 eukaryotes have been sequenced and annotated, including all of the bacteria and fungi that are significant for modern industrial microbiology.

Parallel to the development of microbiological methods, a technology emerged in the 1990s for identifying the entirety of ascertainable metabolites in a cell (metabolomics) and for quantitatively identifying cellular material flows with the help of labeling experiments. Based on the experimental results, it is possible today to prepare models via computer-aided stoichiometric (metabolic) material flow and network analysis for the entire metabolism of a microorganism. These can then serve for modeling the metabolism in its entirety or in part, i.e. using a model to analyze what effects the changes in individual parameters have on the formation of various metabolic intermediates or end products. Since around the year 2000, the term "systems biology" has been used to refer to the modeling of the entire metabolism of an organism. This field owes its existence to the aforementioned capacity for measuring an ever-increasing amount of cellular parameters and to the possibilities opened up by information technology and bioinformatics for processing increasingly large amounts of data. Researchers are hopeful that systems biology will continue to provide more detailed information on how microorganisms with industrial applications can be more efficiently manipulated to synthesize desired compounds.

"Metabolic engineering" refers to the use of genetic engineering methods for modifying metabolic pathways in industrial product strains, thus optimizing and increasing the ability of a cell to produce a desired substance. By inactivating, weakening, or overexpressing one or more genes for enzymes or regulatory proteins, the carbon flow from the substrate can be directed to the desired product, the substrate spectrum can be expanded, and synthetic pathways to undesired side products can be blocked. Totally, new metabolic pathways can be established in bacteria by expressing heterologous genes even originating from cells of higher eukaryotes, e.g. plants.

The first product to be industrially manufactured by use of genetically modified (GM) microorganisms was human insulin. The production procedure was developed in 1978, as was the recombinant *Escherichia coli* strain that was used. This scientific success led to the development of further procedures using genetically modified organisms to produce other proteins and numerous other active ingredients, especially in the pharmaceutical sector. Since approximately 1986, it has become increasingly common to use genetically engineered strains to synthesize various products, including alcohols and solvents, organic acids, some L-amino acids, vitamins, and antibiotics. Up to then, these products had been manufactured using bacteria and fungus strains obtained via classical mutagenesis and screening. GM microorganisms have been used since the late 1970s for the production of enzymes that are used in the textile, paper, and detergent industries (technical enzymes, e.g. proteases, lipases, and amylases), in particular *Bacillus* and *Streptomyces* as well as *Aspergillus* and *Trichoderma* species. The enzymes used in research and diagnostics are also for the most part obtained with the help of recombinant microorganisms. Concrete examples of products and procedures in which genetically modified microorganisms are used in industrial microbiology will be presented in detail in the following chapters of this book.

1.5 Future Perspectives: Synthetic Microbiology

"Synthetic biology" is a new emerging scientific field and in microbiology refers to a subfield of metabolic (or genetic) engineering, in which microorganisms (or other biological systems) are engineered with characteristics and capabilities that naturally do not exist in organisms. This ultimately includes the engineering of microorganisms with minimal genomes, which only possess metabolic pathways necessary for producing a desired substance. Craig Venter and his staff got the ball rolling in this field when they successfully replaced the natural genome of *Mycoplasma capricolum* with one that was synthetically produced in 2010.

A relevant example for a synthetic biology approach in industrial microbiology is the semisynthetic synthesis of artemisinin. Artemisinin is an antimalarial drug recommended for the first-line treatment of malaria by the World Health Organization WHO. It is naturally produced by the plant *Artemisia annua*, but the supply and price have fluctuated greatly. To smooth out these fluctuations, a semisynthetic process was developed whereby a late-stage precursor is produced by fermentation, then chemically converted to artemisinin (hence the designation of "semisynthesis," as opposed to complete chemical synthesis).

Artemisinin is a terpene molecule. This class of molecules is derived from acetyl-CoA, which is used to produce five-carbon atom building blocks that are assembled sequentially (i.e. C5, C10, C15, etc.). Artemisinin contains 15 carbon atoms and is derived from the universal precursor farnesyl diphosphate (FPP). In the plant, FPP is converted to the alkane precursor of artemisinin, amorphadiene, by the enzyme amorphadiene synthase (ADS), and the amorphadiene is oxidized to dihydroartemisinic acid. It undergoes spontaneous photochemical rearrangement to produce artemisinin.

Preliminary planning to develop a fermentative semisynthesis of artemisinin indicated that the simplest route was to produce artemisinic acid by fermentation rather than dihydroartemisinic acid, as is made in the plant.

The enzyme converting amorphadiene to artemisinic acid was discovered to be a cytochrome P450 monooxygenase. The discovery of the cytochrome P450 enzyme dictated that the production microorganism is *Saccharomyces cerevisiae*, as yeast is known to express P450 enzymes well. Initial engineering of yeast was focused on producing amorphadiene at a concentration that would make the semisynthetic process economically competitive.

FPP is naturally made in yeast *via* the mevalonate pathway as a precursor to biosynthesis of ergosterol (ERG), but in low amounts (Figure 1.9). To increase the production of FPP, the genes encoding every enzyme of the mevalonate pathway, also known as the ERG pathway, required for FPP production were overexpressed. Transcription of integrated copies of each of the eight genes occurred from strong, inducible promoters.

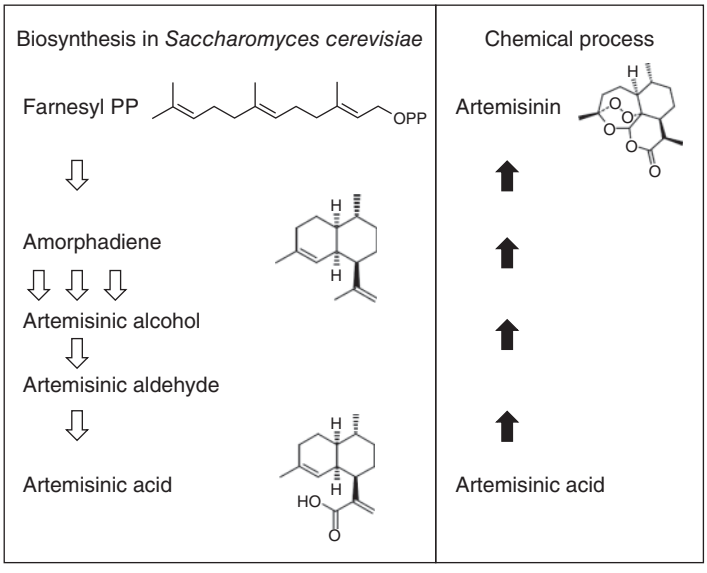


Figure 1.9 Semisynthetic Artemisinin production. Farnesyl-PP is provided as precursor by the *S. cerevisiae* anabolism after overexpression of 10 genes (not shown). Six enzymes (⬆) encoded by genes originating from the plant *Artemisia anna* catalyze the intracellular conversion to Artemisinic acid. Subsequently, four chemical steps (⬆) deliver the antimalarial drug artemisinin.

FPP was converted to amorphadiene by expressing *A. annua* ADS. Over the course of strain development, amorphadiene production was increased from 100 mg/l to over 40 g/l in fermenters.

Engineering of the yeast strain to oxidize amorphadiene to artemisinic acid (Figure 1.9) involved considerable effort. Production had to be brought into balance with viability of the culture.

Oxidation of amorphadiene to artemisinic acid involves three oxidation reactions (amorphadiene → artemisinic alcohol → artemisinic aldehyde → artemisinic acid). For the first oxidation, three proteins, two of them working with the cytochrome P450 enzymes, are needed. Two additional enzymes, dehydrogenases ADH1 and ALDH1, perform the second and third reactions in the oxidation sequence (Figure 1.9). Expression of ADH1 and ALDH1 in amorphadiene-producing yeast enabled production of 25 g/l artemisinic acid by fermentation.

Parallel development of industrial-scale chemistry resulted in a process for conversion of artemisinic acid to artemisinin. The final package provided a commercial source of semisynthetic artemisinin as an alternative to plant-derived artemisinin.

References

Cohen, S.N., Chang, A.C.Y., Boyer, H.W., and Helling, R.B. (1973). Construction of biologically functional bacterial plasmids *In Vitro*. *Proc. Natl. Acad. Sci. USA*. 70: 3240–3244. https://doi.org/10.1073/pnas.70.11.3240.

Demain, A.L. (1971) Overproduction of microbial metabolites and enzymes due to alteration of regulation. *Advances in Biochemical Engineering/Biotechnology* 1: 113–142.

Erker L. (1574) Probirbuch, Library of Leipzig University

Gillam, S. and Smith, M. (1979). Site-specific mutagenesis using synthetic oligodeoxyribonucleotide primers: I. Optimum conditions and minimum ologodeoxyribonucleotide length. *Gene* 8: 81–97.

Kornberg, A., Bertsch, L.L., Jackson, J.F., and Khorana, H.G. (1964). Enzymatic synthesis of desoxyribonucleic acid, XVI. Oligonucleotides as templates and the mechanism of their repication. *Proc. Natl. Acad. Sci. USA*. 51: 315–323. https://doi.org/10.1073/pnas.51.2.315.

Maxam, A.M. and Gilbert, W. (1977). A new method for sequencing DNA. *Proc. Natl. Acad. Sci. USA*. 74: 560–564. https://doi.org/10.1073/pnas.74.2.560.

Mullis, K.B. and Faloona, F.A. (1987). Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol.* 155: 335–350.

Pasteur, L. (1876). *Etudes sur la biere. Avec une theorie Nouvelle de la Fementation*. Paris: Gauthier-Villars.

Saiki, R.K., Gelfand, D.H., Stoffel, S. et al. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239 (4839): 487–491.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*. 74: 5463–5467. https://doi.org/10.1073/pnas.74.12.5463.

Further Reading

Adrio, J.-L. and Demain, A.L. (2010). Recombinant organisms for production of industrial products. *Bioeng. Bugs* 1 (2): 116–131.

Buchholz, K. and Collins, J. (2010). *Concepts in Biotechnology*. Wiley-VCH.

Erickson, B., Nelson, E.J., and Winters, P. (2012). Perspective on opportunities in industrial biotechnology in renewable chemicals. *Biotechnol. J.* 7: 176–185.

Flaschel, E. and Sell, D. (2005). Charme und Chancen der Weißen Biotechnologie. *Chem. Ing. Tech.* 77 (9): 1298.

Paddon, C.J. and Keasling, J.D. (2014). Semi-synthetic artemisinin: a model for the use of synthetic biology in pharmaceutical development. *Nat. Rev. Microbiol.* 12: 355–367.

Pelzer, S. (2012). Maßgeschneiderte Mikroorganismen. *Biol. unserer Zeit* 42: 98.

Ulber, R. and Soyez, K. (2004). 5000 Jahre Biotechnologie. *Chem. unserer Zeit (ChemiuZ)* 38: 172–180.

2

Bioprocess Engineering

Michael R. Ladisch[1,2], Eduardo Ximenes[1], Nathan Mosier[1], Abigail S. Engelberth[1,3], Kevin Solomon[1], and Robert Binkley[1]

[1] Purdue University, Laboratory of Renewable Resources Engineering, Department of Agricultural and Biological Engineering, 500 Central Drive, West Lafayette, IN 47907-2022, USA
[2] Purdue University, Weldon School of Biomedical Engineering, 500 Central Drive, West Lafayette, IN 47907-2022, USA
[3] Purdue University, Environmental & Ecological Engineering, 500 Central Drive, West Lafayette, IN 47907-2022, USA

CHAPTER MENU

2.1 Introduction

Bioprocess engineering pairs unit operations familiar to chemical engineers, including bioreactors and bioseparations, with enzymes and microbial kinetics, bioprocess design for production of proteins, biomolecules, polymers, biofuels, and food products in amounts ranging from hundreds of grams to kilotons annually produced. This broad field is addressed by current and previous textbooks (Aiba et al. 1973; Bailey and Ollis 1986; Mosier and Ladisch 2009; Shuler et al. 2017). The purpose of this chapter is to provide the reader with an introduction to this important field, starting with the key metabolic pathway for glucose utilization (i.e. glycolysis) by living cells, modeling of cell growth using unstructured models, and reactors and reactions that are central to bioprocess engineering.

The types of unit operations for a bioprocess will vary with the type of the bioproduct. These range from small molecules (fuels and chemicals) with molecular mass less than 50 g/mol to polysaccharides whose molecular mass may exceed

This chapter includes excerpts from Mosier and Ladisch (2009).

Industrial Microbiology, First Edition.
Edited by David B. Wilson, Hermann Sahm, Klaus-Peter Stahmann, and Mattheos Koffas.

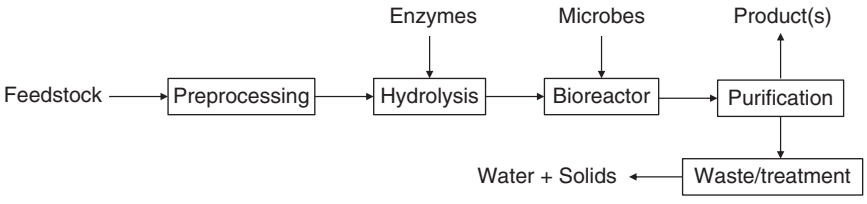


Figure 2.1 Schematic representation of key processing steps for biologically based production of fuels, bioproducts, proteins, and/or microorganisms from carbohydrates. All inputs assumed to be sterile. Other medium components including proteins, peptides, minerals, and growth factors are not shown. Example: for feedstock, e.g. corn, for preprocessing, e.g. milling, and for hydrolysis by enzymes, e.g. maltose release by amylase. Source: From Mosier and Ladisch (2009), with kind permission from National Research Council.

10 million g/mol (xanthan, discussed in Chapter 11). Regardless of product, the sequence of processing steps will generally follow the steps outlined in Figure 2.1, where the type of feedstock determines the number of processing steps that precede the bioreactors. For solid feedstocks such as starch (from maize) or cellulose (from lignocellulosic biomass), preprocessing (i.e. pretreatment) before hydrolysis is performed to generate sugars for fermentation, where the sugars are converted into various types of biomolecules. Bioproducts may also be produced directly from sugars. Some fermentations require oxygen while others are carried out at anaerobic conditions. One possible process is illustrated in Figure 2.1.

A recent study of the US National Research Council on Industrialization of Biology (National Research Council 2015) reports that bio-based markets have business-to-business revenues for industrial biology exceeding 125 billion US$. Bio-based products generated revenue of 353 billion US$ in 2012. According to this report, Lux Research has estimated that industrial chemicals produced through synthetic biology represent a market of 1.5 billion US$, with an annual growth rate of 15–25%. "Bio-based chemicals are neither entirely new, nor are they a historic artifact and … bioprocessing techniques (such as fermentation, baking, and tanning) have been used throughout much of human industrial history" (National Research Council 2015). This report also makes the case that the advanced "manufacturing of chemicals through biology can help address global challenges related to energy, climate change, sustainable and more productive agriculture, and environmental sustainability." Bioprocessing is a key tool to scaling developments based on advances in DNA technology and products where biology offers synthetic pathways that are better than conventional chemical synthesis. In this context, the report points out that fermentation and processing are one of the major engineering considerations for production of biological systems. The importance of bioprocess engineering has been historically noted by the National Research Council (National Research Council 1992), and the subject of bioprocessing is presented in this chapter with emphasis on introducing bioreactors and the microbial basis of biocatalysis that occurs within these bioreactors.

The products derived through bioreactors, or a combination of bioreaction and chemical synthesis, address a wide range of molecules. Biofuels constitute

a familiar bioproduct that has widespread use in large volumes but is characterized by low economic margins as defined by difference between price and cost of a raw material and manufacturing. Intermediate value products are defined based on fatty acid metabolism in *Escherichia coli* and baker's yeast to produce high-value oils, fatty alcohols, fatty acid methyl esters, diols alkanes, and olefins directly from carbohydrates. Other products include industrial enzymes (with markets that exceed 5 billion US$ worldwide). Organic acids are finding uses as livestock feed additives that replace growth-promoting antibiotics (McCoy 2017) while bio-based aniline has potential for an alternate route that would result in the same functionality as its chemically synthesized counterpart for the production of anilines (Tullo 2017). Bioreactions will also be a factor in the manufacturing of biomolecules and biomaterials from sugar and lignin, as the demand for paper drops and the existing pulp and paper mills seek to manufacture other products from wood to make up the lost revenues (Scott 2017). Manufacture of protein biopharmaceuticals is the most lucrative market segment with high margins. Again, bioreactors are a key to achieving a scale, with products being derived through both microbial fermentations and culture of mammalian cells and a sequence of protein purification steps (Ladisch 2001; Harrison et al. 2003; Le et al. 2016).

2.1.1 Role of Bioreactors

In the simplest sense, a bioreactor is any vessel where biological reactions take place. From there, reactors grow in variety and complexity, in order to accommodate the variety of microorganisms, processes, and products being formed. These biological reactions can range from catalytic conversion of reactants by enzymes in batch reactors to the production of biomolecules, accomplished through cell growth and the accompanying formation of small molecules (e.g. ethanol) or secondary metabolites (such as penicillin). A more modern concept of a bioreactor is a vessel where a microorganism is monitored, proliferated, and maintained in order to modify a chemical into a product, typically in some types of nutrient solutions (these solutions are typically called "media"). Centuries ago, a beer brewing barrel could be considered a bioreactor, with the microorganism being yeast and the chemical to modify being the sugars in the barley and rye into the product, ethanol. Since then, reactors have become much more sophisticated, in terms of both design and integrated technology, which allows for monitoring of condition parameters. Modern reactors can be made out of many materials, including, glass, steel, concrete, and plastics, and can range from less than 1 ml to over 1000 m^3 in volume. The largest vessels are associated with wastewater treatment processes where anaerobic digesters may exceed 5000 m^3 in volume. As such, a fundamental understanding of the physical properties and biochemical responses of reactor processes is vital for the economic scale-up and production of bio-based molecules.

In an industrial setting, the production step in a reactor is often referred to as being an "upstream" process, whereas the processing and product recovery, purification, and packaging or formulation are broadly defined as being "downstream." This section introduces the upstream concepts of bioreactor design and bioprocess engineering defined within the context of bioreactors that use

microorganisms, enzymes, or immobilized cells for generation of biomolecules that have a broad field of use and which account for economic activity approaching 350 billion US$ in the United States alone (National Research Council 2015).

2.1.2 Basic Bioreactor Configurations

The most basic form of a bioreactor is a small glass vessel, such as a flask or a beaker, commonly used in biology and research labs. Although these vessels can be considered as bioreactors in a broad sense, their simplicity often precludes them from the designation of a bioreactor. This type of culture is often described as a "shake flask" culture and is widely used, because of its simplicity, for initial culture validation-type studies, such as screening of mutant strains, or initial studies of media and nutrient culture requirements. Vessels are first sterilized in an autoclave, sterile media are added to the vessels using an aseptic technique (to minimize the contamination), and then a microorganism is added to the vessel for growth and production studies. Often, these vessels are run in batch process mode, where no solutions are added once the growth begins. These flasks are typically placed in orbital shakers (Figure 2.2) in order to mix the cultures, maintain the sample culture homogeneity, and prevent settling of the cells. Many shaker models also allow for temperature control, where flasks are placed in a temperature-controlled environment to remove temperature variation between experiments (or conversely to study the effects of different temperatures). Typically, these flasks are between 5 and 5000 ml in volume. For aerobic cultures, an air-permeable stopper or membrane is often placed over the opening of the flasks to allow for oxygen transfer while maintaining aseptic conditions in the flask. Shaking and mixing also allows for enhanced oxygen transfer when compared to stagnant cultures. Although oxygen transfer can vary greatly because of flask geometry and shaking characteristics, typically only 20% of the flask's nominal volume is used in flask cultures because of oxygen transfer limitations. Although these flasks are often simple to be used as an implement, they are also limited in the parameters that can be controlled. They are often run in batch mode, and therefore, automated control of process parameters is not available. New technologies have allowed for direct measure of dissolved oxygen and pH by use of specialized flasks and platforms, making these even closer to true bioreactors.

The culture of strict anaerobes (i.e. microorganisms that require oxygen-free conditions as they are inactivated by oxygen in the ppb – parts per billion – range due to the fragility of essential Fe–S clusters) requires that inoculation of the microorganism be carried out in a special hood, known as an anaerobic chamber, so that oxygen is excluded and prevented from dissolving in the broth. The enclosed chamber resembles a glove box through which the oxygen-free gas (typically N_2 gas) is passed. Traces of oxygen are scavenged from the chamber gas by passing the gas over hot (200–300 °C) copper filings that react with trace oxygen to form copper oxides and result in an oxygen-free nitrogen sweep gas.

Controlled laboratory fermentations at a mid-scale are typically carried out in sealed glass vessels of the type shown in Figure 2.3. Volumes of these vessels typically range from 1 to 20 l, with the working (broth volume) typically being about

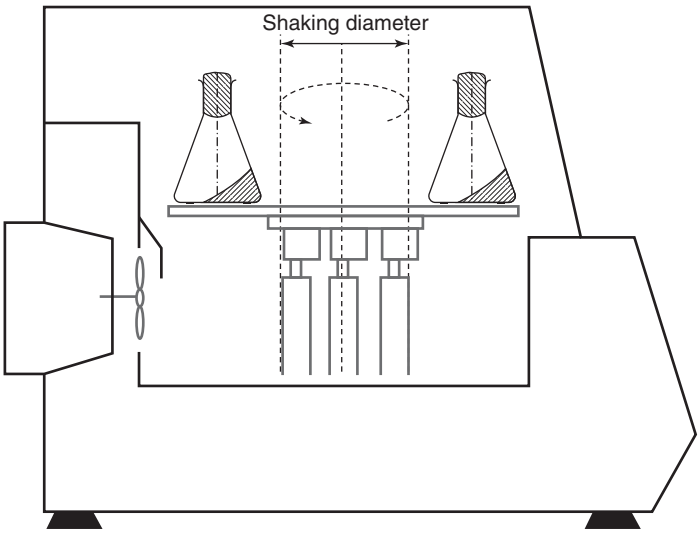


Figure 2.2 Schematic drawing of an incubator shaker for growing bacteria and fungi in the laboratory. Triple eccentric drive of the shaker plate induces the liquid in the flasks to consistently mix the fluid. Most of the liquid forms a wave-like bulk remaining a film (not shown) with a large surface for gas transfer. Source: Mosier and Ladisch (2009). Reproduced with permission of John Wiley and Sons.

20–80% of the fermenter volume. The entire vessel, including head plates and fermentation media, can be placed into an autoclave for sterilization. These reactors have sealed ports for pH and O_2 probes, as well as ports for addition of acid or base for purposes of pH control. Aeration of the fermentation broth is carried out by bubbling compressed air that has been sterile filtered through a filter, into the bottom of the vessel. Typically, a filter with a 0.2 or 0.45 μm cutoff is used, as the pores are small enough to block any airborne spores or microorganisms from entering the fermenter with the air. Ports enable samples to be taken for intermittent analysis by chromatography or other methods. These data generate fermentation time courses and products for purification characterization and testing (Mosier and Ladisch 2009).

2.1.3 Types of Growth Media

Basic components of growth media provide energy, carbon, nitrogen, and mineral sources. Growth media can be either complex or defined. Complex media contain nutrients that are chemically undefined, such as water-soluble, enzymatically digested proteins. The exact make up of nutrients is unquantified, and thus, the medium is considered to be complex. A defined medium contains carefully measured pure components that are added to water. The nutrients and energy source can be chemically quantified and thus the medium is defined.

Traditionally, the development of an economic medium for a particular bioreaction process requires trial and error experimentation. The selection, discovery, and/or engineering of microorganisms that are able to utilize inexpensive media

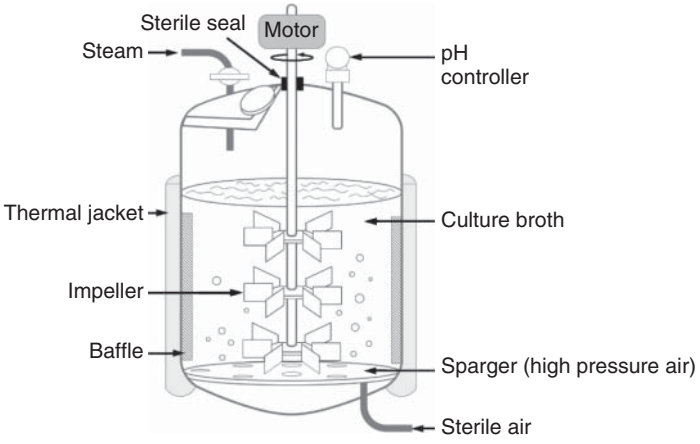


Figure 2.3 Diagram of a bench-scale fermenter used for submerged bacterial and fungal fermentations. The fermenter can control temperature, gas exchange, and pH. Not shown are instruments to measure temperature, dissolved oxygen (DO), and pH. Source: Bjurstrom (1985). Reproduced with permission of Chemical Engineering.

components and optimization of culture conditions including pH, temperature, and/or the rate at which oxygen is supplied (i.e. aeration rate) and other factors must be considered. Typical media components include glucose (carbon and energy source), growth factors, nitrogen source (i.e. proteins or peptides), and minerals including phosphates.

Anorganic nitrogen sources can be ammonia or nitrate. Organic sources of nitrogen, such as proteins (i.e. polymers of amino acids), nucleic acid bases (purines and pyrimidines), as well as individual amino acids, are often components of media (these are typically referred to as "nitrogen sources," as they supply N even though they contain C, H, O, and sometimes S in their structure). Costs of complex nitrogen sources for culture can be expensive compared to other substrates (usually hexoses, pentoses, or lipids). Complex sources of nitrogen include soybean, peanut, and fish meals; yeast extract; whey; casein; and hydrolyzed proteins. Complex sources of nitrogen do not have a clearly defined composition and often contain other growth factors that are defined on the basis of their effect, rather than by identification of their chemical structure. By-products from sugar processing, such as molasses from sugarcane or corn steep liquor from corn wet-milling, contain such nutrients and are therefore to supplement water in making up a major part of the liquid fraction of the starting culture medium.

2.2 Nonstructured Models

2.2.1 Nonstructured Growth Models

A mechanistic model was defined by Krist V. Gernaey (Bjurstrom 1985) as a mathematical formulation of the internal operation of a system in terms of its

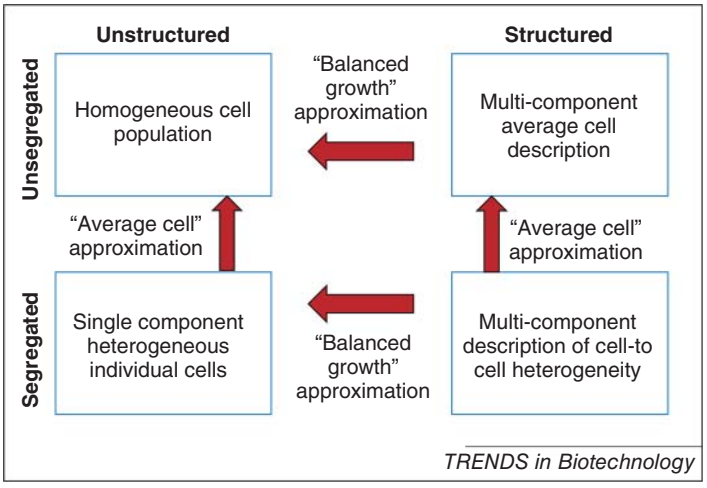


Figure 2.4 Model classifications for mathematical representation of cell populations (Bailey 1998), where "structured" indicates a model representing cell material that consists of multiple chemical components, and "segregated" designates a model describing the presence of individual cells in a heterogeneous population. Source: Bailey (1998). Reproduced with permission of Elsevier.

constituent parts and mechanisms and is based on deterministic principles, which stipulate that at given initial conditions, future system behavior can be predicted.

Figure 2.4 shows a generally accepted classification of mechanistic models of cell populations, when assuming a homogeneous reactor environment. When not possible to assume the homogeneous reactor environment, a distributed model is needed. This is a model where not only time, but also space (1-, 2-, or 3D), forms an independent variable. Unsegregated models, which rely on average cell description, are most common. Unstructured models are the simplest unsegregated models that use a single variable to describe biomass (Roels 1980; Essener et al. 1983; Sin et al. 2008; Gernaey et al. 2010).

2.2.1.1 Unstructured Models

These are reproduced from models in which the variation of biomass composition in response to environmental changes is totally ignored. When coupled with macroscopic methods, which originate from the widely accepted balancing of extensive properties method of chemical engineering, these models provide the biotechnologist with a convenient starting point for the analysis of microbial systems and for design purposes, although these models are not suitable for extrapolation.

Unstructured models are intrinsically simplified. Changes in the quality of growing cells, arising mainly through a differential gene expression that gives a spectrum of translated enzymes, differences in chemical composition of cells, and shape and size of cells, are ignored. Unstructured models only give a simple manifestation of the growth phenomena with Monod-type models describing only balanced and steady-state growth (Panikov 2011). The population of

microorganisms or cells is viewed as a homogeneous biomass and its properties are time invariant. Cell mass or its concentration is the only determining variable, and its properties are described by kinetics for substrate uptake, growth, and product formation, all depending only on the concentrations in the reactor (Essener et al. 1983). These models have found applications in the modeling of natural populations or filamentous microorganisms and for biotechnical processes where there is no information about substrates (Weiss and Ollis 1980) or growth restriction by the available substrate (Frame and Hu 1988; Monbouquette 1992; Bellgardt 2000).

2.2.1.2 Biotechnical Processes

Unstructured models (Mosier and Ladisch 2009) reflect fermentations as proposed by Bailey and Ollis (1986) and Gaden (1959). The interrelation of growth rate and product synthesis is an important characteristic of any biotechnical process. Gaden (1959) classified biotechnical processes according to their growth and production kinetics roughly into three different types, demonstrating a fairly distinctive rate pattern.

In processes of type I, which are related to the production of primary metabolites, the main products appear directly as final products of catabolism, electron flux, or energy metabolism. The production and growth are directly coupled. There is a coincidence in the rates of substrate uptake, growth, and product formation, with the concentrations of biomass and product showing a very similar pattern. In this case, these products are called growth-associated products. Therefore, only one key reaction or bottleneck is considered by this type of mathematical model (Gaden 1959; Bellgardt 2000). Examples include the aerobic propagation of microorganisms, accumulation of ethanol, and production of lactic acid.

Processes of type II, where overproduction of primary metabolites occur, are those in which the formation of the main product is also coupled to catabolism or energy metabolism, but the product is not a final end product of the pathway. Rather, it results from transformation of metabolites, such as citric acid derived through the citric acid cycle or the synthesis of amino acids by the cell. Product formation is low under optimal growth conditions. Although there may still be a positive correlation of growth and product formation, maximum product formation proceeds on the expense of growth rate. The enhanced production is often due to the limitation of a certain growth factor, regulatory deficiencies of the cell, or it can be initiated by addition of a precursor. The production phase occurs with exponential growth at a slow rate. This phase can be clearly distinguished from the first exponential phase of fast growth and might also be preceded by an additional transient or lag phase.

In processes of type III (non-growth-associated products), the main product does not result from central growth processes – such as catabolism or energy metabolism – and it may have no obvious function in the metabolism (i.e. it is not directly derived from the glycolytic or citric acid cycle pathways shown in Figure 2.5). For example, antibiotics are often synthesized under impaired growth conditions or in connection with morphological differentiation of the cells, with no direct and simple coupling of growth rate and product formation. It is often observed that at high growth rates, there is no product synthesis. Maximum

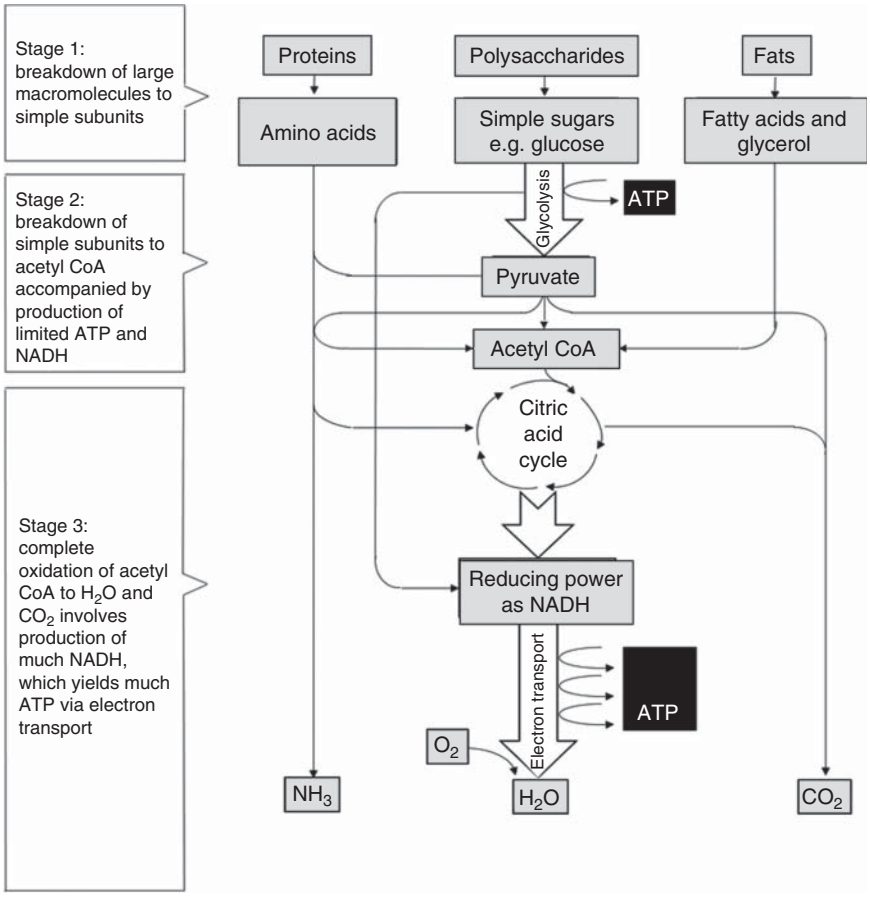


Figure 2.5 Overview of catabolism for macromolecules via building blocks, acetyl CoA, NADH, and ATP to CO_2 and water. Source: Mosier and Ladisch (2009). Reproduced with permission of John Wiley and Sons.

product formation occurs at low or even without growth. The production phase is usually very short and the final product concentration is low in a typical batch process that utilizes a single substrate. Extension of the production phase can be achieved by fed-batch operation or in batch operation when the microorganisms are grown on a mixture of two substrates. In this case, the first phase leads to high growth rate. When the first substrate is exhausted, the microorganisms are then switched to the second substrate and low growth rates. Production is then induced. The production may continue into the stationary or declining growth phase.

The glycolytic pathway is central to the functioning of most applied microorganisms and is addressed in Chapter 5. It is briefly introduced here as glucose is the most frequently used source of carbon and energy. Glucose metabolism processes include glycolysis under anaerobic conditions, complete oxidation under aerobic conditions, and the pentose phosphate pathway (Li et al. 2014). Glycolysis

is the metabolic pathway that converts glucose into pyruvate in the cytoplasm, with production of adenosine triphosphate (ATP), which is the most important high-energy phosphate compound in living organisms. It consists of two phosphoric acids that bond as energy-rich anhydrides to a third phosphoric acid coupled via an ester bond 5-prime at the ribonucleoside adenosine. ATP is the primary energy carrier in living organisms. It is generated during exergonic reaction and used to drive endergonic reaction. The free energy of high phosphate energy bonds is often used to drive biosynthetic reactions and other parts of cell function in a carefully regulated process, with the ATP-released energy from its hydrolysis coupled to energy requiring reactions (Madigan et al. 1996).

2.2.2 Modeling Fermentations

Many microorganisms grow in a bioreactor through binary division. One cell becomes two; two cells become four, and so on. Each doubling of cells is called a generation. Mathematically, the total number of cells in a bioreactor (C) after n number of generations can be determined using Eq. (2.1):

$$C = C_0 2^n \tag{2.1}$$

where the initial number of cells is given by C_o, and the number of generations by n. A nonzero amount of time, 20–60 minutes for some bacteria, must pass between generations as the cells metabolize nutrients from their environment to provide the raw materials and energy necessary for cellular division. This amount of time is called the doubling time (t_d) for the cell. If we know the doubling time, we can determine the number of generations (n) that have occurred during a time span t by dividing t by t_d. Through substitution, Eq. (2.1) becomes

$$C = C_0 2^{t/t_d} \tag{2.2}$$

At this point, an assumption is helpful. If we assume that all of the cells are more-or-less the same, then the total *mass* of cells (X) is directly proportional to the number of cells (C), and we can rewrite Eq. (2.2) in terms of mass instead of number of cells:

$$X = X_0 2^{t/t_d} \tag{2.3}$$

We can rearrange Eq. (2.3) and apply the natural logarithm to both sides of the equation:

$$\ln\left(\frac{X}{X_0}\right) = {}^{t}/_{t_d} \ln(2) = \mu t \tag{2.4}$$

where the specific growth rate μ is equal to $\ln(2)/t_d$. The natural logarithm is useful here as it allows us to further simplify by finding the derivative of Eq. (2.4) where we assume that μ is a constant.

$$\frac{1}{X}\,\mathrm{d}X = \mu\,\mathrm{d}t \tag{2.5}$$

It is from the rearranged form of this Eq. (2.6) that μ gets its name: specific growth rate

$$\mu = \frac{1}{X}\frac{\mathrm{d}X}{\mathrm{d}t} \tag{2.6}$$

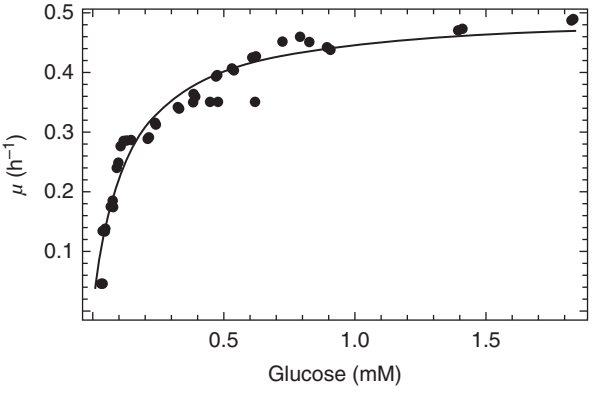


Figure 2.6 Specific growth rate (μ) of *Saccharomyces cerevisiae* constrained by glucose concentration. The maximum specific growth rate ($\mu_{max} = 0.5\ h^{-1}$) and the concentration of ½ maximum substrate ($K_m = 0.3$ mM) vary significantly between microorganisms. Source: Alberts et al. (1989). Reproduced with permission of Garland Publishing.

The specific growth rate (μ) is a constant that is equal to the rate of growth (dX/dt) normalized by, or made specific to, the mass of cells (X) present at any given time. Equation (2.6) is often called the Monod equation after the pioneering microbiologist Jacques Monod who mathematically described microbial growth. Although exponential growth had been described previously, Monod's key contribution to the field was identifying that microbial growth is exponential but also constrained. Therefore, μ is not strictly a *constant* but rather a parameter that changes as the resources available for growth are consumed.

In the simplest case, cell growth is limited by a single critical nutrient, usually the major source of carbon and energy, e.g. glucose, although other nutrients can be rate limiting. To use glucose as an example, when sufficient glucose is available, the specific growth rate is at its maximum (μ_{max}). The addition of even more glucose does not result in faster cell division. The cells cannot grow any faster as their growth is now constrained by the biochemical limits of their metabolism rather than the availability of nutrients. On the other hand, when the amount of glucose is insufficient, the rate of growth is slowed. At the limit, no glucose is available, and growth stops (Figure 2.6).

This relationship between specific growth rate and limiting nutrient concentration can be described mathematically:

$$\mu = \frac{\mu_{max} S}{K_m + S} \tag{2.7}$$

where μ_{max} is the maximum specific growth rate and K_m is the concentration of substrate (S) where the specific growth rate (μ) is half of the maximum (μ_{max}). When Eq. (2.7) is combined with (2.6), the resulting Monod equation can be used to model cell growth (dX/dt) as a function of substrate concentration.

$$\frac{dX}{dt} = X \frac{\mu_{max} S}{K_m + S} \tag{2.8}$$

The Monod equation is the basis upon which nearly all unstructured models are developed to describe the time course of cell accumulation during the exponential growth phase. This type of model is useful for many industrially relevant processes where exponential growth occurs. To be useful for this purpose, the differential equation for cell growth must be coupled to a differential equation that describes consumption of the substrate. The simplest form of this equation uses a yield coefficient ($Y_{S/X}$) to relate the rate of substrate consumption to the rate of growth

$$\frac{\mathrm{d}S}{\mathrm{d}t} = -Y_{S/X}\mu X \tag{2.9}$$

The yield coefficient, $Y_{S/X}$, is therefore defined as the incremental mass of substrate that is consumed for each incremental biomass that is produced:

$$Y_{S/X} \equiv \frac{\mathrm{d}S}{\mathrm{d}X} \tag{2.10}$$

We can write a similar ordinary differential equation (ODE) that describes formation of the product (P) as a function of growth. This assumes a type 1 fermentation where the product is formed as a result of the energy metabolism of the microorganisms that convert the substrate into energy and a chemical by-product which is the desired product of the process (P). We must also assume that the substrate is only consumed for growth. If those assumptions are valid, we can use a similar approach to that shown above:

$$\frac{\mathrm{d}P}{\mathrm{d}t} = -Y_{P/S}\frac{\mathrm{d}S}{\mathrm{d}t} \tag{2.11}$$

where $Y_{P/S}$ is the yield coefficient of product formation in units of grams of product produced per gram of substrate consumed ($\mathrm{d}P/\mathrm{d}S$). Equations (2.8), (2.9), and (2.11) taken together make a system of ODEs that model cell growth and product formation constrained by substrate availability.

Table 2.1 provides some example values for the parameters $\mu_{\max}$, K_{m}, $Y_{P/S}$, and $Y_{X/S}$. The bacterium *Zymomonas mobilis* has been studied for potential applications for increased ethanol production efficiency. This bacterium naturally produces ethanol through the metabolism of glucose via the Entner–Doudoroff (ED) pathway rather than the more common Embden–Meyerhof–Parnas (EMP) pathway utilized by *Saccharomyces cerevisiae*. The ED pathway produces only 1 mol of ATP per mol of glucose metabolized, compared to the 2 mol produced via the EMP pathway. The result of this is a greater specific yield of ethanol ($Y_{P/S}$) as shown in Table 2.1 (0.49 versus 0.46). Another approach to modeling a type 1 fermentation is given by the Luedeking–Piret model, which was developed for lactic acid fermentation. *Lactobacillus delbrueckii* was found to produce lactic acid semi-independently of bacterial growth. In this model Eq. (2.11), the product (P), lactic acid in this case, is produced as both a function of growth (μX) and a function of the amount of biomass present in the fermenter at any given time (X).

This non-growth-associated product formation can be thought of as the formation of product by cells metabolizing sugars just to maintain their life functions but not to generate the necessary molecular components for cell division

Table 2.1 Monod model parameters for *Saccharomyces cerevisiae* and *Zymomonas mobilis* concerning ethanol production.

Microorganism	Substrate concentration (g/l)	K_m (mg/l)	μ_{max} (1/h)	$-Y_{P/S}$ (g/g)	$-Y_{S/X}$ (g/g)
S. cerevisiae	100 Glucose	—	0.27	0.46	20
Industrial[a)]	20 Glucose	—	0.29	—	20
424A (LNH-ST)[b)]	20 Xylose	—	0.21	—	—
Unidentified[c)]	100 Glucose	250	—	—	—
ATCC 4226[d)]	100 Glucose	315	—	—	—
Bacteria					
Z. mobilis[a)]	100 Glucose	—	0.37	0.49	50
E. coli[c)]	100 Xylose	<5	—	—	—

a) Davis et al. (2006).
b) Govindaswamy and Vane (2007).
c) Tao et al. (2010).
d) *Papiliotrema laurentii* (Kufferath).

(growth). It is because of this that the parameter β is sometimes known as the maintenance coefficient, as it describes the rate of product formation that occurs to maintain the cells that are currently alive in the reactor but not to produce any more cells.

$$\underset{\text{product accumulation}}{\frac{dP}{dt}} = \underset{\text{growth associated}}{\alpha(\mu X)} + \underset{\text{nongrowth associated}}{\beta X} \tag{2.12}$$

Because substrate consumption and product formation are directly linked in a type 1 fermentation, the ODE for the rate of substrate utilization will be proportional to the rate of product formation using a yield coefficient in a similar manner as in Eq. (2.11). However, in this case, the yield coefficient, $Y_{S/P}$, is defined as the incremental substrate consumption per incremental product formed (dS/dP).

$$\frac{dS}{dt} = -Y_{S/P}\frac{dP}{dt} \tag{2.13}$$

From these basic models, more complex models that mathematically describe industrial fermentations can be derived. In addition to substrate availability constraining growth, the accumulation of the product in the fermentation media can also inhibit cellular growth. Ethanol fermented from plant sugars in a batch reactor is one example of this. In a batch process, sterilized media that contains a primary and limiting nutrient, such as glucose from corn starch or sucrose from cane sugar, along with other nutrients is used to fill the fermenter along with an initial inoculum of *S. cerevisiae* yeast cells. Over the course of the process, the sugar is consumed and cells and ethanol are produced. A by-product of the fermentation of sugars to ethanol is carbon dioxide (CO_2), which leaves the reactor as a gas. Once the exponential growth phase begins, vigorous generation of CO_2 occurs with one mol of CO_2 generated for each mol of ethanol produced. In addition, heat is generated as a by-product of the metabolism of the cells. At

large scale, active measures such as heat exchangers are required to remove the heat to prevent the temperature of the fermenter from rising above a level that can be tolerated by the cells Eq. (2.14)):

$$\text{Glucose} + \text{nutrients} + \text{few cells} \rightarrow \text{many cells} + \text{ethanol} + \text{CO}_2 + \text{heat} + \text{by-products} \tag{2.14}$$

Trace amounts of oxygen are essential for synthesis of unsaturated fatty acids and steroids required for growth of brewer's yeast, although the fermentation itself is microaerobic (not aerated). The experimental conditions resulting in the constants for the model were based on measurements at a constant temperature of 30 °C, a pH range of 3.9–4.15, and a limiting amount of substrate (1% glucose). The composition of the fermentation medium consisted of 1 l of tap water, 10 g of glucose, 1.5 g of yeast extract, 2.5 g of NH_4Cl, 5.5 g of Na_2HPO_4. $7H_2O$, 3 g of KH_2PO_4, 0.25 g of $MgSO_4$, 0.01 g of $CaCl_2$, and citric acid/sodium citric at approximately 5 and 2.5 g, respectively, to control the pH at about 4.

The accumulation of ethanol in the reactor has the effect of slowing the growth rate of the cells; thus, the Monod model must be modified to account for this:

$$\frac{dX}{dt} = X\frac{\mu_{max}S}{K_m + S}\left(1 - \frac{P}{P_{max}}\right)^n \tag{2.15}$$

where X is the biomass concentration (g/l); μ_{max}, maximum specific growth rate (h^{-1}); K_m, Monod constant; S, substrate (glucose) concentration (g/l); P, product (ethanol) concentration (g/l); P_{max}, maximum tolerable ethanol concentration (g/l); n, toxicity coefficient.

In the model given by Eq. (2.15), there is a maximum concentration for the product, ethanol, above which no growth will occur (the function $1 - P/P_{max}$ becomes 0). Product formation and substrate consumption is given in Eqs. (2.8) and (2.11).

For many large-scale processes, significant economic advantages can be gained by converting a batch process into a semicontinuous process – generally fed-batch in operation. At the most basic level, a continuous process is more productive than a batch process because of the fact that a fermenter used for a batch process must be emptied, cleaned, and refilled between batches. That turnaround time is the time when the equipment is not making a product that can be sold to support the purchase and operating costs of the equipment. Disadvantages of a continuous process include low product concentration, handling of large water volumes, and substrate remnants in the harvested broth. One continuous process used for fermentations is known as a continuous stirred tank reactor (CSTR) where the substrate solution is fed into a reactor at a volumetric rate equivalent to the rate that the medium is being removed such that the reactor volume remains constant. The concept, shown in Figure 2.7, is a tank of fixed volume that is under constant mixing to quickly incorporate the new medium flowing into the reactor.

In the ideal case, a key assumption can be made to develop the equations describing growth and substrate consumption in a CSTR. We must assume that the reactor contents are homogeneous because of efficient and rapid mixing. Thus, there are no gradients in concentration or temperature within the reactor

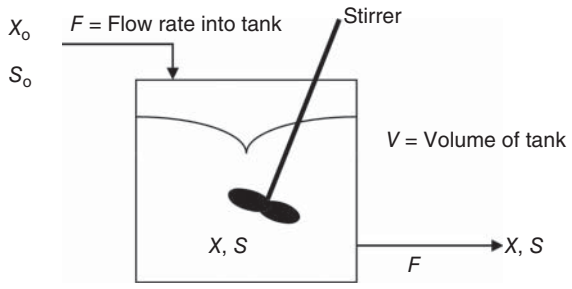


Figure 2.7 A continuous stirred tank reactor (CSTR). The biomass (X) and substrate (S) concentrations in the reactor and effluent are affected by the growth rate, substrate consumption, and the concentration of biomass (X_0) and substrate (S_0) fed into the reactor as described in Eqs. (2.16)–(2.19). Source: Mosier and Ladisch (2009). Reproduced with permission of John Wiley and Sons.

and the fresh media being introduced is "instantaneously" incorporated into the reactor volume. This assumption also allows us to conclude that the concentrations of biomass (X), substrate (S), and product (P) in the effluent leaving the reactor are the same as the concentrations inside the reactor. Finally, because the reactor is homogeneous, we can consider the entire reactor volume as our control volume for developing the continuity equation (2.16) for the purpose of imposing a mass balance constraint on the process.

$$\text{Accumulation} = \text{In} - \text{Out} + \text{Generation} - \text{Consumption} \tag{2.16}$$

The continuity equation forms the basis for deriving our biomass and substrate equations. Starting with biomass concentration we find:

$$[\mathrm{d}X \cdot V]_{\text{acc}} = [F \cdot X_0 \cdot \mathrm{d}t]_{\text{in}} - [F \cdot X \cdot \mathrm{d}t]_{\text{out}} + \left[X \frac{\mu_{\max} S}{K_{\text{m}} + S} \cdot V \, \mathrm{d}t\right]_{\text{gen}} - [0]_{\text{con}} \tag{2.17}$$

where X is the biomass concentration in the reactor (g/l); X_0, biomass concentration in the influent (g/l); V, volume of the reactor (l); F, volumetric flow rate into/out of the reactor (l/h); $\mu_{\max}$, maximum specific growth rate (h^{-1}); K_{m}, Monod constant; S, substrate (glucose) concentration (g/l).

If we assume that the influent solution is sterile ($X_0 = 0$) and that the cells are not dying (loss = 0), we can divide through by $V\,\mathrm{d}t$ (volume multiplied by differential time) to find the rate change of biomass concentration in the reactor Eq. (2.18).

$$\frac{\mathrm{d}X}{\mathrm{d}t} = -[D \cdot X] + \left[X \frac{\mu_{\max} S}{K_{\text{m}} + S}\right] \tag{2.18}$$

The ratio of flow rate to reactor volume (F/V) is replaced with the parameter D, called the dilution rate, which has units of inverse time (h^{-1}). Following a similar derivation, the rate expression for substrate change in the reactor can be derived and simplified to

$$\frac{\mathrm{d}S}{\mathrm{d}t} = (S_0 - S)D - \left[Y_{S/X} \cdot X \frac{\mu_{\max} S}{K_{\text{m}} + S}\right] \tag{2.19}$$

where S_0 is the substrate concentration in the influent (g/l); D, dilution rate (h^{-1}); $Y_{S/X}$, yield coefficient, dS/dX.

Under steady-state conditions, the concentrations of cells and substrate within the CSTR do not change (i.e. $dX/dt = dS/dt = 0$) and Eqs. (2.18) and (2.19) can be solved algebraically. The cell balance equation (2.18) simplifies to

$$\mu = D \tag{2.20}$$

where μ is the function for specific growth rate (e.g. Eq. (2.10)) and D is the dilution rate. If we rearrange Eq. (2.17) with the full expression for μ, we find the steady-state solution for S in the reactor.

$$S = \frac{K_m D}{\mu_{max} - D} \tag{2.21}$$

Substituting the expression for the steady-state value of S into Eq. (2.19), we can solve for the expression that gives the steady-state value of X:

$$X = Y_{X/S}\left(S_0 - \frac{K_m D}{\mu_{max} - D}\right) \tag{2.22}$$

where $Y_{X/S}$ is the inverse of the yield coefficient for substrate utilization ($1/Y_{S/X}$). As μ is bounded between 0 and μ_{max}, there is a dilution rate (D) where the steady-state concentration of biomass (X) will go to 0 (Eq. (2.22)). This *critical* dilution rate (D_c) can be found by setting X equal to 0 in Eq. (2.22) and solving for D

$$D_c = \frac{\mu_{max} S_0}{K_m + S_0} \tag{2.23}$$

The behavior of the CSTR can be shown graphically (Figure 2.8), where the productivity of the reactor as a function of cell biomass output (concentration times flow rate) increases with dilution rate (D) until reaching the critical dilution

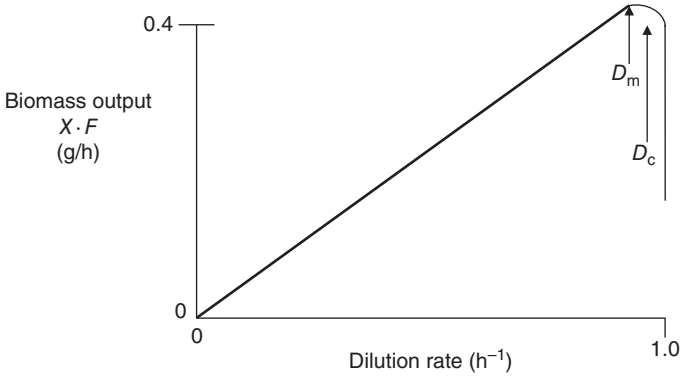


Figure 2.8 Illustration of biomass output as a function of dilution rate typical for continuous stirred tank reactor cultivations. D_m means maximal dilution rate equal to maximal growth rate, D_c means critical dilution rate, a rate where washing out is observed because it is above the maximal growth rate of the microorganism. Source: Mosier and Ladisch (2009). Reproduced with permission of John Wiley and Sons.

rate (D_c). Beyond the critical dilution rate, the cell concentration in the reactor will go to zero in a condition known as "washout," so called because the flow of liquid through the reactor washes the cells out of the reactor faster than cell growth can replenish the cells within the reactor.

These CSTRs are sometimes referred to as a chemostat or turbidostat bioreactors (Novick and Szilard 1950a,b). The term turbidostat probably arose from the observable changes in turbidity that occurred when the rate of addition of substrate (liquid) changed, causing the steady-state population of growing cells to change in number. As the number of cells increased, the turbidity also increased. The principle of the chemostat is based on the observation that a reactor operated in this manner has a constant output (CO_2, soluble products, etc.). This is analogous to the assumption that underlies the model of a CSTR. The time to mix a small volume of medium should be small relative to the replacement time, t_r, given by V/F, where V is the culture volume and F is the flow rate at which the medium is added to the fermentor. The dilution rate is inversely proportional to the replacement time. Furthermore, many of the equations developed from the models only account for biomass production and do not take into account product formation. While limited, these studies can be vital in providing information on many fundamental aspects of bioprocesses.

A major difference between a CSTR and a CSTBR (continuous stirred tank biological reactor) is the occurrence of biomass growth and the difficulty in stoichiometrically accounting for the disappearance of substrate directly into products, since the product includes living biomass as well as individual biochemical species.

Uses of a CTSBR for research purposes may include

1. study of biomass growth rate with respect to changes in substrate concentration;
2. study of biomass growth rate in response to environmental parameters such as pH and temperature;
3. substrate-limited growth with a constant flow rate; and
4. study of metabolic flux for systems biology models of cellular metabolism.

Theories attributed to Monod (1950) and Novick and Szilard (1950a,b) first showed that the specific growth rates could be fixed at any values ranging from 0 to the maximum specific growth rate, μ_{max}.

2.2.3 Metabolic Pathways

Bio-based products are the intermediates or end products of biochemical reactions within the cell known as metabolism (Table 2.2). Metabolism encompasses more than 1000 different enzyme-catalyzed reactions performed simultaneously by microbes. Series of metabolic reactions, or metabolic pathways, are used to derive energy and essential cellular building blocks and are highly regulated and interconnected. Metabolic reactions are stereospecific, with the potential to produce compounds with several stereocenters such as pharmaceuticals that are uneconomical to produce by traditional chemical synthesis. These reactions also use renewable feedstocks as starting substrates to sustainably produce common

Table 2.2 Common bio-based products derived from microbial metabolic pathways.

Metabolite	Metabolic pathway	Cellular function	Typical product
Alcohols	Fermentation	By-product of energy generation	Biofuel (Chapter 4)
Organic acids	Citric acid cycle and fermentation	By-product of biomass and energy generation	Food additive (Chapter 5)
Amino acids	Amino acid metabolism	Building block of proteins (biomass generation)	Animal feed (Chapter 6)
Riboflavin	Purine biosynthesis	FAD precursor	Animal feed (Chapter 7)
(Aromatic) polyketides	Polyketide metabolism	Cell defense	Antibiotics (Chapter 8)

commodity chemicals. Thus, the study and control of metabolism by bioprocess scientists and engineers enable new opportunities to improve process efficiency and develop new bioproducts.

Metabolism is highly regulated to conserve cellular resources and produce metabolites as needed. However, this regulation is often counter to industrial applications where one wants to maximize product formation. Consequently, scientists and engineers have developed creative approaches and innovative tools to manipulate microbial metabolism. In the early days of fermentations, scientists based their processes around microbial mutants where formation of a targeted product was unregulated. With increased understanding of biology and DNA, scientists realized that overproducing mutants could be generated via chemical- or radiation-induced mutagenesis and isolated via subsequent functional screens. These techniques were successfully used to overproduce in commercial quantities many nutrients such as the amino acids L-lysine, L-glutamate, L-tryptophan, and L-threonine, which also have value as food flavors and animal feed components.

2.2.4 Manipulation of Metabolic Pathways

Advances in molecular biology and recombinant DNA technology enable scientists to directly manipulate metabolism. As enzymes that drive metabolism are encoded within the DNA of the microbial production platform, scientists can control metabolism with specific alterations to this genetic code. DNA is organized in functional subunits, termed "biological parts," that control (i) the relative expression level of genes encoding enzyme (promoter and RBS), (ii) how production of the enzyme is regulated by externally supplied chemicals and internal metabolites (promoter and operator sites), and (iii) encode the specific function of the enzyme (coding domain sequence – CDS). Recent advances in DNA sequencing and synthesis now allow scientists to read and rewrite this code in a high-throughput manner. For more than a decade, computer-optimized DNA design and chemical synthesis have been used to assemble *in vitro* new combinations of biological parts from diverse species that introduce new metabolic pathways or otherwise optimize production. Microbial cells are

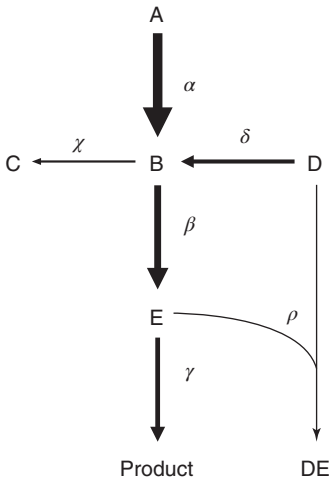


Figure 2.9 Metabolic pathways for product are interconnected with competing reactions in the cell. Each reaction is catalyzed by an enzyme, represented by a Greek letter, to form intermediates, represented in bold. The relative flux to each intermediate is indicated by the arrow line weight. In this hypothetical example, the desired product is the major metabolite produced.

then transformed with the newly programmed DNA via the introduction of self-replicating circular DNA, known as plasmids, that contain the synthetic DNA, or through direct integration of the DNA into the host genome at a specific chromosomal location (locus). These approaches have grown tremendously in power with the advent of gene-editing technologies such as CRISPR/Cas9 that can be readily programmed by scientists to target essentially any DNA sequence within the genome for modification.

The ability to directly manipulate microbial metabolism has revolutionized the field, giving birth to the discipline of *metabolic engineering* with paradigms borrowed from classical engineering disciplines. The interconnected nature of microbial pathways form networks where the *metabolic flux* of an intermediate, or the turnover rate of a metabolite through a pathway, is controlled by the relative abundance of enzymes and their intrinsic kinetic properties (Figure 2.9). The objective of the metabolic engineer is to optimize pathway flux toward the product while minimizing the impact of these manipulations on cellular health. Common approaches include disruption or deletion of competing pathways (e.g. deleting the gene encoding enzyme χ) or increasing the amount of pathway enzymes (e.g. by upregulating genes α, β, and/or γ). Modifications of this form are most beneficial at bottlenecks or metabolites common to multiple pathways that form control points for the distribution of metabolic flux. Metabolic pathways may also be susceptible to feedback inhibition where excess product downregulates its formation by inhibiting an upstream enzyme (e.g. product inhibits α). In these cases, engineers may replace the enzymes with evolutionary cousins of a similar function from another organism that does not experience feedback inhibition. Similar approaches may be used to supply pathway enzymes with superior kinetic properties (e.g. higher k_{cat}). An example is given in the schematic of Figure 2.10, where removal of enzyme *c* allows product *P* to be accumulated. Addition of the compound *E* (required for growth and functioning) to the fermentation broth enables the cell to stay viable and/or proliferate, in the absence of enzyme *C*.

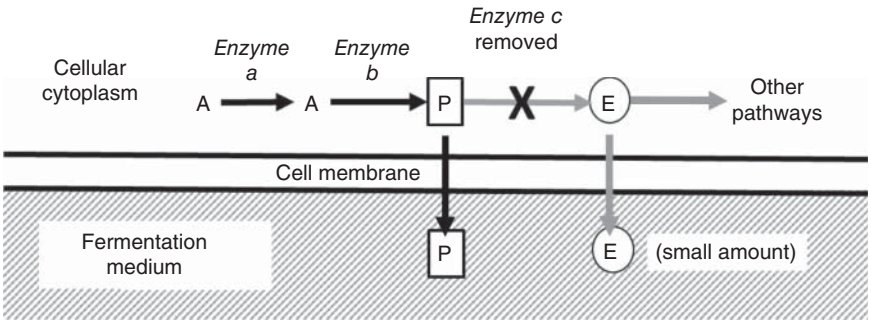


Figure 2.10 Representation of effect of gene editing to remove pathway from P (desired product) to E (undesired product). Source: Aiba et al. (1973) and Wang et al. (1979). Reproduced with permission of Elsevier and John Wiley and Sons.

2.2.5 Future of Pathway Design

With current understanding of biochemistry, and growing knowledge of the biosynthetic potential encoded within organisms, scientists can now develop non-natural metabolic pathways to sustainably produce bio-based products. Enzymes have historically been thought of only catalyzing a reaction with a specific metabolic precursor. However, enzymes are promiscuous and, more accurately, catalyze a *type* of reaction (e.g. a Claisen condensation) with a *preference* for a given precursor. These preferences may be loose or restrictive, making it possible for an enzyme to form an array of products dependent on the precursor fed. Thus, engineers and scientists may devise reaction schemes to upgrade the value of a common metabolite to a value-added chemical by identifying the types of chemistries needed and the enzymes that are capable of that type of transformation. Libraries of these enzymes from different organisms that complete a similar chemistry are then screened to identify variants that will accept the required precursors. Similarly, transporters for the feedstock and the product are needed to move materials across the cell membrane at a sufficiently high production rate. Enzyme variants and transporters that successfully transform the precursors and transport the products as desired may be permanently introduced to a microbe via CRISPR/Cas9 or other recombinant methods to build pathways for non-natural compounds. This approach, also called retro-biosynthesis, has successfully created 4-methyl-1-pentanol that might be applied as a next-generation biofuel and didanosine, a dideoxyinosine whose activity as a nucleoside reverse transcriptase inhibitor may be leveraged as an HIV antiviral agent (Birmingham et al. 2014; Sheppard et al. 2014).

These non-natural products, however, may be volatile, nonaqueous, and/or otherwise inhibitory and require unique bioprocess innovations such as two-phase bioreactors or stripping fermentations to recover product and maximize yield. In two-phase bioreactors, toxic nonaqueous products and by-products partition from the aqueous production phase rich in cells to a flowing organic overlay to recover the product (e.g. biofuels). Stripping fermentations, in contrast, continuously condense the fermentation headspace to recover and remove volatile products that may inhibit product formation (e.g.

styrene production). Other products in certain species (e.g. *Shewanella*) are redox dependent and may benefit from electrofermentation or direct electron injection/removal via electrodes. In all these approaches, however, knowledge of thermodynamic equilibria, product toxicity, and more importantly microbial metabolism is essential to design the fermentation process to maximize product formation.

Metabolic pathways exist within the context of a cell that constrains the ability of bioprocess scientists and engineers to manipulate metabolism. Fundamentally, metabolism exists to fulfill important cellular functions. Thus, competing pathways that produce essential cellular building blocks cannot be removed even though they may limit product formation. To better identify and regulate these critical metabolic nodes, scientists build genome-scale metabolic (GSM) models of metabolism that create a flux balance for every metabolic reaction in the cell. For healthy cells, the fluxes are at steady state. Scientists may use linear programming techniques to solve the optimization problem of product flux with a constraint of sufficient biomass formation (e.g. amino acid production and nucleotide production are high and above a specific threshold) to predict the specific interventions that will improve product formation while maintaining cellular viability. Similarly, metabolic intermediates may interfere with other cellular processes if allowed to accumulate. To address this challenge, engineers and scientists build genetic circuits using the tools of synthetic biology that provide negative feedback by reducing pathway enzyme expression in response to the accumulation of toxic intermediates. These circuits are also frequently triggered by environmental triggers that are controlled as part of the bioprocess (e.g. light exposure, introduction of chemical inducers, and changes in temperature).

2.3 Oxygen Transport

2.3.1 Aerobic versus Anaerobic Conditions

Bailey and Ollis (1986) succinctly define "the sequence of steps that extract energy under anaerobic conditions" as fermentations. This convention follows that of Pasteur who called fermentation "life without air." As most early microbiological processes such as wine making were anaerobic, the term was also used to describe these processes. Microbial conversion processes are usually called fermentations in industry even though many of these processes are aerobic. The exact meaning of fermentation must often be inferred from context (Bailey and Ollis 1986, p. 232). This chapter, as well as other sections of the book, attempts to clearly define the nature of the transformations carried out by living microorganisms, although the term fermentation will be used in an industrial context, i.e. to denote microbial cultures and transformations under both aerobic and anaerobic conditions.

Strictly speaking, fermentative conditions refer to metabolism that occurs in the absence of oxygen. The metabolism leading to alcohols is referred to as being anaerobic ("in the absence of oxygen"). However, ethanol is actually generated in the presence of trace amounts of oxygen, at microaerobic conditions. Aerobic bioreactors are a key component of the existing and emerging biotechnology

industry. They provide numerous valuable products ranging from proteins and peptides to antibiotics and organic acids. They also begin to motivate our desire for a more detailed fundamental knowledge of how cells use oxygen to make products that are industrially important. *S. cerevisiae* grows at both aerobic and microaerobic conditions, although the end products are different at the two conditions. Aerobic conditions are achieved by introducing air into fermentation media, while anaerobic conditions are the ones where air is excluded (or when an inert gas like N_2 is used). Strictly anaerobic conditions refer to microbial culture where oxygen must be almost completely absent because it is toxic to the microorganisms.

The definition of respiration in the context of industrial processes is also sometimes unclear. Although respiration is a process where organic or reduced inorganic compounds are oxidized by inorganic compounds to produce energy, oxidants other than molecular oxygen (O_2) can be involved. For example, sulfate (SO_4^{2-}) is the oxidant for *Desulfovibrio* and nitrate (NO_3^-) is used by denitrifying bacteria. Bacterial sulfate reduction is believed to be possible at temperatures of up to 110 °C, based on the measurements made at deep ocean hydrothermal vents (Wang et al. 1979).

Most industrial fermentations are aerobic and have metabolisms that can be characterized by aerobic respiration. Anaerobic respiration refers to metabolism where an oxidant other than oxygen is involved. Aerobic respiration refers to processes where O_2 is the oxidizing agent.

2.3.2 $k_L a$ – Volumetric Mass Transfer Coefficient

The maximum solubility of oxygen in water decreases from about 1.7 mM at 10 °C to 1.03 mM at 40 °C (Bailey and Ollis 1977). The concentration of electrolytes in a fermentation medium is known to be a controlling parameter in oxygen diffusion and also suppresses solubility. At 25 °C, oxygen solubility is 1.26 mM in the absence of NaCl and 1.07 mM at 500 mM NaCl. The measurement at which air is taken up during fermentation requires knowledge of a mass transfer coefficient as well as the area of the air bubbles through which air may diffuse.

A vigorously growing cell fermentation may contain 10^9 cells/ml in mid-exponential growth phase (discussed in Chapter 5). Bailey and Ollis (1977) calculated oxygen requirements at 375 mmol/l/h:

$$\frac{0.3\ \text{g O}_2}{\text{g dry cell mass/h}} \left| 10^9 \frac{\text{cells}}{\text{ml}} \right| 10^{-10} \frac{\text{cm}^3}{\text{cell}} \left| 1 \frac{\text{g cell mass}}{\text{cm}^3} \right| 0.2 \frac{\text{g dry cell mass}}{\text{g wet cell mass}} \Bigg| = 0.006 \frac{\text{g O}_2}{\text{ml h}} = 375 \frac{\text{mmoles}}{\text{l h}}$$

Transfer of oxygen occurs from the inside of an air bubble into the liquid that surrounds it. The major resistance is the film of liquid that surrounds each air bubble and through which the oxygen must be transferred. The volumetric oxygen transfer, for liquid side controlling, is given by Eq. (2.24) and is illustrated in Figure 2.11:

$$N_A = Hk_L a(\overline{p} - p^*) = k_L a(\overline{C} - C^*) \tag{2.24}$$

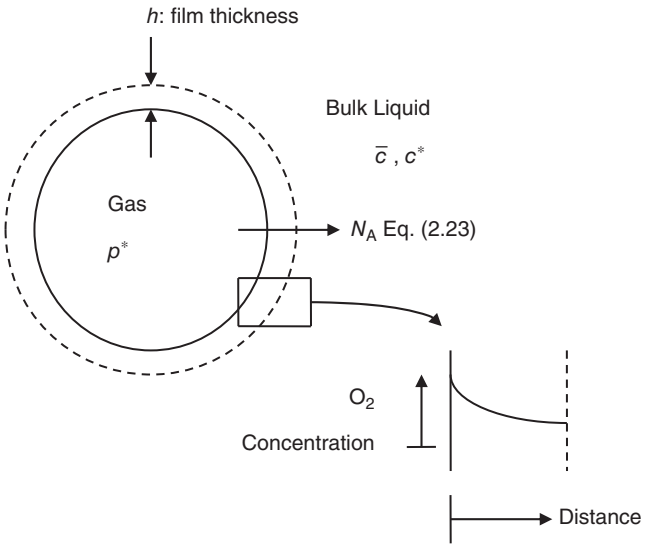


Figure 2.11 Schematic representation of a gas bubble where N_A represents mol of oxygen transferred and typical dimensions are (mm) for gas bubbles and (μm) for film thickness (=distance). Source: With permission from Mosier and Ladisch (2009).

where k_L is the mass transfer coefficient on liquid side; H, Henry's constant; a, interfacial area between liquid and gas per unit volume of liquid; A/v, interfacial area/liquid volume; $\overline{p}$, partial pressure of O_2 in bulk gas phase; p^*, equilibrium concentration of O_2 in gas phase with respect to $\overline{p}$; $\overline{C}$, dissolved O_2 concentration in bulk liquid phase; C^*, equilibrium concentration of O_2 in the bulk (liquid) phase.

The product of mass transfer rate and interfacial area is important to successfully scale up a fermentation vessel. Because C^* and $\overline{C}$ are usually known or readily estimated, the measurement of k_L and a, separately, or $k_L a$ as a group enables Eq. (2.28) to be used.

Determination of k_L may be achieved by sparging N_2 through a tank filled with liquid medium. Once this is done, air is injected and dissolved oxygen as a function of time of sparging is measured. The change in concentration of oxygen in the liquid phase is based on the (linear) difference between equilibrium (C^*) and operational or bulk phase ($\overline{C}$) oxygen concentrations:

$$\frac{dc_L}{dt} = k_L a(C^* - \overline{C}) \tag{2.25}$$

Equation (2.25) is graphically represented in Figure 2.12, where the slope is equivalent to $k_L a$. Interfacial area is proportional to hold-up in a tank. In this case (Eq. (2.26)):

$$a = \frac{\overset{\text{Hold-up}}{\text{total volume of bubbles}}}{\text{total volume of broth}} \frac{\overset{\text{Surface to volume}}{\text{area of bubble}}}{\text{volume of bubble}} = \frac{nF_o t_b}{V} \quad \frac{\pi D^2}{\left(\frac{\Pi D^3}{6}\right)} \tag{2.26}$$

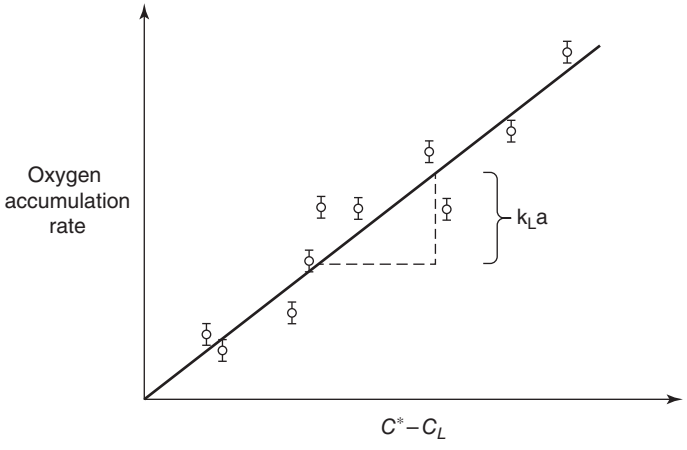


Figure 2.12 Schematic representation of definition of $k_L a$ showing how an increase in dissolved oxygen concentration in an oxygen-free (previously nitrogen sparged) aqueous solution may be used to estimate $k_L a$. Source: From Mosier and Ladisch (2009), with kind permission from National Research Council.

where n is the number of orifices in a sparging tube; F_o, volumetric air flow rate per orifice; t_b, residence time of bubble in liquid; D, average diameter of air bubble.

Equation (2.27) is directly expressed in terms of hold-up and bubble diameter:

$$a = H\left(\frac{6}{D}\right) \tag{2.27}$$

Hence, a direct method for estimating a in a tank is enabled by measuring the increase in the liquid level when air is bubbled through the broth of a specified rate.

2.4 Heat Generating Aerobic Processes

The breaking of high-energy bonds that occurs during cellular metabolism generates heat[1]. Heat generation and dissipation are important considerations for all fermentations. For large-scale fermentations, understanding the biochemical formation of heat is important for engineering bioprocesses to remove that heat from the reactor. Studies with *E. coli* K12, grown on succinic acid, showed that the enthalpy change accompanying the growth process that involves both anabolism and catabolism was nearly the same as catabolism alone (Battley 1991). For the purpose of these calculations, the formula mass of the cells was 24.19 Da with an atomic composition of $CH_{1.595}O_{0.374}N_{0.263}P_{0.023}S_{0.006}$ (Battley 1991). The number of available electrons in a unit carbon formula mass is 4.16. There is one carbon atom in the molecular formula from which the unit carbon formula mass is derived, and hence, the degree of reduction of the biomass is given by the number

1 (Parts Excerpted From Mosier and Ladisch 2009)

Table 2.3 Comparison of heats associated with growth of *E. coli*. Basis of calculation: 1 M succinic acid at pH = 7 and temperature of 25 °C (data from Battley 1991). Succinic acid, MW = 118.

Process	Succinic acid (kJ/mol)	Succinic acid (kJ/g)
Anabolism	+15	0.12
Catabolism	−780	−6.6
Metabolism	−770	−6.5
Total	−1500	−13
Oxidation	−1600	−13

of available electrons.[2] The yield coefficient for the cells (includes ions, as well as organic matter) was 41.4 g of dry microbial biomass per mol of succinic acid (or about 0.35 g of cells/g substrate). The stoichiometry for this conversion, as experimentally measured in an aerated fermentation, was

$$\begin{aligned} C_4H_6O_4\,(aq) + 0.438\,NH\,(aq) + 0.038\,HPO_4{}^{2-}(aq) + 0.010\,HSO_4{}^{-}\,(aq) \\ + 0.086\,H^+\,(aq) + 1.747\,O_2\,(g) + 1.664\,\text{Ions} \\ \rightarrow 1.664\,[CH_{1.595}O_{0.374}\,N_{0.263}\,P_{0.023}\,S_{0.006} + \text{internal ions}] \\ + 2.336\,CO_2\,(g) + 2.397\,H_2O\,(1q) \end{aligned} \quad (2.28)$$

Aqueous is abbreviated as aq, whereas liquid and gas are abbreviated as lq and g, respectively.

These data resulted in calculation of heats in catabolism, anabolism, and metabolism (sum of catabolism and anabolism) for *E. coli* grown on succinic acid. The calculations support the concept that a carbon substrate can be almost completely oxidized to CO_2, water, and heat through microbial transformations as represented by Eq. (2.29) (Battley 1991).

$$\begin{aligned} 0.505\,C_4H_6O_4\,(aq) + 1.747\,O_2\,(aq) + 0.010\,HSO_4{}^{-}\,(aq) \\ \rightarrow 2.020\,CO_2\,(aq) + 1.515\,H_2O\,(1q) + 0.010\,HS^-\,(aq) + \text{heat} \end{aligned} \quad (2.29)$$

These results generally apply to aerated, microbial fermentations that can give a net heat release that is close to the heat generated if the molecule (succinic acid, in this example) was completely oxidized as shown in Table 2.3.

Measurements of biomass and heat generated for the yeast, *Kluyveromyces fragilis*, grown aerobically on deproteinized whey permeate[3] at 30 °C with pH

2 A detailed analysis of methods, results, and impacts of different assumptions for calculating structure is discussed in Battley (1991). The formula, briefly summarized here, should be further interpreted in the context of the detailed discussion in Battley (1991) if exact calculations of the relatively small heat of anabolism are to be carried out.

3 Whey remains after casein has been coagulated for the manufacture of cheese. Deproteinized whey permeate refers to the liquid stream that passes through a membrane system, in which sugars and other lower molecular weight molecules pass while proteins are retained. The fluid that passes through the membrane is the permeate, while the fluid that is retained is referred to as the retentate.

Table 2.4 Measured yield coefficients for *K. fragilis* grown in batch culture on different carbon sources.

Carbon source	Lactose	Lactose	Glucose	Galactose
Type of medium	Whey permeate	Defined	Defined	Defined
$Y_{X/S}$ $\frac{\text{g cells generated}}{\text{g carbon source consumed}}$	0.51	0.52	0.45	0.59
$Y_{Q/O}$ $\frac{\text{kJ heat generated}}{\text{mol of } O_2 \text{ consumed}}$	440	410	410	450
$Y_{Q/X}$ $\frac{\text{kJ heat generated}}{\text{g cells generated}}$	9.4	11	13	13
Closure on carbon balance (%)	91	97	90	110
Closure on energy balance (%)	85.6	90.0	89.8	102.7

Source: Marison and Von Stockar (1988). Reproduced with permission of Wiley-VCH.

controlled at 5.5 resulted in the consumption of 45 g/l sugars to zero in less than nine hours, with a proportional increase in biomass during the same time period. The rate of heat generated corresponded to the rate of cell growth, with the total heat generated being directly proportional to the amount of biomass generated and the amount of oxygen consumed, expressed as molO_2/l broth.[4] The heat evolved fits a straight line with a slope of about 10 kJ Heat evolved/g cell mass or 545 kJ Heat evolved/mol O_2. This corresponds to about 2.4 kcal/g and 130 kcal/mol O_2, respectively. The heat generated is higher than the 444 kJ/mol reported for *K. fragilis* grown in defined media containing lactose, glucose, and galactose (Table 2.4) by other researchers but still falls within the expected range. Similar results were shown by strains of *Candida lipolytica* (grown on citrate, succinate, and hexadecane), *Candida utilis* (grown on glucose, acetate, ethanol, and glycerol); and *Candida boidinii* (grown on ethanol) (Marison and Von Stockar 1988). The data indicated that some variability is likely to be encountered in the literature because of different growth conditions and substrates.

Heat is not only generated by biological activity in fermentation. Mechanical work loss through agitation causes heat generation while heat loss occurs through evaporating water or heat transfer from the sides of the fermenter. These sources and sinks must be included in a complete energy balance for a fermentation process. A comprehensive study by Cooney et al. (1969) and Cooney (1979) accounted for heat generated by agitation of the vessel and heats of evaporation, sensible heat loss (due to heat-up of fluid), and heat lost to surroundings, as well as the heat of fermentation. The sum of the heats of agitation, evaporation, sensible heat loss, and loss to surroundings were about 17 kJ/l/h for a 10 l fermenter filled with 9 l of media. Their data were obtained for both complex and defined media

4 Oxygen is only sparingly soluble in aqueous media, with concentrations typically found at the 6 to 8 ppm level. Consequently, the heat evolved based on oxygen consumption is calculated by dividing the total oxygen consumed (experimentally measured) by the volume of the fermentation broth.

over a range of oxygen consumption rates and total oxygen consumption. A linear correlation was obtained for the Q_{heat}, the total heat produced as a function of oxygen consumption rate, q_{ox} for *E. coli* grown on succinic acid (Eq. (2.30)):

$$Q_{heat} = K_{ox} q_{ox} \tag{2.30}$$

The value of 460 kJ/mol is similar to the value of 540 kJ/mol calculated for *Kluyveromyces fragilis* that was described above. A similar linear relation between rates and extents of CO_2 evolution was observed with $K_{CO_2} = 0.46 \pm 0.02$ kJ/mmol CO_2.

The *rate* of heat production, q_h, was found to be a linear function of the rate of oxygen consumption q_{ox} as well, with:

$$q_h = k q_{ox} = 0.519 q_{ox} \tag{2.31}$$

where k is in dimensions of $\left(\frac{\text{kJ heat generated}}{\text{liter fermentation broth·h}}\right) / \left(\frac{\text{mmol } O_2 \text{ consumed}}{\text{liter fermentation broth·h}}\right)$, or kJ/mmol O_2. This important and practical series of experiments showed that heat production and oxygen consumption are directly proportional, and relatively constant, for a range of growth rates, substrates, and types of microorganism (Cooney et al. 1969; Cooney 1979).

2.5 Product Recovery

2.5.1 Basics

Microbial process modes of operation can be categorized in groups including batch, fed-batch and continuous. For the first (batch), the nutrients needed to the growth are initially added to the vessel, and the products are removed once the period of growth is completed. Although in a continuous process nutrients are continually fed to and product is withdrawn from the vessel (liquid flow rates are generally the same and in this sense constant volume is maintained), fed-batch processes fell between batch and continuous processes. In the fed-batch process, an initial concentration is added to the fermenter, and during this time, the cultivation is a batch process, with microorganisms growing at their maximum specific growth rate. After that, nutrient is added into the vessel and the product is removed once the process is completed. In this process, it is possible to vary the feed rate, so that the concentration of nutrients can be controlled in the bioreactor.

2.5.2 *In Situ* Product Recovery (ISPR)

In Situ Product Recovery (ISPR) techniques are based on the removal of the product from the vicinity of the cell as soon as it is formed (Freeman et al. 1993) with the expected outcome of leading to increased productivity and overall titers for inhibited fermentations, as well as reduced wastewater treatment costs (Roffler et al. 1984; Outram et al. 2017). The medium composition can influence growth and product yields, and they can be separated into three broad classes. Nutrients that are not chemically defined, such as yeast extract, tryptone, peptone, and casamino acids, are part of the complex medium. A defined medium, for its

turn, is chemically defined, whereas a semicomplex or semidefined medium is a combination of the two (Yee and Blanch 1992).

ISPR technologies include various types of techniques, and the application will depend mainly on the nature of the product (Takors 2004). Although it is predominantly used to remove the target product from the production medium, in some cases, ISPR can also be used to separate inhibitory by-products, which, when present in high concentrations, affect the titer of the target compound (Wong et al. 2009, 2010). The different possible configurations of ISPR, depending on the location of the separation unit, are internal-direct (ID), internal-indirect (II), external-direct (ED), and external-indirect (EI). The majority of investigations (60%) apply the internal-direct configuration, with 35% being of the external type. Indirect configuration is preferred when the presence of microorganisms adversely affects the performance of the ISPR technology (Van Hecke et al. 2014). Figure 2.13 shows heuristics for selection of the proper ISPR technology.

Some bio-based processes are limited by product titers and volumetric productivities because of product inhibition, whereas others suffer from side reactions decreasing the yield (total product produced to total substrate consumed) of the process. As a consequence, this will lead to major downstream processing costs, high wastewater volumes and fermenter costs, as well as increased substrate cost in the case of a decreased yield.

In this sense, an investment in a recovery technology that allows the selective separation of the product during fermentation can be advantageous, so that it is possible to enrich the product. This will lead to a decrease in downstream processing costs, improve the volumetric productivity when product inhibition is alleviated, reduce the process flows (decrease the amount of wastewater per mass unit of product), and by removal of the target product from the fermentation broth, improve the yield, while also rendering it unavailable

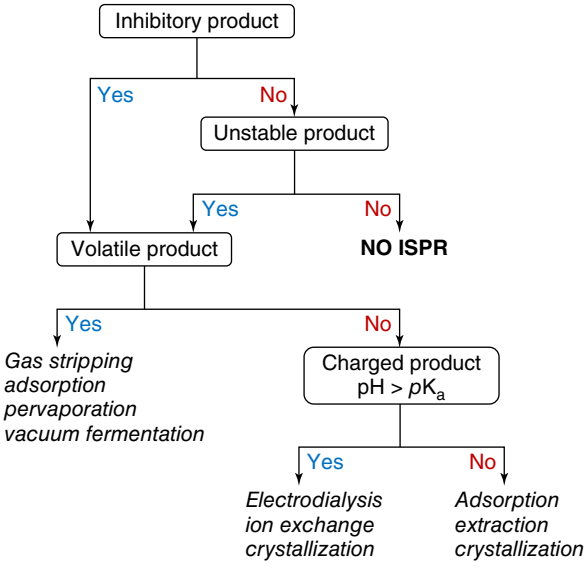


Figure 2.13 Heuristics for selection of appropriate ISPR technique based on (Wong et al. 2010). Source: Wong et al. (2010). Reproduced with permission of Springer.

for side reactions. These considerations apply to bioseparations including chromatography, adsorption, and tangential flow filtration.

2.6 Modeling and Simulation of Reactor Behavior

2.6.1 Basic Approaches and Software

Bioreactor modeling is necessary to gain a holistic understanding of the reactions occurring and to be able to design the process at the desired scale. As the initial experimental phase occurs in the 20–200 ml range, a valid theoretical model must be developed to enable scale-up and incorporation into a bioprocess. Bioreactor modeling is generally more complex than simulation of chemical reactions because of the capricious nature of microorganisms.

Initial software packages that were developed to simulate bioreactors include Intelligen SuperPro and Aspen Batch Plus (Shanklin et al. 2001). Traditional chemical unit operations software packages were not suitable for bioreactor simulations as the processes are governed by physical and biological principles and not solely by phase equilibria. Additionally, bioreactors generally contain a variety of materials that are not readily characterized (proteins, enzymes, etc.) or have fully understood behavior. Aspen Batch, now part of the Aspen One package, is a fully integrated process modeling software program that has evolved over the years to handle biological components along with organic chemicals. Aspen is especially useful when designing an entire bioprocess, not solely a single reactor (Dutton 2006).

The limitation of Aspen is the lack of biological components in the available component databases; however, users are allowed to add in their own components and supply the thermodynamic properties necessary to determine material and energy balances. Intelligen SuperPro was designed specifically to handle biological processes including food, fuel, pharma, and wastewater applications. SuperPro and Aspen both track material balances well, but Aspen is generally considered to be better equipped to simulate thermodynamic interactions.

2.6.2 Numerical Simulation of Bioreactor Function

The calculation and visualization of time courses of biomass accumulation, substrate depletion, and product formation during microbial fermentation depend on simultaneously solving a series of first-order differential equations (ODE). Time-dependent concentration profiles can be generated once the equations have been developed and the values of constants and yields are well defined. The Runge–Kutta method is the easiest to set-up and may be solved using a Visual Basic™ program that is embedded in an Excel™ spreadsheet. Alternatively, software such as MatLab™, MathCAD™, Maple™, or other such software – capable of numerically solving systems of ordinary differential equations – may also be used.

The basic approach for solving an ODE with the Runga–Kutta method using a numerical solver requires that the equation must be reduced to first order;

systems of ODEs can be represented in a vector form. Simpson's rule is a special case of the Runge–Kutta method and is applied when numerical integration of a single, separable ODE is in the form $dx/dt = f(t)$ with initial condition $x = x_0$ at $t = 0$. S. Simpson's rule is a standard integration technique used for bioreactor solution (Alhumaizi and Ajbar 2006). A more complex equation with additional reliance on time, $dx/dt = f(x,t)$, with the initial condition $x = x_0$ at $t = t_0$, can be solved numerically using a fourth-order Runge–Kutta method. Examples of fourth-order Runge–Kutta method as applied to the bioreactor simulation include modeling oscillations of yeast behavior, biomass distribution, and bioethanol production in an unconventional bioreactor (Sharifian and Fanaei 2009; Mendoza-Turizo et al. 2017). Matlab is generally employed to employ the fourth-order Runge-Kutta method.

Growth kinetics – or biomass accumulation – are generally described using equations based on balanced growth because of the assumption that balanced growth is independent of cell age. The kinetic model is unstructured because it does not relate specific changes of metabolic pathways to changes in cell division or production of given metabolites. Monod kinetics are used to describe simple growth, which can be solved using the Runge–Kutta method.

2.6.3 Contamination of Bioreactors

Fermentation processes are susceptible to contamination (presence of unwanted organisms). Product loss due to contamination will result in a costly economic penalty, so contamination should be avoided at all costs. Routinely, 3–5% of industrial fermentations are wasted because of failed sterilization (Doran 2013). All parts of the bioreactor must be sterilized and remain sterile throughout the fermentation and aseptic technique must be strictly adhered to. Sterilization of fluid streams can occur by either physical means, such as filtration or physical separation, or by inactivating particles using heat, radiation, or chemicals. Industrial bioreactors should be designed to decrease points of stagnation and eliminate crevices or places where microbes may accumulate.

In an effort to model cell death, the extinction equation, Eq. (2.32), demonstrates that the probability of extinction of the total cell population, $P_0(t)$, is an exponential function.

$$P_0(t) = [1 - p(t)]^{N_0} \tag{2.32}$$

where $p(t)$ is the probability that an individual cell will still be viable at time t, and N_0 is the number of individual cells initially present. The expected number of individual cells to survive to time t, $E[N(t)]$, is shown in Eq. (2.33).

$$E[N(t)] = N_0 \cdot p(t) \tag{2.33}$$

The expected survival rate is then used to determine the death rate, k_d (Eq. (2.34)), which is necessary to calculate the death kinetics required for sterilization modeling.

$$k_d = \frac{1}{-E[N(t)]} \cdot \frac{d}{dt} E[N(t)] \tag{2.34}$$

k_d can be determined using chemical, thermal, or radioactive techniques (Shuler et al. 2017). Steam sterilization is the most common technique employed, although some temperature-sensitive equipment cannot withstand this type of sterilization. Ultraviolet radiation is commonly used for membrane bioreactors but it is not able to fully penetrate fluids – especially those with a large quantity of suspended solids (Shuler et al. 2017). Chemical agents for sterilization often include ethylene oxide gas and sodium hypochlorite or formaldehyde – all chemical agents used must leave no adverse residue.

2.7 Scale-up

Scale-up of processes using bioreactors requires recognition of the effect of engineering parameters on design of larger vessels or systems (Figure 2.14). Important parameters that impact scale-up but are often not recognized at the laboratory scale are aeration, sterility, viscosity, and shear (i.e. mixing), and fluid transfer between unit operations. Scale-up is sometimes expressed in terms of technology readiness levels (TRLs) that range from 1 to 7. TRL 1 represents fundamental laboratory research such as involved in microorganism discovery, metabolic pathway engineering, gene editing, and propagation of modified cells at a culture tube level. This is followed by scale-up to shake flasks and proof of concept, both for recovery and purification of the products (ranging from cells to small molecules, depending on the application). This occurs at TRL 2 to 3. At TRL 3 to 4, the biology is scaled from shake or spinner flask to an agitated bioreactor (TRL 3 to 4, representing a first prototype) and is then scaled further where the indicated parameters must now be accounted for. This occurs in a sequence, where

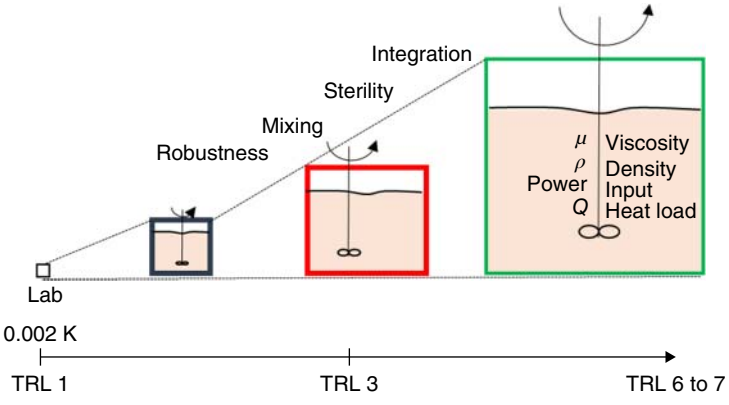


Figure 2.14 Key considerations in scale-up of bioreactors. Laboratory-scale reactors (or shake flasks) are used for basic research at a technology readiness level 1 (TRL1). Scale-up in larger volume bioreactors moves through increasing technology readiness levels. Runs carried out in an operational environment where effects of parameters such as viscosity, density, heat loads, oxygen transfer, agitation effects, and media optimization are studied and would correspond to a TRL 7. Source: Ladisch et al. (2010). Reproduced with permission of American Institute of Chemical Engineers Publication.

engineering data and process design occur as the technology (bioproduct) moves from TRL 4 or 5 to 6 to 7. Ultimately, a process ready for validation will be constructed and testing carried out (TRL 6 to 7) resulting in a commercial process (TRL 7). These steps represent a huge effort, with costs that may exceed the initial cost of research by a factor of 10 or more.

References

Aiba, S.H., Humphrey, A.E., and Millis, N.F. (1973). *Biochemical Engineering*, 2e. Academic Press.

Alberts, B., Bray, D., Lewis, J. et al. (1989). *Molecular Biology of the Cell*, 1219. New York, London: Garland Publishing.

Alhumaizi, K. and Ajbar, A.H. (2006). Optimization of an unstructured first-order kinetics model of cyclically operated bioreactors. *J. Environ. Eng.* 132 (5): 453–462.

Bailey, J.E. (1998). Mathematical modeling and analysis in biochemical engineering: past accomplishments and future opportunities. *Biotechnol. Progr.* 14 (1): 8–20.

Bailey, J.E. and Ollis, D.F. (1977). *Biochemical Engineering Fundamentals*, 70. New York: McGraw-Hill.

Bailey, J. and Ollis, D. (1986). *Biochemical Engineering Fundamentals*, 2e. McGraw-Hill.

Battley, E.H. (1991). Calculation of the heat of growth of *Escherichia coli* K-12 on succinic acid. *Biotechnol. Bioeng.* 37 (4): 334–343.

Bellgardt, K.-H. (2000). Bioprocess models. In: *Bioreaction Engineering: Modeling and Control* (eds. K. Schügerl and K.-H. Bellgardt), 44–105. Berlin, Heidelberg: Springer-Verlag.

Birmingham, W.R., Starbird, C.A., Panosian, T.D. et al. (2014). Bioretrosynthetic construction of a didanosine biosynthetic pathway. *Nat. Chem. Biol.* 10: 392–399. https://doi.org/10.1038/nchembio.1494.

Bjurstrom, E. (1985). Biotechnology; fermentation and downstream processing. *Chem. Eng.* 92: 126–158.

Cooney, C.L. (1979). Continuous culture of microorganisms: an overview and perspective. *Ann. N.Y. Acad. Sci.* 326 (1): 295–314.

Cooney, C.L., Wang, D.I., and Mateles, R.I. (1969). Measurement of heat evolution and correlation with oxygen consumption during microbial growth. *Biotechnol. Bioeng.* 11 (3): 269–281.

Davis, L., Rogers, P., Pearch, J., and Peiris, P. (2006). Evaluation of *Zymomonas*-based ethanol production from a hydrolyzed waste starch stream. *Biomass Bioenergy* 30: 809–814.

Doran, P.M. (2013). *Bioprocess Engineering Principles*. Academic Press.

Dutton, G. (2006). Bioprocess simulations gain traction-solutions for dealing with variability and bottlenecks are among the benefits. *Genet. Eng. News* 26 (16): 34.

Essener, A.A., Roels, J.A., and Kossen, N.W.F. (1983). Theory and applications of unstructured growth models: kinetic and energetic aspects. *Biotechnol. Bioeng.* 25: 2803–2841.

Frame, K.K. and Hu, W.S. (1988). A model for density-dependent growth of anchorage-dependent mammalian cells. *Biotechnol. Bioeng.* 32: 1061–1066.

Freeman, A., Woodley, J.M., and Lilly, M.D. (1993). In-situ product removal as a tool for bioprocessing. *Nat. Biotechnol.* 11: 1007–1012.

Gaden, E.L. Jr, (1959). Fermentation process kinetics. *J. Biochem. Microbiol. Technol. Eng.* 1 (4): 413–429.

Gernaey, K.V., Lantz, A.E., Tufvesson, P. et al. (2010). Application of mechanistic models to fermentation and biocatalysis for next-generation processes. *Trends Biotechnol.* 28 (7): 346–354.

Govindaswamy, S. and Vane, L.M. (2007). Kinetics of growth and ethanol production on different carbon substrates using genetically engineered xylose fermenting yeast. *Bioresour. Technol.* 98: 677–685.

Harrison, R.G., Todd, P., Rudge, S.R., and Petrides, D.P. (2003). *Bioseparations Science and Engineering*, 406. New York: Oxford University Press.

Ladisch, M. (2001). *Bioseparations Engineering: Principles, Practice, and Economics*, 735. Wiley.

Ladisch, M., Mosier, N., Kim, Y. et al. (2010). Converting cellulose to biofuels. *Chem. Eng. Prog.* 106 (3): 56–63.

Le, T.S., McCann, M., Azarin, S.M., and Hu, W.-S. (2016). An introduction to mammalian cell culture. *CEP* 112 (4): 34–40.

Li, B., Qiu, B., Lee, D.S. et al. (2014). Fructose-1,6-bisphosphatase opposes renal carcinoma progression. *Nature* 513: 251–255.

McCoy, M. (2017). Organic acids grow on the farm. *CEN* 95 (23): 11.

Madigan, M.T., Martinko, J.M., Parker, J., and Brock, T.D. (1996). Brock biology of microorganisms. In: *Nutrition and Metabolism*, 8e, 124–125. Upper Saddle River, NJ: Prentice Hall, Chapter 4.

Marison, I.W. and Von Stockar, U. (1988). Fermentation control and the use of calorimetry. *Biochem. Soc. Trans.* 16 (6): 1091–1093.

Mendoza-Turizo, M.G., Justo, O.R., Perez, V.H. et al. (2017). Modeling of bioethanol production in unconventional bioreactor assisted by electromagnetic field. *Int. J. Energy Res.* 41 (1): 103–112.

Monbouquette, H.G. (1992). Modeling high-biomass-density cell recycle fermentors. *Biotechnol. Bioeng.* 39: 498–503.

Monod, J. (1950). La technique de culture continue: théorie et applications. *Ann. Inst. Pasteur.* 79: 390–410.

Mosier, N.S. and Ladisch, M.R. (2009). *Modern Biotechnology: Connecting Innovations in Microbiology and Biochemistry to Engineering Fundamentals*, 433. New York: Wiley.

National Research Council (1992). *Putting Biotechnology to Work: Bioprocess Engineering*. National Academy of Sciences.

National Research Council (2015). *Industrialization of Biology: A Roadmap to Accelerate the Advanced Manufacturing of Chemicals*. Washington, DC: National Academies Press.

Novick, A. and Szilard, L. (1950a). Description of thermostat. *Science* 112 (2920): 715–716.

Novick, A. and Szilard, L. (1950b). Experiments with the chemostat on spontaneous mutations of bacteria. *Proc. Natl. Acad. Sci.U.S.A.* 36 (12): 708–719.

Outram, V., Lalander, C.-A., Lee, J.G.M. et al. (2017). *Biotechnol. Prog.* https://doi.org/10.1002/btpr.2446.

Panikov, N.S. (2011). The biological basis microbial growth dynamics. In: *Comprehensive Biotechnology*, 2e, vol. 1 (ed. M. Moo-Young), 257–283. Amsterdam, Netherlands: Elsevier.

Roels, J.A. (1980). Application of macroscopic principles to microbial metabolism. *Biotechnol. Bioeng.* 22: 2457–2514.

Roffler, S.R., Blanch, H.W., and Wilke, C.R. (1984). In situ recovery of fermentation products. *Trends Biotechnol.* 2: 129–136.

Scott, A. (2017). Switching paper for chemicals. *CEN* 95 (23): 28–29.

Shanklin, T., Roper, K., Yegneswaran, P.K., and Marten, M.R. (2001). Selection of bioprocess simulation software for industrial applications. *Biotechnol. Bioeng.* 72 (4): 483–489.

Sharifian, M. and Fanaei, M.A. (2009). Dynamic simulation and control of a continuous bioreactor based on cell population balance model. *Iran. J. Chem. Chem. Eng. (IJCCE)* 28 (2): 15–27.

Sheppard, M.J., Kunjapur, A.M., Wenck, S.J., and Prather, K.L.J. (2014). Retro-biosynthetic screening of a modular pathway design achieves selective route for microbial synthesis of 4-methyl-pentanol. *Nat. Commun.* 5: 5031. https://doi.org/10.1038/ncomms6031.

Shuler, M., Kargi, F., and DeLisa, M. (2017). *Bioprocess Engineering: Basic Concepts*, 3e. Prentice Hall.

Sin, G., Ödman, P., Petersen, N. et al. (2008). Matrix notation for efficient development of first principles models within PAT applications: integrated modeling of antibiotic production with *Streptomyces coelicolor*. *Biotechnol. Bioeng.* 101: 153–171.

Takors, R. (2004). Complete cell ISPR process development: chances and risks. *Chem. Ing. Tech.* 76 (12): 1857–1864.

Tao, H., Gonzalez, R., Martinez, A. et al. (2010). Engineering a homo-ethanol pathway in *Escherichia coli*: increased glycolytic flux and levels of expression of glycolytic genes during xylose fermentation. *J. Bacteriol.* 183 (10): 2979–2988.

Tullo, A. (2017). Covestro pursues biobased aniline. *CEN* 95 (23): 10.

Van Hecke, W., Kaur, G., and De Wever, H. (2014). Advances in in-situ product recovery (ISPR) in whole cell biotechnology during the last decade. *Biotechnol. Adv.* 32: 1245–1255.

Wang, D.I.C., Cooney, C.L., Demain, A.L. et al. (1979). *Fermentation and Enzyme Technology*. New York: Wiley.

Weiss, R.M. and Ollis, R.F. (1980). Extracellular microbial polysaccharides. I. Substrate, biomass, and product kinetic equations for batch xanthan gum fermentation. *Biotechnol. Bioeng.* 22: 859–873.

Wong, M., Wright, M., Woodley, J.M., and Lye, G.J. (2009). Enhanced recombinant protein synthesis in batch and fed-batch *Escherichia coli* fermentation based on removal of inhibitory acetate by electrodialysis. *J. Chem. Technol. Biotechnol.* 84 (9): 1284–1291.

Wong, M., Woodley, J.M., and Lye, G. (2010). Application of bipolar electrodialysis to *Escherichia coli* fermentation for simultaneous acetate removal and pH control. *Biotechnol. Lett.* 32 (8): 1053–1057.

Yee, L. and Blanch, H.W. (1992). Recombinant protein expression in high cell density fed-batch cultures of *Escherichia coli*. *Nat. Biotechnol.* 10: 1550–1556.

Further Reading

dos Santos, A.C., Ximenes, E., Kim, Y., & Ladisch, M.R. (2018). Lignin–enzyme interactions in the hydrolysis of lignocellulosic biomass. *Trends Biotechnol*. 37 (5): 518–531.

Fredrickson, A.G., Megee, R.D. III, and Tsuchiya, H.M. (1970). Mathematical models in fermentation processes. *Adv. Appl. Microbiol.* 13: 419–465.

Jørgensen, B.B., Isaksen, M.F., and Jannah, H.W. (1992). Bacterial sulfate reduction above 100°C in deep-sea hydrothermal vent sediments. *Science* 258 (5089): 1756–1757.

Snoep, J.L., Mrwebi, M., Schuurmans, J.M. et al. (2009). Control of specific growth rate in Saccharomyces cerevisiae. *Microbiology* 155 (5): 1699–1707.

3

Food

Gülhan Ünlü[1,2,3] *and Barbara Nielsen*[1]

[1] *University of Idaho, School of Food Science, 875 Perimeter Drive, MS 2312, Moscow, ID 83844-2312, USA*
[2] *Washington State University, School of Food Science, PO Box 646376, Pullman, WA 99164-6376, USA*
[3] *University of Idaho, Department of Biological Engineering, 875 Perimeter Drive, MS 0904, Moscow, ID 83844-0904, USA*

CHAPTER MENU

3.1 Fermented Foods

In 1857, Louis Pasteur published *Mémoire sur la fermentation appellee Lactique (Extrait par l'auteur) – Report on the lactic acid fermentation (Author's abstract).* This event is often credited as being the birth of the science of microbiology. However, it is by no means the beginning of the use of microbiological methods in food production. Centuries before Pasteur and others discovered the microorganisms responsible, humans had learned that certain foods, if encouraged to ferment, would not spoil as quickly and thus could be prepared in times of plenty for use when food was scarce. Often fermented food (Figure 3.1) would also develop pleasing aromas, flavors, and textures, as well as enhanced nutritional traits. With industrialization, familial and local fermentation techniques were scaled up to feed an ever-increasing population. Scientific advances helped to modernize the fermentation industry and increase efficiencies.

3.1.1 Food Preservation

Throughout history, man has harnessed the power of the microbe for preservation of food. Some bacteria, most notably the lactic acid bacteria (LAB) and

Industrial Microbiology, First Edition.
Edited by David B. Wilson, Hermann Sahm, Klaus-Peter Stahmann, and Mattheos Koffas.

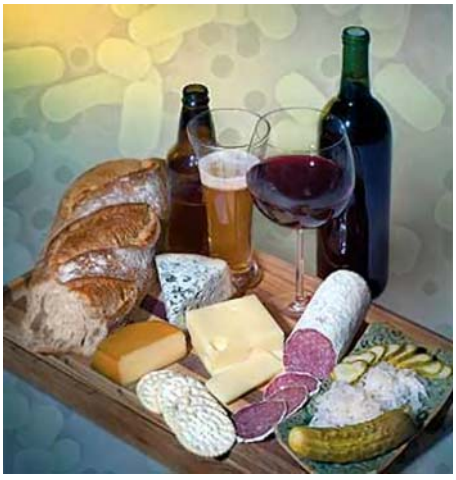


Figure 3.1 Fermented foods and beverages. The bread, cheese, fermented sausage, beer, wine, and fermented vegetables in this image are all fermentation products. The backdrop is the electron micrograph of select microorganisms involved in the manufacture of fermented foods and beverages and represents their diversity.

yeasts, found naturally on food products, convert sugars to organic acids and alcohol by way of various catabolic pathways. These metabolic products, in turn, are able to discourage or prevent the growth of microorganisms, often including the ones that produced them. This allows food that would normally be subject to spoilage and/or growth of pathogenic bacteria to remain safe for consumption for an extended period of time. An example of this can be shown in the fermentation of cabbage to sauerkraut, described later in this chapter. Raw cabbage is covered with a wide range of bacteria carried in from the field. Salt provides a competitive advantage to the LAB, which quickly metabolize sugars and produce lactic acid, dropping the pH. The low pH levels (<4.4) in combination with the lactic acid entering the cells in the protonated (un-dissociated) form prevents the growth of harmful bacteria such as *Salmonella* spp.

3.1.2 Flavor and Texture

Eating is definitely a multisensory activity. The human perception of flavor is influenced not only by taste but also by visual cues, textures, and aromas. During the fermentation process, many changes take place in the food because of both direct microbial action and indirect microbial effects of metabolic products on the food components. Toxic, bitter, or nondigestible components of the food may be reduced. Compounds, such as lactic and other organic acids, acetaldehyde, diacetyl, acetoin, ethanol, and other flavor and aroma compounds are produced, as well as CO_2 for carbonation and texture. These metabolic products provide the consumer with dietary interest and variety in flavor, aroma, and texture. Some fermented foods may exhibit only a few of these aroma and flavor compounds, leading to a clean, simple sensory experience. Others may have hundreds of these compounds, with much more complex aromas and flavors (Table 3.1).

3.1.3 Health Benefits

While fermenting microbes catabolize food components such as sugars, the resulting fermented foods often retain some of the health benefits of the

Table 3.1 Selected aromatic compounds produced by microorganisms during food fermentations.

Aroma Compounds	Sample aroma	Beer	Italian Salami	Sourdough Bread	Swiss Cheese	Sauerkraut	Wine	Yogurt
Alcohols	Alcohol, ethereal, chemical	x	x	x		x	x	
Aldehydes	Fruity, floral, green, sweet		x	x	x	x	x	
Diacetyl	Buttery				x	x	x	x
Esters	Fruity, floral, sweet, green	x	x	x	x	x	x	x
Furans	Sweet, almond, bread			x			x	
Ketones	Fruity, sweet, solvent, nut		x	x			x	x
Lactones	Sweet, tropical		x	x			x	
Organic acids	Sour, pungent, sharp		x	x	x	x	x	x
Organosulfides	Garlic, sulferous, vegetable		x		x		x	
Phenols	Chemical, smoky, spicy	x	x				x	
Pyrazines	Coffee, nutty, earthy				x		x	
Terpenes	Woody, floral, citrus, spicy		x				x	

The x indicates that these aromatic compounds are produced during the fermentations of the indicated foods.

starting material. For example, sauerkraut retains most of the high vitamin C content of cabbage (34.7 mg/100 g, 35–50% World Health Organization (WHO) recommended dosage). However, even more, fermentation enriches the foods, making them more easily digested and nutritious by increasing the availability of proteins, amino acids, fatty acids, and vitamins. Harmful or toxic compounds in raw foods may be reduced or eliminated through fermentation. Patulin, a toxic chemical produced in damaged apples by molds such as *Penicillium* or *Aspergillus*, can be eliminated in apple juice by fermentation with *Saccharomyces cerevisiae*. Fermentation reduces some antinutritional factors such as phytic acid in beans and seeds, allowing for better mineral absorption. In addition, fermented foods may be a good source of probiotics, live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Food and Agriculture Organization of the United Nations (FAO) 2001). Fermented foods can thus often be classified as "functional foods," delivering specific non-nutritive physiological benefits that may provide health benefits to the consumer.

3.1.4 Economic Impact

Fermented foods account for a large share of the consumer market. They can be considered "value-added" products of the food industry. Most fresh produce and dairy have a limited shelf life of days to weeks. Because of the preservative action of the acid environment produced and other antimicrobial effects, fermented foods extend the shelf life to months or even years in many cases. For example, milk has a shelf life of a couple of weeks at refrigeration temperatures. Yogurts stay fresh for over a month. Cheeses properly stored can stay wholesome for months or years. This reduces food waste and thus affects the bottom line. Fermented foods allow for more efficient uses of food products to feed an expanding world population.

Alcoholic beverages account for the largest economic impact. According to the Beer Institute, the brewing industry in the United States has a combined economic impact of 252.6 billion US$, producing 591 million hectoliters (Mhl) while directly or indirectly employing 1.75 million workers, with sales of 105.9 billion US$ (2014 statistics). A study conducted by Regioplan Policy Research and Ernst and Young (EY) showed 2012 sales of beer in the EU to be €111 billion, producing 390 Mhl in 4460 breweries while directly or indirectly employing 2 million workers. About 29 Mhl wine was produced in the United States in 2015, 80% of which was produced in California. The wine market in the United States (domestic and imported) is 55.8 billion US$, while wine exports total 1.61 billion US$. EU countries collectively produced 141 Mhl wine in 2012, about 60% of worldwide production, exporting 22.3 Mhl, valued at 11.3 billion US$.

Cheese production has the largest economic impact among dairy fermentations. In 2011, US production was 10.6 billion pounds (4.8 million metric tons [MT]) and 224 306 MT of cheese was exported at a value of 958 million US$. Around 142 146 MT of cheese was imported at a value of 1.07 billion US$. Per capita consumption of cheese in the United States was 32.8 pounds (14.9 kg). The global cheese market in 2012 was 75.57 billion US$, expected to rise to 105.13 billion US$ by 2019 (Table 3.2).

3.2 Microorganisms and Metabolism

LAB (Figure 3.2) is the most important bacterial group for fermentations, as these bacteria have the ability to metabolize sugars and produce lactic acid, as well as other acids and metabolic products. These include mainly the genera *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, and *Lactobacillus*. Other bacterial genera important for specific fermentations are the acetic acid bacteria, including the genera *Acetobacter*, *Gluconobacter*, and *Gluconacetobacter*, for acetic acid production, *Bacillus* for natto production, *Bifidobacterium* for probiotic functions, *Brevibacterium* for surface-ripened cheese, and *Propionibacterium* for "eye" formation in Swiss-type cheese. *Kocuria*, *Micrococcus*, and *Staphylococcus* species are also used in meat fermentations for aroma, flavor, and texture development.

Table 3.2 A brief world market of fermented foods.

Product	Substrate	Primary microorganisms	Region	Economic impact in US$	Market	(Year)
Beer	Barley, grains	*Saccharomyces* ssp.	Worldwide	>100 billion	USA	(2014)
Bread	Wheat, grains	*Saccharomyces cerevisiae,* lactic acid bacteria	Worldwide	> 10 billion > 1 billion	USA	(2017)
Cheese	Milk	Lactic acid bacteria	Worldwide	<100 billion	World	(2012)
Kimchi	Cabbage, vegetables, seafood	Lactic acid bacteria	Korea	> 1 billion	Korea	(2015)
Miso	Soybeans, Rice	*Aspergillus oryzae, Rhizopus* spp.	Japan	> 1 billion	Japan	(2016)
Sauerkraut	Cabbage	Lactic acid bacteria	Germany	< 0.1 billion	Germany	(2016)
Soy sauce	Soy beans, wheat	*Aspergillus* spp., lactic acid bacteria, *Zygosaccharomyces rouxii*	Asia	< 1 billion	World	(2009)
Wine	Grapes	*Saccharomyces cerevisiae*	Worldwide	<100 billion	USA	(2015)
Yogurt	Milk	Lactic acid bacteria	Worldwide	< 10 billion	USA	(2017)

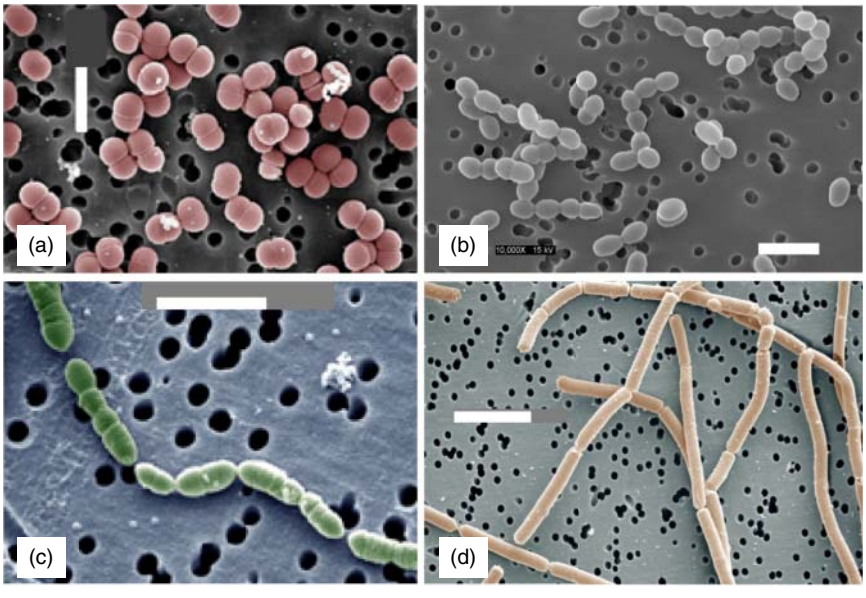


Figure 3.2 Scanning electron micrographs of lactic acid bacteria associated with food fermentations. (a) *Pediococcus pentosaceus*, appear as cocci in pairs or in tetrads but never in chains; (b) Leuconostoc mesenteroides, appear as coccoid or even short rod-like; (c) *Oenococcus oeni*, appear as ellipsoidal to spherical cocci, often in pairs or chains; (d) *Lactobacillus delbrueckii* subsp. *bulgaricus*, appear as long rods in chains. White scale bars are 2 μm. With kind permission from F. Breidt, R.W. Hutkins (University of Nebraska) and J.R. Broadbent (Utah State University).

The most important yeast for fermentation belongs to the genus *Saccharomyces* for its role in the production of beer, wine, and bread. Important mold genera include *Penicillium* for its role in cheese making and *Aspergillus* for soy sauces and pastes.

3.2.1 Fermentation Processes

Classically, fermentation is the anaerobic metabolic process whereby sugars are converted to acids, alcohols, and/or gasses, usually involving the metabolic action of yeasts and/or bacteria. A broader definition could be the aerobic or anaerobic process where a substance is broken down into a more simple substance. This is accomplished using various glycolytic pathways, including the Embden–Meyerhof–Parnas (yields highest amount ATP), the Entner–Doudoroff (important in dairy fermentations for its ability to fully utilize lactose,) and the heterofermentative (can also utilize 5-carbon sugars) pathways. (Figure 3.3) Industrial fermentation also involves the highly aerobic process by which fungal mycelia are grown in large batches in tanks known as fermenters.

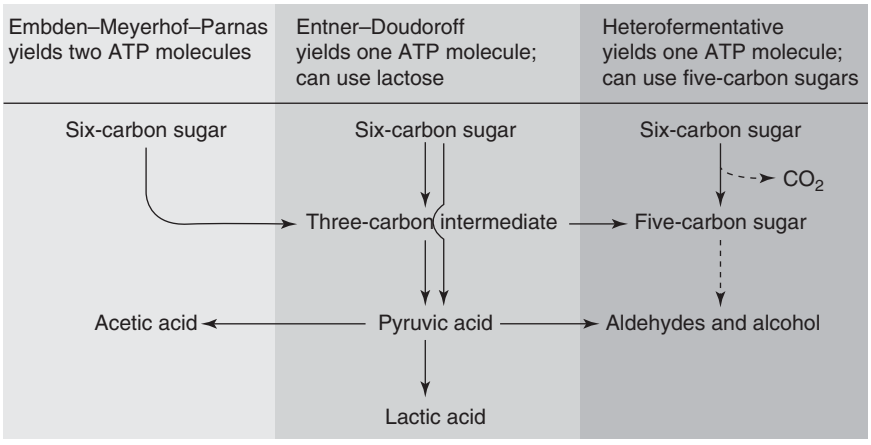


Figure 3.3 Simplified catabolic pathways used in fermented foods. With kind permission from Matthews et al. (2017), p. 313; ASM Press.

3.2.2 Starter Cultures

Success in producing fermented foods depends completely on the presence, growth, and metabolism of the specific bacteria and/or fungi responsible for the desired fermentation. Although often an array of microorganisms indigenous to the material to be fermented or its environment will accomplish the fermentation, sometimes resulting in superior sensory properties, starter cultures are the best way to make sure that the necessary microorganisms are present in sufficient numbers to quickly and efficiently complete the desired fermentation, while inhibiting any contaminating organism from taking over. This results in a more consistent product, meeting the needs of large-scale industrial production.

Starter cultures need to have carefully selected microorganisms with properties specific for the intended fermentation. For instance, starter cultures for dairy fermentations, needing consistent and predictable controlled acidification, flavor production, texture modification, and phage resistance, would be different than that for meat fermentations, which need rapid acidification to quickly inhibit any unwanted contaminants to produce the proper results.

3.3 Yeast Fermentations – Industrial Application of *Saccharomyces* Species

Yeast means fungi with a single-cell growth phenotype. Although they can come from different phylogenetic branches, they are often discussed as a group

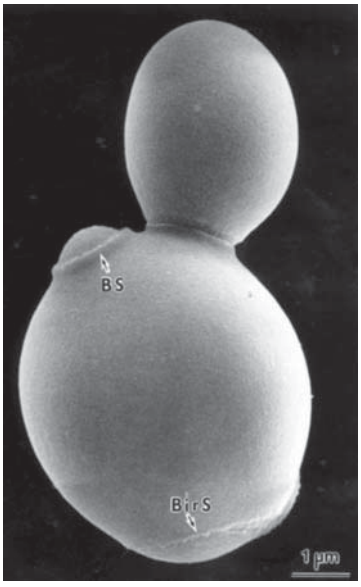


Figure 3.4 Scanning electron microscope image of *Saccharomyces cerevisiae* cell division, birth scar (BirS), and bud scar (BS), on formation. The budding of each daughter cell leaves a ring-shaped residue, referred to as a bud scar, on the cell wall of the mother cell. Source: With kind permission from Elsevier (from: Dr. Masako Osumi).

of fermentative microorganisms. Industrial products involving brewer's yeast or baker's yeast, mostly strains of *S. cerevisiae*, include alcohol and bread, respectively (Figure 3.4).

3.3.1 Grain Fermentation for Ethanol Production – Beer

Beer making is a grain fermentation that emphasizes ethanol production, secondarily producing various flavor compounds and CO_2 (often sufficient to result in carbonation). The resulting carbonated grain-based beverage generally has an alcohol content between 4% and 6%. Figure 3.5 outlines the many steps in beer production.

The main ingredients of beer are water, hops, and malt. Malt is prepared by germinating the desired grain, usually barley or wheat, to activate the plant enzymes that cleave starches and disaccharides into simple sugars to be used for alcohol production in the fermentation process. Other enzymes degrade proteins into peptides and free amino acids to be precipitated out and removed. The grains are then dried to stop the germination process, leaving the enzymes intact. The catabolism of *Saccharomyces* sp. is limited to mono- and disaccharides (Table 3.3).

The dried malt is milled and mixed with water in the **mash tun** to form the mash. This is heated to various differential temperature levels in the **mash kettle** to allow the enzymes in the mash to solubilize the carbohydrates, proteins, and other components of the malt. This is then transferred to the **lauter tun** where the wort (solubilized liquid component) is separated from the mash debris (insoluble plant material component), alternatively in many breweries, the soluble sweet wort is separated from the insoluble portions of the mash by filtration through a **mash filter**. Hops, the cone-shaped flowers of the hop plant (*Humulus lupulus*),

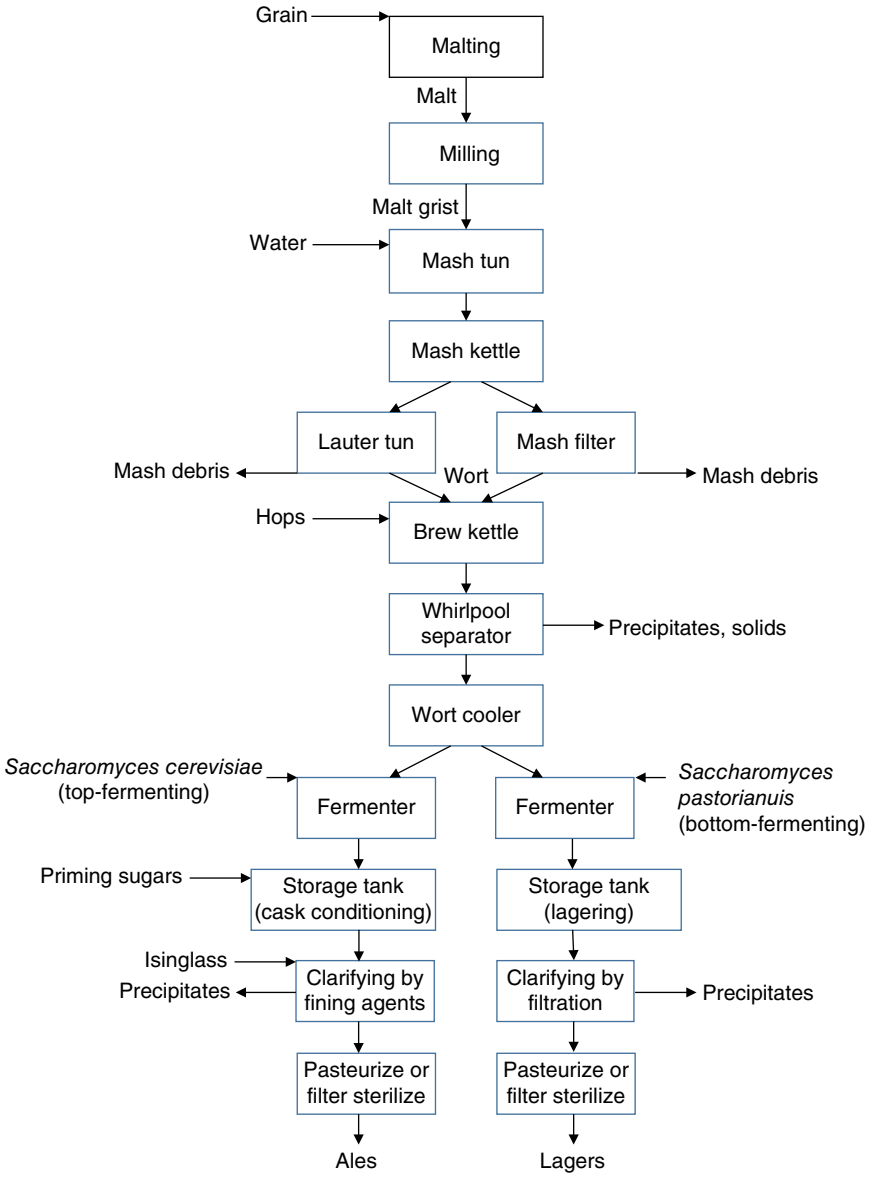


Figure 3.5 Schematic for making beer.

are used to add flavors to the beer and also provide antimicrobial properties; these are added to the wort in the **brew kettle**, and the mixture is boiled for about one to two hours to concentrate the wort, inactivate the enzymes, extract soluble bitter compounds from the hops, precipitate proteins, remove volatile compounds such as fatty acids, kill all undesirable microorganisms, and form hydroxymethylfurfural and other colorants.

Table 3.3 Number of physiologically relevant enzymes and uptake systems found for starch and protein catabolism of *Saccharomyces cerevisiae* and *Aspergillus oryzae*.

Macromolecular substrate	Dimer	Monomer	Protein function	*Saccharomyces cerevisiae* number of genes	*Aspergillus oryzae* number of genes
Starch			Starch hydrolases (secreted)	0	27
	Maltose		Maltose hydrolases (secreted)	2	3
			Maltose transporters	3	2
	Sucrose		Sucrose hydrolase (secreted)	4	2
			(intracellular)	3	2
		Hexose	Hexose transporters	7	4
Protein			Protein hydrolases (secreted)	0	16
		Amino acids	Amino acid uptake systems	16	21

The hot wort is strained to remove the hops and then cooled to about 50 °C to facilitate precipitation of proteins and tannins. The wort is again separated from the formed precipitates (trub) using a **whirlpool separator**. The clear wort is then cooled and passed into the **fermentation tanks** where it is inoculated with the appropriate brewer's yeast (*Saccharomyces* sp.) The species of brewer's yeast employed is dependent on the fermentation process desired. Fermentation takes place over the next 4–10 days, as the glucose and maltose in the wort are converted to ethanol and CO_2.

Saccharomyces cerevisiae is used for **top fermenting** ales, e.g. Ale, Stout, Altbier, Porter, and Wheat Beer. In fermentation, the yeast cells flocculate, trapping CO_2 bubbles, which lift the yeast mass to the surface, where it can be scooped off for use in subsequent batches. This fermentation requires a temperature of 15–20 °C and results in higher concentrations of alcohol and a fruity aroma because of the formation of fruity esters.

The young (green) ale is placed in **sealed storage tanks** and matured by "cask conditioning," adding priming sugar and allowing the remaining yeasts a secondary fermentation to carbonate the beer in sealed casks. Hops may be added for flavor. The beer is stored up to seven days. Isinglass finings, derived from fish swim bladders, are then added to precipitate yeast, tannins, and protein complexes clarifying the product. The beer is then ready for pasteurization or sterile filtration and packaging.

*Saccharomyces pastorianu*s (formerly classified as *Saccharomyces carlsbergensis* and others) is the species of yeast used for **bottom fermenting** lagers, e.g. Lagers, Pilsners, Dortmunders, Bocks, Malt Liquor, and low-alcohol beers. The

yeast remains disbursed in the wort during fermentation, sinking to the bottom as nutrients in the wort become depleted. This allows a simple, cost-effective removal of the yeast from the fermentate. This fermentation is achieved at a temperature of 4–9 °C, resulting in fewer fruity esters and a clearer flavor profile.

The young (green) beer is then subject to "**lagering**." It is transferred to storage tanks and held at 8–14 °C. During this time, residual sugars are converted to alcohol. The CO_2 generated purges unwanted volatile compounds such as diacetyl, hydrogen sulfide, mercaptoethanol, and acetaldehydes. The tanks are then sealed and held at 0 °C for a period of one week to six months. The CO_2 is retained, carbonating the beer. More CO_2 may be added, if needed. Desirable flavor enhancing esters increase and tannins, proteins, hops resins, and other chill-haze particles precipitate out. The beer is clarified by filtration, then pasteurized or filter sterilized, and packaged in cans, bottles, or other packaging containers.

3.3.2 Grain Fermentation for CO_2 Production – Bread

In contrast to beer making, bread making is a grain fermentation specializing in the production of CO_2 while secondarily producing ethanol and flavor compounds important for the finished bread. Interestingly, neither the CO_2 nor the ethanol produced during the fermentation step (proofing) remain in the finished product.

Until the mid-nineteenth century, bakeries were often built adjacent to the breweries for convenience as bakeries would commonly use waste yeast from beer or spirit making as leavening for their breads and pastries. The inconsistencies in the resulting products were improved with the introduction of a starter culture of a selected strain of *S. cerevisiae* optimized for baking. This selected strain produced ample CO_2 necessary for the leavening of the bread, along with good bread-flavoring compounds and good viability under storage conditions. These strains of *S. cerevisiae* are known as Baker's yeast, to differentiate them from the strains used in brewing and wine making, known as Brewer's yeast. Individual breweries and bakeries often use proprietary strains of yeast developed and cultivated in-house that contribute to the unique taste, texture, or other characteristics that define their products.

3.3.2.1 Yeast Preparation

Baker's yeast begins as a pure stock culture, scaled up in series until the biomass is large enough to seed a production fermenter. Conditions in the industrial fermentation tanks (up to 250 000 l) are optimized for yeast cell growth. Molasses, augmented with yeast nutrients such as ammonium salts, trace minerals, and iron, makes an excellent inexpensive growth media, provided on a continuous basis for nonstop yeast production. Aerobic conditions (aeration and agitation) are necessary as maximum yeast cell yield, and not metabolic product production, is the desired outcome of this fermentation. A temperature around 30 °C and a pH between 4.0 and 5.0 also encourage maximal growth.

Care must also be taken to maintain glucose levels low enough in the medium that any Crabtree effect is minimized. *S. cerevisiae* can metabolize sugars by

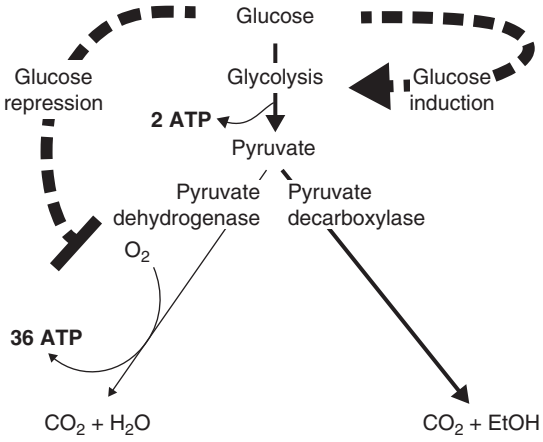


Figure 3.6 Regulation of sugar metabolism in *Saccharomyces cerevisiae*. With kind permission from Johnston and Kim (2005) (from: Biochemical Society Transactions 33 [1]: 247-252, with kind permission of Portland Press).

both respiration, producing biomass, and fermentation, producing ethanol and CO_2. Usually, the pathway used is determined by the presence of oxygen (Pasteur effect); however, in yeast metabolism, substrate availability can also regulate gene expression. If the glucose concentration is too high, over 0.2%, the respiratory pathway is repressed and the fermentative pathway is expressed (Crabtree effect), resulting in reduced yeast yields. This is great for alcohol production but inhibitory for biomass production (Figure 3.6).

When maximal growth is achieved, the yeast broth is centrifuged and the collected yeast "cream" has a cellular density of approximately 10^{10} yeast cells per gram and about 18–20% yeast solids. This "cream" can be used directly in commercial bread production only if it can be used immediately because of its highly perishable nature. Because of this, most commercial bakers use compressed yeast cakes. The yeast "cream" is press-filtered or vacuum-filtered to remove most of the water, formed into moist cakes at 30–32% yeast solids, and then stored under refrigeration condition for up to a few weeks. The yeast cells in the cakes are still metabolically active, ready to quickly begin fermentation upon addition to the dough.

For longer storage needs, the compressed yeast cakes can be further dried to form the active dry yeast (90–96% yeast solids) commonly available to consumers at the retail level. Active dry yeast, properly packaged, is shelf stable, remaining active for up to a year at room temperature. As it requires a rehydration step before it can be used, it is generally used commercially only in bakeries in tropical or subtropical climates or in small-scale baking operations.

A standard bread dough is basically a mixture of wheat flour, water, yeast, and salt, allowed to ferment. Ingredients, such as oil for texture, sugar for enhanced fermentation and color development, and other items that add flavor, aroma, color, or texture to the finished bread may be added if desired. The dough is worked or "kneaded" to drive the water into the wheat particles and distribute the yeast and other ingredients uniformly throughout the dough. This mixing also combines two hydrated flour proteins, gliadin and glutenin, forming gluten, an important component of bread dough.

As the yeast cells begin to grow in the bread dough, they metabolize glucose aerobically, via the tricarboxylic acid (TCA) cycle increasing yeast biomass and generating CO_2 and ATP. This metabolism quickly depletes the oxygen in the dough, causing the yeast to shift to anaerobic metabolism of glucose, then maltose, then sucrose, by way of the Embden–Meyerhof–Parnas pathway, producing ethanol and CO_2 (and much less ATP!). This CO_2 end product is what causes the bread to rise, transforming the stiff, heavy dough into airy, light dough. The CO_2 produced by small colonies of yeast stretches the elastic gluten and becomes trapped in the matrix, forming large gas cells. When the dough is "punched down," this disperses the gasses, forming smaller, more regular cells. The yeast and sugars are redistributed, and the yeast forms more CO_2, improving the texture and uniformity of the bread. Acids and alcohols produced by the yeasts and any LAB present also contribute to flavors, aromas, and textures of the bread.

The bread dough is then divided, shaped, and placed in pans for the final proofing. As most of the CO_2 is dissipated into the air in shaping, the yeast once again metabolizes the redistributed sugars in the loaf, rising to the proper levels for baking.

Most commercial operations utilize continuous conveyer-type ovens, where the proofed bread is subjected to varying temperatures to perfectly cook the bread. When the bread enters the oven, and the dough temperature begins to rise, there is a rapid increase in loaf volume (known as "oven spring") as the CO_2 expands. As the internal temperature rises, all CO_2 dissolved in the dough is released, causing more expansion. At around 55–60 °C, the ethanol produced in the fermentation is volatilized. The yeasts, active until this point, are killed. The starch swells and the gluten dehydrates, causing a rigid structure.

Surface temperatures, of course, rise more rapidly than temperatures in the interior of the loaf, and a crust begins to form. Meanwhile, enzymes remain active inside the loaf until the temperature approaches 80 °C, continuing the denaturation and coagulation of the gluten and the gelatinization of the starch, the increased temperature also facilitating the evaporation of water and volatiles. Finally, as the internal temperature reaches about 95 °C, the interior dough becomes crumb-like and the crust firms up. The crust browns because of both caramelization and Maillard browning reactions.

Sourdough bread is prepared by including a sourdough starter usually consisting of LAB and acid-tolerant yeasts into the bread dough as the leavening agent or in addition to the *S. cerevisiae* commonly used for bread. The LAB ferments the sugars producing lactic acid and other metabolic products, resulting in the distinctive sourdough flavors and aromas. When whole grain flours are used as an ingredient, the sourdough fermentation has a major impact on dough functionality. The other mechanics of sourdough bread production would be similar to that described above.

3.3.3 Fruit Fermentation – Wines and Ciders

Wine is made by a yeast fermentation of fruit juices, usually grape juice. In the process of fermentation, desirable flavors are developed by the production of fermentation by-products such as acetoin, aldehydes, amines, diacetyl, esters,

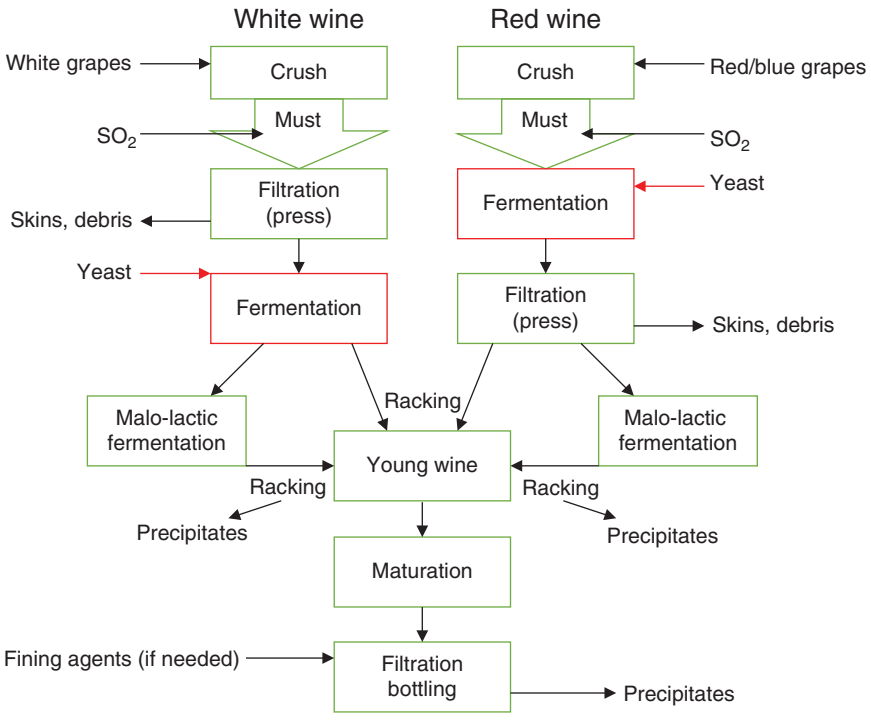


Figure 3.7 Schematic for making wines.

ketones, and organic acids in addition to the production of ethanol and CO_2. Multivalent alcohols (fusel oils) may also form, which, in small amounts, also add to the flavor profile of the wine. Figure 3.7 outlines the steps in the production of both red and white wines.

Historically, the fermentation of wine was achieved utilizing the yeasts and other indigenous microorganisms on grapes (Figure 3.8a) (the "bloom"). This fermented the wine and endowed it with special regional characteristics. Older, established wine-producing regions tend to have a well-developed natural microflora. However, this "natural" fermentation is slow due to the low initial concentration of yeasts and can vary in quality, with flavor and aroma defects due to the action of many microorganisms. To minimize these problems, SO_2 (30–50 mg/l) is added to the grape **must** (destemmed, crushed fruit and juice; Figure 3.8b) to inhibit non-*Saccharomyces* yeasts and spoilage bacteria. Differential temperatures can also be used to encourage fermentation activity of select microorganisms. Balancing all of these factors with multiple microorganisms can lead to desirable flavor and aroma character development resulting in fine, complex wines.

Newer wine-producing regions tend to have a less developed natural microflora. The "bloom" may not be sufficiently developed to carry out an acceptable fermentation. These fermentations normally require the introduction of a "starter culture," selected strains of ***S. cerevisiae*** with defined viability

Figure 3.8 Important aspects of wine making. Different varieties of grapes are used for white (Riesling, chardonnay, and sauvignon blanc) and red wines (merlot, pinot noir, and shiraz; (a)). The crushing, pressing (b), and pretreatment are different for white and red wines. For red wines, the juice and skins are fermented together (c). The skins float, and the juice is pumped over (d) the skins to extract color (because of the red and purple anthocyanins) and phenolics such as tannin.

and fermentation characteristics. These can be added to the grape must in concentrations far higher than would be found naturally in the bloom, quickly dominating the fermentation. Again, non-*Saccharomyces* native microorganisms can be suppressed by adding SO_2 and manipulating the temperature.

The use of starter cultures has several advantages. The fermentation is more uniform and controlled, with even and rapid fermentation rates. The product is less prone to off-flavors because of wild yeasts and bacteria. The wine produced is consistent in flavor and overall quality, but it may not have the complexity of wines produced naturally.

Although wine was traditionally fermented in open wooden vessels, it is now common for fermentation to take place in temperature-controlled stainless steel fermentation vessels. Red wine "must" is placed in the fermentation tanks and fermented at 24–29 °C for a period of three to five days until the level of fermentation desired has been reached. During this time, anthocyanins are extracted from the skins and seeds to color and flavor the wine (Figure 3.8c).

As fermentation progresses, heat and CO_2 generated force the grapes to the surface, forming the "cap." This needs to be recirculated, either manually (punch down) or with a recirculating pump (pump over) once or twice a day for the

duration of the fermentation. This keeps the grapes in good position for optimal fermentation (Figure 3.8d).

When the desired level of fermentation has been reached, the liquid portion of the fermentate (free run) is removed and placed in tanks to settle and clarify. The skins and seeds are placed into a wine press where the remaining liquid is mechanically removed (press run) and again placed in tanks to settle and clarify, a process known as "racking." During this time, most of the yeast and particulates in the wine settle to the bottom of the container (the "lees"). The wine is then pumped out carefully, leaving the lees, and put into vessels for aging.

For white wine, the skins and seeds are removed from the must and the juice is fermented in the tanks at 10–20 °C for one to four weeks, until the level of fermentation desired has been reached. Again, after fermentation, the young wine is transferred to tanks for racking, then to the desired vessel for aging.

The vessels chosen for aging impact the final wine produced. Oak barrels or casks allow for slow access to oxygen and water and ethanol evaporation, so they need to be regularly topped off to prevent the growth of aerobic bacteria or yeasts that may spoil the wine. The oak also imparts important flavoring agents into the wine, adding interesting nuances of toast, vanilla, caramel, almond, and clove. Stainless steel is inert, adding no flavors to the wine, but is easier to keep oxygen out using inert gasses.

A secondary fermentation step, malo-lactic fermentation, may be desirable at this point for some wines. This fermentation commonly utilizes *Oenococcus oenii*, although sometimes other species of *Lactobacillus* or *Pediococcus*, to convert malic acid to lactic acid. This reduces the acidity; imparts a buttery flavor, aroma, and texture; and stabilizes the wine. Wines that have undergone malo-lactic fermentation are less likely to be spoiled in the bottles. Most red wines benefit from malo-lactic fermentation. This secondary fermentation should not be performed on most white wines where acidity and a citrusy fruitiness are desired, as this can make a white wine seem flat. A notable exception is Chardonnay, where a buttery flavor is an expected characteristic. Sweet wines may be thrown out of balance if subjected to malo-lactic fermentation.

Wine typically ages for two to five years, depending on the type of wine being produced. This allows a natural clarification of some of the wines. Other wines may need the use of a fining agent – materials that react with and effectively remove tannins, acids, proteins, or other substances, resulting in a clear, well-flavored wine.

At this point, the wine is now ready for the vintner to blend and either pasteurize, filter-sterilize, or add chemical preservatives to the wine before packaging in bottles or casks for commercial distribution.

Sparkling wines, such as champagne, may be produced either in the traditional method in bottles or in bulk fermentation in tanks. In the traditional method, a quality, finished wine is blended and placed in bottles strong enough to withstand high pressure. Sugar and yeast (usually *Saccharomyces bayanus*, because it can grow at high alcohol and low temperature conditions) are added and the bottles are sealed and incubated horizontally at 15–16 °C for two to three months for the secondary fermentation. As the sugar is depleted, the yeast dies and precipitates out. The young champagne is then held horizontally at 10 °C for 1–5 years.

The champagne is then slowly tilted neck down and rotated for a few weeks to facilitate the collection of all precipitates and yeast debris in the neck of the bottle. The neck of the bottle is then frozen, forming an ice plug that contains the precipitates. When the bottle is turned to approximately a 45° angle and opened, the pressure built up in the bottle due to the fermentation process forces the ice plug out. A sugar and wine mixture (the dosage) is then added to sweeten the champagne and replace any loss in the removal of the precipitate, and the bottles are resealed to retain the CO_2 dissolved in the champagne.

Bulk champagne production, known as the Charmat process to honor its inventor, is completed in 20–950 hl tanks specially designed to withstand pressures of 10–20 atm for safety considerations. Valves control the pressure inside the tanks, while cooling coils control the temperature. The tanks are aerated, allowing for rapid turnover. The fermentation is completed in one to two months. The young champagne is then cold-stabilized to remove tartrates and cold-filtered under pressure to remove yeast cells and precipitates. Dosage is added to sweeten the champagne, and SO_2 is added to kill any stray yeast not removed in the filtration and to prevent oxidation that might darken the champagne. The champagne is then bottled and sealed, retaining its dissolved CO_2.

Cider making is similar to white wine making, as it begins by separating the apple juice from the other plant material (skins, core, and seeds), pasteurizing or filter-sterilizing the juice, and then fermenting it at 20–25 °C using a *S. cerevisiae* starter culture, a process that may take from one to four weeks. The yeast is allowed to settle, and the young cider is raked or centrifuged to remove particulates, then stored, and oxygen is excluded, to mature for several months. During this time, the cider is generally subjected to malo-lactic fermentation to reduce the acidity and the concentration of quinic acid and develop other flavor compounds. The cider is now ready for blending and commercial packaging and will generally have an alcohol content of 3–6%. Cane sugar may be added to the juice before fermenting if a higher alcohol cider is desired.

3.4 Vinegar – Incomplete Ethanol Oxidation by Acetic Acid Bacteria Such as *Gluconobacter oxydans*

Vinegar is produced when alcohol is converted to acetic acid by acetic acid bacteria (*Acetobacter* spp.) in the presence of oxygen.

3.4.1 Substrates: Wine, Cider, and Malt

The types of vinegar produced are dependent on the type of alcoholic fermentation used for the precursor. For example, cider vinegar is made from hard cider, and wine vinegar is made from wine. Malt vinegar is made from unhopped beer. As vinegar is the desired end for these fermentations, the quality of the grain or fruit is not as important as it would be in the beverage industry, although it is not completely unimportant. The final character of the vinegar has its roots in properties of the material from which it is derived. Commercially, these first

alcoholic fermentations are accomplished utilizing yeast (usually *S. cerevisiae* var. *ellipsoideus*) specially selected for vinegar production. Then, a second fermentation is performed, where acetic acid bacteria are introduced to the alcohol, and the ethanol is oxidized to acetic acid.

There are three methods by which alcohol may be converted to acetic acid in the manufacture of vinegar. The first and oldest is known as the **Orleans** method, dating back to the fourteenth century. This is the traditional surface method, where the alcoholic precursor solution is placed in partially filled barrels or open vats and allowed to naturally oxidize, utilizing acetic acid bacteria occurring naturally in the air. The large surface area of the alcoholic fermentate and holes above the level of the liquid in the vat or cask allow for the necessary oxygen exchange. A film consisting of *Acetobacter* spp. bacterial cells and their associated cellulose slime (the "mother of vinegar") develops on the top and can be supported by a wooden grating to keep it from sinking under its own weight, killing the highly aerobic bacteria. This mother of vinegar builds up on the casks and aids in speeding up the acidification of new batches. Or a small amount of vinegar or mother of vinegar may be added to facilitate the conversion by lowering the pH and adding desirable acetic acid bacteria. This method is slow, requiring one to three months, but produces a good quality product with unique flavors and aromas because of the action of several additional bacteria that may be active in the production of other acids and esters. This method is still used to produce small batches of artisan vinegars.

In the eighteenth to nineteenth century, the **Trickling Generator**, a new method for vinegar production, was developed.

The alcoholic precursor was circulated through a tank packed with woody debris (colonized with *Acetobacter* ssp.) repeatedly until the proper concentration of acetic acid was reached. The heat produced in the manufacture caused a draft to be created, which aerated the system and allowed the aerobic reactions to take place. This new method took days instead of weeks to produce vinegar. This evolved into the modern vinegar generator – a tank packed with wood shavings or other inert materials that provide a large surface area on which acetic acid bacteria can adhere and a pump to recirculate the alcohol/acetic acid mixture, with provisions for aeration and cooling. Care must be taken to not allow the alcohol concentration to fall below 0.3–0.5%, as the bacteria on the shavings die quickly in the absence of ethanol. Good yields of high-quality acetic acid, 10–12% (v/v), can be produced in about three days.

Technology developed in antibiotic research during World War II led to the **Submerged Method** of vinegar production, the method most used today. This takes place in a large, highly aerated, temperature-controlled stainless steel tank known as an acetator (Figure 3.9). Specially selected strains of acetic acid bacteria grow in a suspension of very fine air bubbles continuously flowing through the alcoholic medium, oxidizing the ethanol to acetic acid while keeping it thoroughly dispersed. When the ethanol concentration falls to less than 0.3%, 50% of the medium is removed and replaced with fresh alcoholic medium. The bacteria are very sensitive and die quickly under low oxygen or ethanol conditions. The submerged method is inexpensive to set up and fast to run, producing uniform high-quality acetic acid in 24–48 hours. Continuous production and automation

Figure 3.9 Large-scale acetator for the production of vinegar used in most modern facilities (with kind permission from FRINGS brochure).

is easier using the submerged method, as is changing production from one type of vinegar to another.

Many vinegars benefit from a period of maturation to improve flavor or body, especially those produced by the quicker methods. Then, the vinegars are filtered and/or treated with fining agents to clarify. Finally, the vinegar is pasteurized before bottling for retail sale.

3.4.2 Distilled (White) Vinegar

As most uses for vinegar in the food industry are as a component in the manufacturing of other foods, the vinegar needs to be inexpensive and without strong flavors. Distilled white vinegar is the vinegar of choice for most of the food processing industry. The ethanol precursor can be produced from inexpensive starting material, with any unwanted flavors or aromas eliminated in the distillation process. This ethanol is then fermented to vinegar as described above.

3.4.3 Balsamic and Other Specialty Vinegars

Specialty vinegars are becoming increasingly popular with consumers. Some, such as fruit or herbal vinegars, are simply infusions, with fruits, juices, spices, or herbs added to distilled or wine vinegars. Others are complex fermentations. A classic example is traditional balsamic vinegar produced in Modina, Italy.

The traditional method for producing Balsamic vinegar begins with select Trebbiano, Lambrusco, and Sauvignon grapes, harvested as late as possible to maximize sugar content. The juices are concentrated at near-boiling temperatures to a sticky syrup with a sugar concentration of 20–24%. The syrup is inoculated with a specialized "mother of vinegar" containing strains of *Saccharomyces* and *Zygosaccharomyces*, as well as the acetic acid bacterium *Gluconobacter*, and then placed in a wooden barrel for a long and slow fermentation. The alcohol and acetic acid fermentations occur almost simultaneously, as *Gluconobacter* has the ability to oxidize not only ethanol but also sugars directly to acetic acid. The aging of balsamic vinegar is also slow. The vinegar is transferred into a series of barrels, each made from a different wood to add differing characteristics to the aging vinegar. The barrels are generally stored in attics or other areas not insulated from seasonal temperature variation. When the temperatures are generally warm, fermentation takes place; when cold, precipitates firm and settle, clarifying the vinegar. This goes on for at least 12 years and sometimes as long as 25–50 years. The result is a dark brown vinegar with a distinctive complex aroma and sour–sweet flavor. Is it any wonder that such a product can command top dollar at gourmet shops? Artisan vinegar makers in the United States are also responding to the desires of sophisticated consumers with specialty vinegars, adopting traditional methods of barrel fermentations and aging periods.

3.5 Bacterial and Mixed Fermentations – Industrial Application of Lactic Acid Bacteria, With or Without Yeast or Molds

3.5.1 Milk – Cultured Milks – Buttermilk, Yogurt, Kefir, and Cheese

Buttermilk was originally the liquid left over when butter was churned from cultured cream. Environmental LAB would acidify the cream, causing a separation, thus speeding up the churning process and leaving a thin, watery tart beverage. This is known as "traditional" buttermilk and has very little to do with the buttermilk found in shopping establishments.

Cultured buttermilk is made from reduced or low-fat milk, which is homogenized, pasteurized at 85 °C, cooled to 20–22 °C, and inoculated with a starter culture containing a combination of LAB such as *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* and flavor producing bacteria such as *Leuconostoc citrovorum* and diacetyl-producing strains of *L. lactis* subsp. *lactis*. This is incubated for 12–16 hours at 20–22 °C to produce the buttermilk product commercially available. Although buttermilk was very popular in the past, it has steadily lost market share in the United States to yogurt.

Yogurt is a traditional fermented milk originating in central Asia thousands of years ago. Milk was a commodity easily obtained but hard to keep fresh before refrigeration. This easy ferment kept the milk wholesome for an extended length of time. Introduced to consumers in the United States in the early 1970s, yogurt has steadily increased in popularity, until it is now the most popular cultured dairy product in the United States with an average annual consumption of

14.7 pounds per person (2015, United States Department of Agriculture [USDA] Dairy Data).

Yogurt is prepared by blending milk (nonfat, low-fat, or whole) with desired nonfat milk solids or evaporated milk, stabilizers, emulsifiers, and/or preservatives and then homogenizing to a smooth and consistent yogurt mix. This is then pasteurized at 85° for up to 30 minutes. This step kills any spoilage or other competing bacteria that may be in the mix. It also serves to denature the whey proteins, resulting in yogurt with better body and texture. The yogurt mix is then cooled to 40–45 °C, the desired incubation temperature for yogurt bacteria, and inoculated with a starter culture for yogurt.

A starter culture for yogurt needs to include two LAB, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. More than one strain of each are often included but always in a ratio of roughly 1 : 1. Neither make acceptable yogurt alone, but the two microorganisms work well together: the non-proteolytic *S. thermophilus* produces mainly lactic acid, whereas *L. delbrueckii* provides *S. thermophilus* with nitrogeneous compounds and contributes lactic acid and organoleptic compounds such as acetaldehyde.

Here, we reach a division – is this to be a cup-style or a Swiss- style yogurt? For cup-style or "set" yogurt, the inoculated yogurt mix is immediately dispensed into cups for fermentation. Fruit or other flavors are added to the cup before the yogurt mix, with the yogurt mix on top for "fruit on the bottom" style. The cup is incubated at 40–45 °C, then cooled, and prepared for marketing. "Set" yogurt has a firm jelly-like texture. Swiss style, also known as prestirred yogurt, is incubated in a vat at 40–45 °C and allowed to ferment. When the fermentation has completed to the level desired, the yogurt is gently stirred and cooled. Fruit and other flavors are added, and the mixture is dispensed into containers and prepared for marketing. The texture of "stirred" yogurt is generally less firm than "set" yogurt.

A third type of yogurt has gained popularity over the last few years. "Strained" or "Greek" style yogurt has taken over a large share of the overall yogurt market. This yogurt is made by either concentrating the milk before adding the starter culture, by removing the acid whey by filtration or centrifugation after the fermentation is complete, or often by a combination of the two. This results in a yogurt with a thicker, creamier consistency, higher in protein and other nutrients.

Kefir is a fermented dairy beverage produced by the actions of microflora encased in the "kefir grain," a symbiotic association of bacterial and yeast species contained in a protein–polysaccharide matrix, on the carbohydrates in milk. Containing many bacterial species already known for their probiotic properties, it has long been popular in Eastern Europe for its purported health benefits, where it is routinely administered to patients in hospitals and recommended for infants and the infirm. It is beginning to gain a foothold in the United States as a healthy probiotic beverage, mostly as an artisanal beverage, home-fermented from shared grains but also recently as a commercial product commanding shelf space in commercial establishments. This is similar to the status of yogurts in the 1970s when yogurt was the new healthy product. Scientific studies into these reported benefits are being conducted, although not all of the studies have been conclusive.

As traditional kefir can only be made by the fermentative action of the many bacteria and yeasts enclosed in kefir grains added to milk, adapting kefir for

commercial purposes has proven impractical. Quality, consistency, shelf life, and packaging are all challenges that make traditional kefir problematic. Commercial kefir products in the United States are instead prepared by fermenting milk with a starter culture containing a representative mixture of bacteria and yeast, yielding a product that approximates kefir, but without the problems that are associated with traditional kefir.

Russian-style kefir is made by taking the traditional kefir product, removing grains, and inoculating it into pasteurized milk at a concentration of 1–3% and then subjecting it to incubation and maturation. Industrial kefir is made by then taking Russian-style kefir and inoculating it into pasteurized milk at a concentration of 2–3% and then subjecting it to still further incubation and maturation. Every pass results in a change in the microbial composition of the kefir and a decline in the quality of the beverage. After the cycle leading to industrial kefir, the product has lost most of its traditional kefir characteristics.

Cheese has had a long and varied history and appears to have been well established as a food source in the earliest historical records. Hieroglyphic records on tomb walls depict cheese manufacture as early as 3000 BCE. The Hebrew Bible tells of David bringing cheese to his brothers on the battlefield about 1000 BCE. The Greek poet Homer (800 BCE) tells of the loaded cheese racks of the Cyclops. Archeologists have pushed the timing of cheese manufacture back even further with the discovery of apparent ancient cheese strainers dating back to 5500 BCE in ancient cattle-rearing sites in what is now Poland.

The first cheese was probably made accidentally. An ancient travelling ancestor probably filled up his bag (made from an animal stomach) with milk. During the course of his trip, the milk was fermented by microorganisms in the bag or in the atmosphere. The jostling caused the solids to separate from the whey. Upon opening his bag, the traveler was probably amazed to find a new, interesting, and delicious addition to his meal contained therein!

Cheeses are made all over the world from a variety of milks, with varying textures and flavors. About a thousand varieties of cheese have been described, with differences arising from the types and treatment of the milk, methods of production, temperatures, microorganisms in the starter cultures, handling of the curd, seasoning, ripening conditions, and local influences.

There are almost as many variations in cheese making as there are cheeses, but most roughly follow the same process:

(1) *Treatment of raw milk*: The raw milk is analyzed and standardized, then pasteurized, and inoculated with a defined starter culture, usually a mixture of several strains of mesophilic and thermophilic LAB. Coloring and/or flavoring agents may be added at this time. The LAB acidify the milk, which causes the coagulation of casein (pH 4.6) and activates the rennet enzymes that form the curd. The low pH also eliminates other undesirable bacteria. Moreover, the metabolic products of the LAB (ketones, aldehydes, and esters) are important for flavor development of the cheese.
(2) *Formation of curds*: Rennet is added to the treated milk to form the curds. Traditionally, rennet is derived from the fourth stomach (abomasum) of veal and contains roughly 94% rennin (chymosin) and 6% pepsin. Today,

however, much of the rennet used in cheese making is from microbial, usually fungal, sources. Rennin is the enzyme that causes the coagulation of milk by removing surface glucopeptides from κ-casein, making the casein unstable. The casein aggregates in the presence of calcium ions, forming a gel which entraps fats, bacteria, and other particulates. This reaction is accelerated by the action of the LAB on the milk, producing organic acids that lower the pH, and by increasing the temperature of the treated milk up to 45 °C. Although most vegetarian rennet is produced from microbial or fungal sources, a plant-based enzyme coagulant may also be prepared from sources such as thistle, nettle, artichoke, ground ivy, safflower, fig leaves, or melon. This does not provide chymosin, but rather cardosin as the coagulating enzyme, resulting in a curd with a different texture. This may also add bitter flavor, which may be desirable in some cheeses.

(3) *Processing of curds*: The curds are removed from the whey by heating, cutting the curds, lowering the pH, and pressing through cheesecloth. The acids produced by the LAB also make the curds more elastic, a desirable property of cheese. The curds are then salted, which contributes to moisture and acidity control as well as to taste. Other ingredients include herbs, coloring agents, additional enzymes, or additional desirable microorganisms (*Propionibacter* species for Swiss; *Penicillium roqueforti* for mold-ripened blue-veined cheeses, etc.) The cheeses are then pressed and formed, molded, or cut into shape in preparation for aging.

(4) *Ripening and aging of the cheese*: During the ripening period of a cheese, the young curd is converted to the final, full-flavored cheese because of the actions of the original starter microorganisms, the rennet, and the microorganisms mixed in with the processing of the curd. Cheeses are ripened for up to a year, during which time they develop their individual characteristics for which they are known. Soft, surface-ripened cheeses use *Penicillium camemberti*, either originating naturally from the environment or sprayed on the cheese surface. Although the fine white mold is growing on the surface, related hydrolytic enzymes produced by the mold are secreted into the cheese, breaking down the proteins and modifying the texture and flavors (Table 3.3).

3.5.1.1 Bacteriophage Contamination – Death of a Culture

Bacteriophage contamination is a problem in the cheese industry. Bacteriophages are viruses whose host organisms are bacteria. They are generally very specific, attacking only the bacterial host exhibiting the proper receptor sites on their cell wall. Bacteriophages are an important consideration in LAB fermentations, especially in cheese production, as they can spread very quickly, decimating the starter culture before it is able to complete the fermentation. Infection could lead to a decrease or even the complete inhibition of bacterial fermentation by the affected LAB. Even a decrease in fermentation capability is a problem, as it may result in lower quality products or spoilage, or it could disrupt the manufacturing schedule, an important consideration in industry. Bacteriophage infection problems may be combatted in the following ways: (i) Environmental sanitation processes that eliminate all phage points of entry,

(ii) incorporating phage-resistant ingredients into the media, such as phosphate and citrate salts that interfere with the phage's ability to bind to bacterial cells, (iii) using fresh, uninfected starter culture for a few fermentation cycles and then disposing of it before phage infection from environmental sources could become a problem, (iv) isolating and using bacteriophage-resistant variants in bacterial starter culture, and (v) using a starter culture composed of several different strains of the desired bacteria: if one becomes infected, the others still carry out the fermentation.

3.5.2 Meats – Sausages, Fish Sauces, and Pastes

Meat fermentations, again, date back thousands of years. Meat is an excellent growth medium for both LAB and spoilage microorganisms. Rapid, controlled fermentation of meat products acidifies the meat quickly to prevent spoilage and provides interesting flavors and textures.

Sausage production is a fairly simple idea. Ingredients include meat, sugar, salt, curing agent (nitrates or nitrites), and a culture. Spices and other flavoring agents may be added, as may chemicals such as ascorbate or erythorbate to inhibit oxidation. The meat is chopped or ground and mixed with the other ingredients, taking care to minimize oxygen contact that could adversely affect LAB growth or color and flavor development. When well mixed, the sausage meat mixture is extruded into casings, long thin tubes that give the sausages their shape. Casings are either traditional, made from the intestines of animals, or manufactured casings, usually made from cellulose or collagen, and are permeable to moisture and smoke. The shape, size, and diameter of the casing determine how long it takes for the sausage to dry, smoke, or cook, all steps that influence flavor development and texture in the finished sausage.

The encased sausages are then held in environmentally controlled ripening chambers for fermentation. Temperature and relative humidity are important considerations for optimal growth of the LAB inoculum. The fermentation needs to be slow enough to allow for the development of flavors but fast enough to prevent the growth of spoilage microorganisms. When the fermentation is finished, the sausage should have a pH less than 5.1 that, although still needing refrigeration, provides reasonable protection from pathogenic colonization. At pH 5, the flavors would not be very tart. As the pH drops into the high to mid 4's, tangy flavors become more pronounced.

Several additional treatments may be applied once the fermentation is complete. A cooking step will stop the fermentation by killing the microorganisms involved, as well as any pathogenic microorganisms that may have survived the fermentation process. Most fermented sausages in the United States are cooked. If the sausage contains pork, federal regulations make cooking mandatory. Whether the sausage is cooked or not, the fermented sausage can be smoked, dried, or both. Semi-dried products have a final moisture content of around 50% and have a shelf life of around 30 days at ambient temperatures. Dry fermented sausages have a moisture content of approximately 30% and have a shelf life of 75 days or even longer.

Many European sausages undergo another step: mold ripening. The fungi used are not part of the initial LAB fermentation; rather, this is done to enhance the flavor and textural properties of the finished sausage. The selected fungi, often *Penicillium nalgiovense* or *Penicillium chrysogenum*, produce proteinases, peptidases, and lipases, which diffuse into the meat, developing organoleptic products from protein and amino acid metabolism. This mold growth can occur naturally or the sausage can be dipped or sprayed with the desired fungal spores. The advantages to using pure fungal starter cultures are a more consistent quality (including a defined color, e.g. white, not green or blue, which could be seen as spoilage by the consumer) and no danger of mycotoxin development.

Fish sauces and pastes are staples in many Southeast Asian cuisines. Although fresh fish is highly perishable, fish sauces and pastes have long shelf lives, are an inexpensive source of high-quality protein and other essential nutrients, and enhance the flavors of otherwise bland foods.

The manufacture of fish sauce or paste is not complicated: whole small fish, shrimp, squid, or even oysters are salted at about a 3 : 1 ratio, placed in fermentation tanks, and held for a period of six months to one or two years. Occasionally, the tanks are uncovered and stirred. When the fish material is nearing complete hydrolysis, the material is transferred into settling tanks where the solids settle out. The liquid portion is decanted and bottled to sell as the highest quality "first run" product. The sediment is then washed with fresh brine and allowed to steep a few more weeks, producing a lower quality fish sauce. The bones are removed from the sediment, which is packaged as fish paste. Although fish sauce production is technically not a lactic acid fermentation (the hydrolysis of the proteins is accomplished by enzymes in the fish tissues, especially those in the gut), it is probable that fermentation by halophilic bacteria indigenous to the fish contribute to the flavor of the finished fish sauce.

3.5.3 Vegetables – Sauerkrauts and Pickles, Olives

Sauerkraut is a traditional food produced by the fermentation of salted white cabbage. The outer leaves and core are removed and the cabbage is shredded into a "slaw". Salt is added to about 2–3% concentration, and the slaw and salt are carefully mixed to eliminate any pockets of low or high concentration. Although traditionally, the cabbage would be packed in wooden barrels for fermentation; today sauerkraut is fermented in large concrete tanks containing up to 100 tons of shredded cabbage. After packing, the tanks are sealed using thick plastic sheeting, held down by brine or water to hold the cabbage under the brine formed and to exclude any atmospheric oxygen. Generally, the microorganisms needed for the fermentation are already present on the cabbage or equipment – no starter culture is required.

The salt immediately begins to act on the cabbage tissue by drawing out water through osmosis, producing a brine containing salt, sugars, and other dissolved nutrients contained in the cabbage. This produces a medium in which the bacterial microflora can grow. The high salt concentration inhibits the growth of many non-LAB, giving the LAB a competitive advantage for their growth. This starts a well-defined succession of microbial growth, with different strains of bacteria

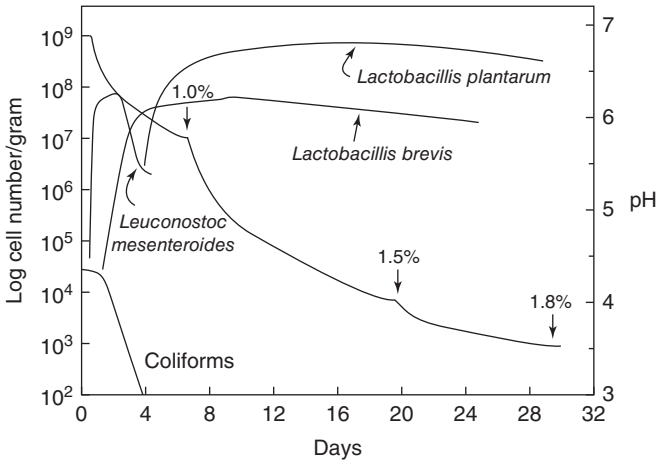


Figure 3.10 Bacterial succession in Sauerkraut. Idealized model for successive growth of lactic acid bacteria during the sauerkraut fermentation. [Figure 7-2 Fermentation succession]. Idealized model for successive growth of lactic acid bacteria during the sauerkraut fermentation. The approximate acidities (as % lactic acid) at varying pHs are indicated (from Hutkins (2019), p. 240, with kind permission John Wiley and Sons).

doing their part, then reducing or disappearing entirely while other microorganisms arise (Figure 3.10).

Many bacteria are present at the beginning of the fermentation. Oxygen is present and the pH is nearly neutral. However, with the exclusion of air entering the fermentation tanks and the residual respiration of the plant cells, the oxygen is quickly used up, eliminating growth of all obligate aerobic microorganisms that can otherwise survive the salt. During the first stage of lactic acid fermentation (initiation, heterolactic, or gaseous phase), *Leuconostoc mesenteroides*, a salt-tolerant LAB with a relatively short lag phase that grows at a relatively low temperature, metabolizes sugars yielding lactic acid, acetic acid, ethanol, and CO_2. This acidifies the salted cabbage, inhibiting other nonlactic competing microorganisms (coliforms drop off abruptly here). The CO_2 generated makes the environment increasingly strictly anaerobic, and the acid environment makes conditions even more favorable for other LAB. However, as the acid concentration approaches 1%, the acid levels become toxic for the *L. mesenteroides*, which drops to barely detectable levels within four to six days of the fermentation.

In the next phase of sauerkraut fermentation (primary, homolactic, or nongaseous phase), other LAB, most often *Lactobacillus plantarum* and *Lactobacillus brevis*, take over, facilitated by the decreasing *L. mesenteroides* levels. These microorganisms are very stable in the acidic environment and are also strong acid producers, reducing the pH below 4. When the fermentation is complete, after about a month, the pH should be around 3.4–3.6, and the acid concentration should be around 1.7%. The finished sauerkraut flavor benefits from several metabolic products of all the bacteria involved: lactic acid, acetic acid, small amounts of diacetyl, acetaldehyde, and other flavor compounds, as well as CO_2 are all important components of sauerkraut. The sauerkraut is now ready for

packaging: it can be either thermally processed and canned or kept fresh, sold in bottles or sealed bags in the refrigerator section.

Most of the **pickles** consumed in the United States are not the products of lactic acid fermentation at all; rather, they are prepared by adding vinegar and salt solution directly to the cucumbers, eliminating the fermentation step. True fermented pickles have different flavor and texture characteristics and take much longer to produce.

Producing fermented pickles is similar in some ways to sauerkraut, as they both rely on salt and the exclusion of oxygen to provide the selective environment for the growth of the necessary fermentation LAB. However, pickles require a far higher concentration of salt, immediately allowing a far less diverse array of fermentation microorganisms. The salt is prepared as a brine of at least 5%, rather than dry salt. *L. mesenteroides* will not grow at this concentration. This is fortunate, as the CO_2 that might have been produced in a heterofermentative fermentation could lead to "bloater and floater" pickle defects. Instead, the initial fermentation is carried out by *Pediococcus* species and *L. plantarum*. Controlled fermentation via the use of a starter culture specially selected to be acid- and salt-tolerant can be used for consistency. At the end of fermentation, the pH will be around 3.5 and the acidity will be between 0.6% and 1.2%.

Bacteriophage infection is usually not a problem in fermented vegetable products, as they are usually not pure culture fermentations. Many rely on spontaneous fermentation by indigenous LAB on the vegetable. If a starter culture is used, it is usually composed of many strains of bacteria.

As in pickle production, the bulk of **olives** produced in the United States are not fermentation products. "California style" or ripe black olives are prepared by treating the olives in a lye (NaOH) solution, aerating to promote darkening, and canned in a 1–3% NaCl brine. However, both Spanish-style and Greek-style olives undergo fermentation.

Spanish-style olives are harvested green and soaked in a lye (NaOH) solution to remove bitter compounds by hydrolysis of oleuropein. The olives are then washed to remove the lye and placed in 10–15% NaCl brine in a fermentation tank or barrel. The first stage of fermentation is a highly variable soup of Gram-negative and Gram-positive bacteria, until the LAB present, especially *L. mesenteroides* and *Pediococcus* ssp., can gain the upper hand by producing acids that drop the pH below 5. After two to three weeks, as the pH continues to drop, the *L. mesenteroides* is replaced by a variety of lactobacilli, settling on a predominance of *L. plantarum* as the pH continues to drop to a final pH of between 3.5 and 4.2, acidity 0.8–1.0%. At this point, the green Spanish-style olives are prepared and packaged for the consumer.

Greek-style olives are naturally dark at harvest. They are not treated with lye to remove bitter compounds. The brine used is at a lower NaCl concentration than for Spanish-style olives, at 5–10%. Thus, the fermenting microflora is much more diverse, including LAB, non-LAB, yeasts, and fungi. Acids produced include lactic, acetic, citric, and malic, as well as ethanol and CO_2. The final pH could be as high as 4.5, titratable acidity less than 0.6%.

3.5.4 Grains and Legumes – Soy Sauce, Miso, Natto, and Tempeh

The carbohydrates in rice and soybeans are mostly locked up in starches and polysaccharides; there are few fermentable sugars in the substrates used for most Asian food fermentations. The production of **soy sauce** begins with the preparation of Koji, an enzyme preparation that is used to convert these nonfermentable raw materials into products that are easily fermentable by selected microorganisms. This is made using *tane koji*, literally "seed mold," a pure culture of *Aspergillus oryzae* or *Aspergillus sojae* spores grown on boiled rice and incubated under conditions to promote fungal growth, with ample air and moisture circulation, and temperature controlled at 30 °C.

To prepare the koji, soybeans (whole or flaked) are soaked for about 12 hours and then cooked for an hour. Roasted, crushed wheat may also be used in soy sauce for color and flavor and to aid in the fermentation process. The prepared soybeans and wheat are usually mixed in a 1 : 1 ratio, inoculated with prepared tane koji, and then placed in tanks with an equal volume of a strong brine (20–25% NaCl) for an extended fermentation time of six to eight months. It is here, during this "mash" or "moromi" step, that the enzymes developed in the koji hydrolyze the proteins, polysaccharides, and starches, and fermentation by halotolerant microorganisms takes place. The fungi are very salt sensitive and quickly die. Contaminants such as *Micrococcus* and most *Bacillus* species also decrease rapidly and are not detectable within the first month of incubation. Only the very salt-tolerant LAB, strains of *L. delbrueckii* and *Tetragenococcus halophilus*, survive to facilitate the early mash fermentation. As the pH drops and the acidity increases, the conditions get too harsh for even these to survive. In the next stage, *Saccharomyces rouxii* takes over fermentation, producing alcohol. Then, in the last phase, *Torulopsis* yeasts produce phenolic compounds important for flavor.

Finally, when the fermentation is complete and sufficiently aged, the moromi is pressed through a hydraulic filter and pasteurized. The heating for pasteurization does more than simply kill all remaining microorganisms: it also inactivates enzymes and develops flavors and color. The finished soy sauce is then ready for commercial packaging.

Miso is a fermented soybean paste, usually also containing a cereal product such as rice or barley. Production is similar to that of soy sauce. The koji is prepared by soaking the carbohydrate source (rice or barley) overnight, cooking, and cooling and then inoculating with tane koji. If no additional carbon source is to be used (soybean miso), soybeans may be used for the koji. For the primary fermentation, the koji is incubated for two to three days at 30–40 °C and aerated to promote fungal growth. The koji is then mixed with soaked and cooked soybeans and dry salt (final salt concentration between 6% and 13%) and mashed into a chunky paste. This is placed in large fermenters and inoculated with starter culture containing yeasts and LAB (or backslopped with raw miso for a traditional fermentation). The enzymes from the koji break down the proteins, polysaccharides, and lipids, while the yeasts and LAB ferment the sugars, forming organic acids, ethanol, and glycerol. When fermentation is completed, the miso may be used immediately or aged for up to two years before being mashed and packaged

for the consumer. The miso may be pasteurized to extend shelf life, but many consumers prefer it to be left raw to take advantage of the potential health benefits of the active cultures.

Natto is a fermented whole soybean product consumed mainly in Japan. Cooked, cooled soybeans are inoculated with *Bacillus subtilis* var. *natto* (formerly *Bacillus natto*) and incubated at 40 °C for 14–18 hours and then held at refrigeration temperatures to retard further growth. *B. subtilis* var. *natto* grows on the surface of the soybean, synthesizing a viscous polysaccharide layer covering the bean surface. Natto is inexpensive, nutritious, and high in Vitamin K.

Tempeh is a product made by fermenting soybeans using a mold. Dried soybeans are boiled, the hulls are removed, and the beans are soaked at ambient temperature for up to 24 hours. During this time endogenous LAB acidify the soak water, restricting the growth of potential spoilage bacteria and pathogens. The soaking step also extracts potential natural mold inhibitors from the soybeans, preparing the beans for optimal mold growth.

The beans are then drained and inoculated with mold culture. This can be done through backslopping from a previous batch of tempeh, using a wild mixed strain culture inoculum or (most common in commercial manufacturing) using a defined starter spore culture of *Rhizopus microsporus* var. *oligosporus*, inoculated directly on the beans or pregrown on steamed rice before inoculating. The beans are then shaped into cakes and traditionally wrapped in banana leaves to promote rapid mold growth. In large-scale fermentation operations, the inoculated beans are placed in trays and incubated in climate-controlled rooms at 25–37 °C for one to two days until the beans are covered with white mycelium which binds the beans together tightly, forming solid cakes. This is the stopping point for the fermentation – further fermentation would allow *Rhizopus* to sporulate, making the product unattractive. The finished product needs to be consumed within a day or two, or preserved by freezing or drying.

Tempeh is easy to manufacture and is an important source of protein. It is also one of the very few plant-based sources of vitamin B_{12}, a nutrient that is difficult to obtain in a vegetarian diet. Vitamin B_{12} is likely synthesized by *Klebsiella pneumoniae* and *Citrobacter freundii* sometime during the fungal fermentation, with production enhanced in the presence of the *Rhizopus* mold. Although modest, at about 6% of the United States Department of Agriculture (USDA) recommended amount in a 100 g serving, it at least partially fills a nutritional gap. Other vitamins present in tempeh in increased amounts over soybeans include riboflavin (B_2), niacin, pyridoxine (B_6), biotin, pantothenic acid, and folic acid, although thiamine (B_1) levels are reduced. Fat-soluble vitamins remain at the same level.

3.5.5 Cocoa and Coffee

Cocoa is derived from the seeds ("beans") of the plant *Theobroma cacao*. The seeds are bitter and astringent in their natural state. To remedy this, the seeds and their surrounding mucilage need to undergo fermentation. The pods are harvested and the seeds and mucilage are heaped on the ground or put into containers for fermenting. The mucilage, containing water, sugars, protein, and minerals, is a rich media source for the fermenting microorganisms, and it is

this pulp, and not the seeds, which actually undergo fermentation. However, the fermentation products profoundly affect the seeds, transforming them biochemically to be able to render the expected flavors, colors, and aromas of chocolate when properly processed.

The first fermentation is a yeast fermentation, where alcohols and aldehydes are produced and an anaerobic environment is established, favorable to LAB. The LAB then produce large quantities of enzymes that aid in the removal of the mucilage from the seeds. After a few days, to prevent too high a level of lactic acid from developing (a chocolate defect), the heaps or containers are turned, reintroducing oxygen and allowing acetic acid bacteria to proliferate. The acetic acid bacteria oxidize the ethanol first into acetic acid, then on to CO_2 and water, causing an increase in temperature. Periodic turning of the mass cools, mixes, and aerates the substrate, allowing the reactions to continue to completion. After fermentation, the beans must be dried to reduce the moisture level from 40% to 50% to around 6% to prevent mold formation during storage.

Fermentation plays a less critical role in the production of coffee. Again, the coffee "bean" is surrounded by mucilage that must be removed. The main function of the fermentation of coffee beans is to release the beans from the mucilage. Flavor is not dependent on the fermentation.

There are two methods of fermentation. The first, known as "wet processing" or "washed coffee," involves depulping to remove most of the mucilage from the bean and then fermenting by holding the berries under water in a wooden or concrete bin to convert the remaining mucilage into water-soluble substances that can be washed away through periodic water changes that minimize the buildup of metabolic products that can cause an off-flavor in the coffee. The beans are then dried to around 12% moisture content. Wet processed coffee beans have a blue-green appearance.

For "dry processing," or "natural coffee," the coffee "cherries" are allowed to partially dry on the plant. When harvested, the fruit is further dehydrated in the sun, during which time fermentations take place within the cherry, secreting enzymes that break down the mucilage around the beans. Care must be taken to minimize mold growth during drying, as that might result in the development of off-flavors. The hull and mucilage can then be removed mechanically, leaving a light brown bean. A final drying then takes place to bring the moisture content to 11–12%.

3.6 Fungi as Food

3.6.1 Mushrooms

Of the thousands of varieties of known mushrooms, only about a third are edible, although not necessarily desirable. Of these, only a relative handful are grown commercially. Some mushrooms currently defy attempts to cultivate. The top three mushroom varieties in cultivation are button (*Agaricus bisporus*), shiitake (*Lentinula edodes*), and oyster (*Pleurotus* ssp.), followed by wood ears (*Auricularia* ssp.), straw (*Volvariella volvacea*), and enokitake (*Flammulina velutipes*).

Worldwide production of mushrooms in 2013 approached 10 million tons, 71% of which was produced in China. The value of the worldwide mushroom crop neared 18 billion US$.

Mushrooms are an economically viable product of lignocellulose fermentation, converting nondigestible plant wastes into edible food. The mycelial form of the fungus penetrates deeply into the host substrate, excreting enzymes that break down the cellular structures of the substrate, releasing nutrients necessary for fungal growth and fruiting body production. This fruiting body, the mushroom, can be easily removed from its substrate for use as food. Edible mushrooms are a source of protein, vitamin D, B vitamins, and minerals (potassium, selenium, and copper).

The most popular mushroom variety in the United States, accounting for over 90% of domestic production, is the common button mushroom, *A. bisporus*, found in meadows and woodlands. It is a secondary decomposer, requiring a primary composting of the substrate before it can further degrade the cellulosic plant debris. It is easily cultured commercially, with a production rate of about six weeks. A solid substrate consisting of straw, manure (or suitable nutrient substitutes), fertilizers, and gypsum is composted for about two weeks to optimize the nutrient level and homogenize the structure. The solid substrate is then pasteurized (peak heating) before being inoculated with a pure culture of the mushroom mycelium. This is allowed to colonize for two to three weeks at 22–25 °C, during which time the fungal enzymes further break down the composted substrate to extract nutrients. The substrate is then covered with a layer of peat-rich soil and gypsum (casing), plus plenty of water, stimulating the mushroom mycelium to form fruiting bodies. Crops begin to reach the harvest stage within a week and continue to produce at weekly intervals for about four weeks.

The Shiitake mushroom is a primary decomposer, naturally growing on dead tree trunks in Asian rain forests. There are records documenting its culture in China for almost a thousand years. Traditional culture of Shiitake mushrooms is laborious and time-consuming, involving drilling holes into 1 m long logs of the shii tree (from which the name "Shiitake" is derived) and then inoculating these holes with fungal "spawn"-wood or sawdust plugs, containing the appropriate fungal mycelium. The holes would be sealed with wax and the logs were held for a six to nine months "spawn run" period to develop the fungal mycelium inside the log. The logs would then be transferred into a moist cool environment for up to an additional year to stimulate the formation of fruiting bodies (Figure 3.11).

Although traditional methods have been adopted worldwide, utilizing wood native to the local areas such as oak, beech, alder, chestnut, or other hardwoods, modern large-scale cultivation usually involves a composite synthetic log solid substrate consisting of sawdust, straw, and corncobs, supplemented with grains such as corn, wheat bran, rice bran, rye, millet, or plant waste to add nutrients that accelerate fungal colonization and increase yields at minimal costs. Moisture in this mixture is adjusted to about 60–65% and then the substrate is placed in plastic bags and steam-sterilized for two hours at 121 °C, cooled, and inoculated with Shiitake spawn. After the mycelium is allowed to fully colonize the logs, a process that can take from 3 to 12 weeks, depending on the log composition and Shiitake strain used, the plastic bags are removed, and the logs are held under controlled

Figure 3.11 Shiitake mushrooms (photograph with kind permission from K.-Peter Stahmann).

moisture and temperature conditions. To stimulate fruiting body production, the logs are soaked in water at 12 °C for several hours. Fruiting bodies begin to form on the exposed substrate logs, sometimes within a few days, with the first crop ready for harvest in about 10 days. Total time for production of shiitake using synthetic logs is around four months, much shorter than the 18 months required for the traditional log method. Shiitake may be used fresh, but are typically dried for use and for preservation.

Oyster mushroom, like Shiitake, are primary decomposers, growing on hardwoods such as beech. Commercial production is generally on a synthetic substrate, similar to that used for Shiitake except the brood is mixed with the moist, semisterilized substrate before packing into specially slit plastic bags. Large oyster mushroom mycelial inoculum, coupled with low air circulation, encourage oyster mushroom growth, while inhibiting fungi that may compete. The colonization step generally takes two to three weeks at a temperature of 24–26 °C.

Lower temperatures, around 15–22 °C, coupled with high relative humidity and light exposure, stimulate fruiting body formation at the slits in the plastic bag. Low light conditions or high CO_2 levels lead to misshaped fruiting bodies. Oyster mushrooms are usually harvested as a cluster and are usually used fresh, although they can be dried or canned.

3.6.2 Single-Cell Protein – *Fusarium venenatum*

In the 1950s and 1960s, scientists sought novel solutions to the "food gap" between the industrialized countries and the developing world. As populations expanded in the less-industrialized areas, coupled with crop failures and limitations on conventional meat production, protein deficiency malnutrition was a

real concern. In the search for a solution that could be produced continuously and that was not subject to external weather conditions, scientists turned to bacteria and fungi. Many different microorganisms were tested, growing in substrates from petroleum products to alcohols to plant wastes and dairy wastes. As the term "microbial protein" did not seem appetizing, in 1966, MIT Professor Carroll L. Wilson coined the term "Single-Cell Protein" (SCP) as a more palatable alternative. Even so, almost all SCP produced currently is used for animal feed. One exception is the *Fusarium* mycoprotein.

In 1967, British researchers scanning fungi for an inexpensive high protein cultivar found that the plant pathogenic mold *Fusarium venenatum* could be grown inexpensively and then processed into a product with flesh-like textures. Growth conditions were optimized to eliminate the production of mycotoxins. The fungal mycelium is grown continuously in a loop reactor in a medium consisting of glucose, peptone, and mineral supplements. Aeration and agitation are accomplished through the introduction of compressed air. Mycelial mass is harvested and heat treated at 74 °C, hot enough to inactivate nucleic acid-forming processes in the cells, but still leave RNases active to eliminate excess RNA, a problem in SCP that could lead to gout and kidney stones in humans. The product has seen commercial success, especially in the vegetarian market, but is not without controversy. All mycoprotein batches must be tested for mycotoxins, a problem that could be eliminated through genetic engineering, but as the current major target consumer has issues with accepting food produced using genetically modified organisms (GMOs), this would be counterproductive. Although it achieved Generally Recognized as Safe (GRAS) status from the Food and Drug Administration (FDA) in the United States in 2002, the Center for Science in the Public Interest, a public advocacy action center advocating for safer and healthier foods, has lobbied for its removal or at least for prominent warnings to be included on packaging because of allergic reactions and gastrointestinal distress experienced by some consumers.

3.7 Conclusions and Outlook

Food production is a major emphasis of industrial microbiology. Fermentation extends the harvest, providing a safe and reliable food supply for the world's expanding population. Food variety is also increased, providing more consumer interest, better digestibility, and greater nutritive capability. Substrates that might otherwise be indigestible may contribute to the production of nutritious, consumable foods to enhance diets. As research continues, efficiencies may be increased to deliver quality-fermented foods to the world more safely and economically. New technologies are being developed and utilized using molecular biology techniques to enhance food quality and safety. For example, a common white button mushroom, *A. bisporus*, was recently genetically modified with the gene-editing tool CRISPR-Cas9 to resist browning. This involved the deletion of a handful of base pairs in the mushroom genome, knocking out one of six genes that encode isoenzymes of the browning polyphenol oxidase. As no foreign DNA from viruses or bacteria was inserted into the genome,

this gene-editing procedure appears to not require USDA regulation. Perhaps, another breakthrough in mushroom research will uncover the intricate molecular mechanisms of symbiotic fungi/plant interactions, allowing the cultivation of stone mushrooms, chanterelles, or even truffles.

Consumer demand has led to increasing research into food safety and natural food-related health benefits. The dramatic rise in understanding of the microbiome and its variety of roles over the past few years has led to increased consumer and medical interest in probiotics and prebiotics. Food microbiology will continue to increase in importance over the coming decades as our understanding of these vital issues grows. Industrial food microbiology will continue to contribute greatly to humanity's overall quality of life.

References

Food and Agriculture Organization of the United Nations (FAO) (2001). Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. In: http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf.

Hutkins, R.W. (2019). *Microbiology and Technology of Fermented Foods*, 2e. Ames, IA: IFT Press, Wiley Blackwell Publishing.

Johnston, M. and Kim, J.-H. (2005). Glucose as a hormone: receptor-mediated glucose sensing in the yeast *Saccharomyces cerevisiae*. *Biochem. Soc. Trans.* 33 (1): 247–252.

Matthews, K.R., Kniel, K.E., and Montville, T.J. (2017). *Food Microbiology: An Introduction*, 4e. Washington, DC: ASM Press.

Further Reading

Doyle, M.P. and Buchanan, R.L. (eds.) (2013). *Food Microbiology: Fundamentals and Frontiers*, 4e. Washington, DC: ASM Press.

Hutkins, R.W. (2019). *Microbiology and Technology of Fermented Foods*, 2e. Ames, IA: IFT Press, Wiley Blackwell Publishing.

Katz, S.E. (2012). *The Art of Fermentation*. White River Junction, VT: Chelsea Green Publishers.

Kumar, P., Chatli, M.K., Verma, A.K. et al. (2017). Quality, functionality, and shelf life of fermented meat and meat products, a review. *Crit. Rev. Food Sci.* 57 (13): 2884–2856.

Liu, Y., Rousseaux, S., Tourdot-Marechal, R. et al. (2017). Wine microbiome: a dynamic world of microbial interactions. *Crit. Rev. Food Sci.* 57 (4): 856–873.

Matthews, K.R., Kniel, K.E., and Montville, T.J. (2017). *Food Microbiology: An Introduction*, 4e. Washington, DC: ASM Press.

Nielsen, B., Gurakan, G.C., and Ünlü, G. (2014). Kefir: a multifaceted fermented dairy product. *Probiotics Antimicrob. Proteins* 6: 123–135. https://doi.org/10.1007/s12602-014-9168-0.

Patra, J., Das, G., Paramithiotis, S., and Shin, H. (2016). Kimchi and other widely consumed traditional foods of Korea, a review. *Front. Microbiol.* 7: 1–15. https://doi.org/10.3389/frmiob2016.01493.

Petel, C., Onno, B., and Prost, C. (2017). Sourdough volatile compounds and their contribution to bread: a review. *Trends Food Sci. Tech.* 59: 105–123.

Sanlier, N., Gokcen, B., and Sezgin, A. (2017). Health benefits of fermented foods. *Crit. Rev. Food Sci.* https://doi.org/10.1080/10408398.2017.1383355.

Tamang, J.P. (2010). *Fermented Foods and Beverages of the World*. Boca Raton, FL: CRC Press/Taylor and Francis.

4

Technical Alcohols and Ketones

Peter Dürre

Institut für Mikrobiologie und Biotechnologie, Universität Ulm, Albert-Einstein-Allee 11, 89081 Ulm, Germany

CHAPTER MENU

4.1 Introduction

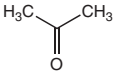
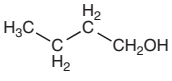
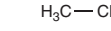
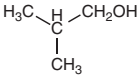
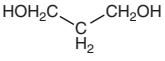
The term "**alcohol**" refers to a family of hydrocarbons with a hydroxyl group (OH group). However, this expression is often used synonymously, albeit imprecisely, to indicate the most prominent member of this group – ethanol (CH_3–CH_2–OH). Polyalcohols or polyols contain several OH groups, whereas ketones are characterized by carrying a C=O group.

Ethanol is a biological product that has long been known to mankind. Until recently, it was only used for beverages (initially in beer and wine, later also in spirits; Chapter 1). The process of making vinegar out of ethanol has also been in practice for a long time (Chapter 3). It was not discovered until the middle of the nineteenth century, however, that butanol, acetone, propanediol, and isobutanol are also microbial products. In 1862, Louis Pasteur described the formation of butanol during fermentation with an anaerobic mixed culture. In 1881, it was demonstrated that 1,3-propanediol could be produced from a glycerol fermentation and in 1904 that *Bacillus macerans* was able to produce acetone. The only substances that came to be produced by means of industrial biotechnical processes at a technical scale, however, were acetone and butanol. The heyday of this technology was between 1915 and 1950 (for historical details, see Section 4.4.1).

The microbially produced compounds with industrial relevance today are ethanol, 1,3-propanediol, butanol, isobutanol, and acetone. Their respective properties are listed in Table 4.1.

Industrial Microbiology, First Edition.
Edited by David B. Wilson, Hermann Sahm, Klaus-Peter Stahmann, and Mattheos Koffas.

Table 4.1 Properties of technical alcohols and ketones stemming from microbial production.

	Acetone	**Butanol**	**Ethanol**	**Isobutanol**	**1,3-Propanediol**
Structural formula					
Molecular weight	58.08	74.12	46.07	74.12	76.1
Melting point (°C)	−94.6	−89	−114	−101.9	−26
Boiling point (°C)	56.1	118	78	107.9	213
Density (g/cm^3)	0.79	0.81	0.79	0.80	1.05
Vapor pressure (kPa)	24.7	0.56	5.95	0.95	0.9
Water solubility (at 20 °C)	Completely miscible	7.9 g in 100 g water	Completely miscible, but azeotrope formation (96% ethanol, 4% water)	8.5 g in 100 g water	10 g in 100 g water

4.2 Ethanol Synthesis by *Saccharomyces cerevisiae* and *Clostridium autoethanogenum*

4.2.1 Application

Eighty percent of the world's ethanol stemming from fermentation is used as biofuel for automobiles. It is mixed with gasoline in different concentrations to make it suitable for this purpose. In Europe, typically 10% ethanol is added; in countries such as Brazil, higher concentrations are used (up to 25%). This type of fuel mixture requires the so-called *flexible fuel* engines, which are capable of processing any ethanol/gasoline mixtures (up to 85% ethanol, E85) as well as pure ethanol. The share of ethanol in the fuel sector clearly shows an upward trend. Table 4.2 compares the properties of ethanol and other biofuels to those of gasoline.

The remaining 20% of the world's ethanol produced by fermentation is used in the chemical/technical sector and in the food sector. In the former, the alcohol is used as a solvent, disinfectant, carrier of scents (e.g. in perfumes, deodorants, and fragrances), liquid fuel in ethanol fireplaces and ethanol burners, and an additive in cleaning and antifreezing agents. A denaturant is added to ethanol in concentrated form (e.g. for use as in ethanol burners) in order to inhibit improper consumption. The increase in crude oil prices has again triggered an economic reaction that has been familiar for many years: the conversion of ethanol to ethene (**ethylene**). Ethene is one of the most important basic components in the chemical industry. Until now, it has been produced almost exclusively from crude oil (**naphtha**) by means of steam cracking. Worldwide production of ethene amounts to more than 100 million tons per year. The Brazilian company Braskem opened a facility in 2010 in Triunfo, in which ethanol, manufactured by fermenting molasses, is converted to ethene via catalytic dehydration with aluminum oxide or silicate/aluminum oxide as catalysts. The plant has a capacity of 200 000 tons per year.

The food sector (alcoholic beverages and vinegar) was already addressed in Chapter 3.

4.2.2 Metabolic Pathways and Regulation

Ethanol can be produced biologically by fermenting sugary or starchy substrates. Cane sugar (sucrose) and starch are first converted into glucose, which is then metabolized into pyruvate (Figure 4.1). Two different pathways are possible: **glycolysis** and the **KDPG (2-keto-3-desoxy-6-phosphogluconate) pathway** (also

Table 4.2 Fuel properties of biofuels in comparison to gasoline.

	Gasoline	Ethanol	Butanol	Isobutanol
Energy density (MJ/kg)	43.5	29.7	36	33
Motor octane number (MON)	85	92	87	93.6
Research octane number (RON)	95	129	96	111

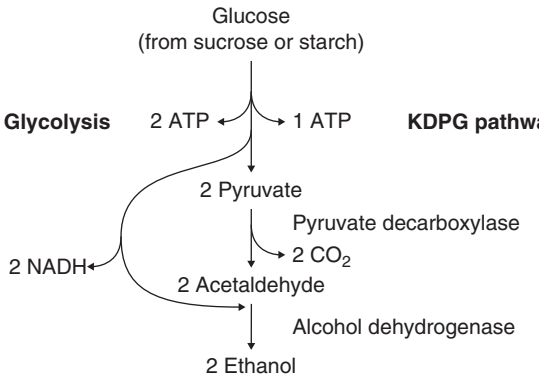


Figure 4.1 Metabolic pathway of alcoholic fermentation. *Saccharomyces cerevisiae* uses the glycolysis pathway and *Zymomonas mobilis* uses the KDPG (2-keto-3-desoxy-6-phosphogluconate) pathway.

known as the Entner–Doudoroff pathway). The ATP yield is significantly higher with glycolysis. Baker's yeast, *Saccharomyces cerevisiae*, (eukaryote) uses glycolysis, whereas the bacterium *Zymomonas mobilis* (prokaryote) uses the KDPG pathway. The resulting pyruvate is then converted into ethanol by both microorganisms with the help of the key enzymes pyruvate decarboxylase and alcohol dehydrogenase (usually, several isoenzymes are present). With *S. cerevisiae*, a facultatively anaerobic microorganism, the **Pasteur effect** comes into play, i.e. significantly more glucose is converted under anaerobic conditions than under aerobic conditions. This is due to a high ATP content, as it occurs in the cell during aerobic growth, which inhibits phosphofructokinase. The anaerobic conditions of fermentation lead to improved sugar consumption and thus to increased ethanol production.

4.2.3 Production Strains

Because of their robustness, *Saccharomyces* strains (primarily *S. cerevisiae*) are used for the fermentation. Admittedly, the bacterium *Z. mobilis* ferments sugar more rapidly than yeast, and it also provides a higher ethanol yield, but the substrate requirements are more complex and the fermentation is very susceptible to contamination because of its neutral pH level. *S. cerevisiae*, however, also exhibits a very limited substrate spectrum. From an industrial production perspective, strains that could also consume pentoses would be particularly useful. The hemicellulose (**xylan**) part of the plant material would then also be available for use as a substrate once it had been split into individual molecules. Intensive research is currently being conducted on options for engineering such strains by means of genetic manipulation.

4.2.4 Production Processes

In Europe, the substrates for alcoholic fermentation are either starches from grains or sugar from sugar beets. In the United States, cornstarch is the main substrate, and in Brazil, the predominate substrate is sugar from sugar cane. The worldwide production of ethanol in 2015 amounted to approximately 77 million tons, roughly 85% of which was produced in Brazil and the United States. One of

Figure 4.2 Ethanol fermentation plant of CropEnergies AG in Zeitz. Source: Courtesy of CropEnergies AG, Mannheim; © Martin Jehnichen.

the largest plants in Europe is operated by CropEnergies AG in Zeitz in Germany (Figure 4.2). This factory produces ethanol using grains and sugar syrup (from sugar beets) and has a capacity of roughly 285 000 t/yr.

The typical ethanol fermentation process with grain as a substrate consists of the following steps: **grinding**, **liquefaction**, **saccharification**, **fermentation**, and **distillation**. Grains are first ground in a dry state and then mixed with hot water and α-amylase (mashing). In the process, starch liquefaction occurs (production of soluble oligosaccharides). These molecules are then split into glucose units through the addition of glucoamylase (saccharification). This sugar is then converted by *S. cerevisiae*, whereby the yeast initially consumes the existing oxygen and produces some biomass before initiating the fermentation process. Ethanol is then extracted from the liquid via the process of distillation. The nonfermentable residue, including the yeast cells, is referred to as mash. Dehydration and evaporation of mash result in a dried, high-protein material in pellet form, which is known as **DDGS** (dried distillers' grains with solubles) and is used as animal feed.

In Brazil, a mixture of sugar cane juice and **molasses** is typically used as a substrate. The reason behind this is that, although sugar cane juice has a low concentration of certain nutrients, it serves as a dilution for inhibitors that are present in molasses. Fermentation is performed in batch, fed batch, and continuous cultures. The advantage of the continuous culture method is higher productivity, whereas the batch cultures undergo an acid treatment following fermentation (addition of sulfuric acid down to a pH level of between 2.5 and 2) and are then used for further fermentations. The acid treatment means that the risk of contamination can be eliminated (lactobacilli, in particular, play a significant role, as they are capable of surviving at a low pH level and in high concentrations of ethanol). With a continuous culture, this is only possible for a fraction of the population, and the risk of contamination is therefore greater. Following a longer period of operation, the starter cultures become dominated by wild *Saccharomyces* strains. Strains from different plants demonstrated volumetric productivities between 2.2 and 2.8 g ethanol/1/h.

When using sugar syrups and molasses, sucrose serves as the substrate. In this case, there is no need for a separate enzymatic decomposition, as *S. cerevisiae* possesses extracellular and intracellular **invertases**, which hydrolyze the disaccharide sucrose into glucose and fructose.

4.2.5 Ethanol – Fuel of the Future?

The partial success in substituting oil with ethanol prompted hopes for replacing the finite supplies of naphtha with renewable resources. This, however, will not be totally possible. With their climatic and agricultural conditions, countries such as Brazil can certainly count on ethanol as a fuel (and also as a base material for ethene), but for many other countries, this is not the case. Nevertheless, worldwide ethanol use does help to stretch the reserves of petroleum. Experts predict that biofuels could make up about 15% of fuels by the year 2030.

The use of ethanol as a fuel for gasoline engines is, however, totally ambivalent. On the positive side, there is the exploitation of renewable resources and the reduced amount of petroleum products being burned, leading to a reduction in the emission of CO_2 into the atmosphere. However, as the fermentation is dependent on substrates that are also required for sustenance (grains, corn, sugar cane, and sugar beets), a conflict of interests between striving for mobility and securing basic food supplies is inevitable (the so-called "food versus fuel" controversy). The considerable rise in ethanol production has indeed led to a sizable price increase for corn and grains. In January 2007, there were massive protests in Mexico opposing the high prices for corn flour (which ultimately led to an increase in subsidization). A satisfactory solution, however, will only be found if it becomes possible to identify fermentation substrates that are not used as food supplies (see the following section).

4.2.6 Alternative Substrates for Ethanol Fermentation by Cellulolytic Bacteria and *Clostridium autoethanogenum*

There are currently two options for alternative substrates to be used in microbial ethanol production that are available in large quantities and do not pose any competition to the food sector: **lignocellulosic hydrolysates** and **synthesis gas**.

Biomass is readily available in large quantities and consists of cellulose (a glucose polymer), xylan or hemicellulose (which is primarily composed of pentoses), and lignin, which serves as a supportive frame (a net of connected aromatic compounds). Although lignin is currently not interesting from an economic standpoint (with the exception of thermal applications involving burning), the other two components would be ideal substrates for microbial fermentation after they have been broken down into their monomers (hexose and pentoses). However, the biological decomposition of biomass containing lignocellulose is extremely slow. In order to make industrial application a feasible option, it is necessary to combine chemical and biological processes. Pretreatment involves either physical or chemical approaches, or a combination of the two. These include heat and water (steam explosion and hydrothermal pretreatment), thermochemical disruption, extraction with solvents or ionic liquids, and pretreatments with acid

or ammonia. So far, it has not been possible to develop a process that is ideal in all respects. To name just a few problems, completion of disruption, possibility of separating individual components, sensitivity of the steel walls in the reactor vessels to sulfuric acid (phosphoric acid is less corrosive), and formation of substances that inhibit further fermentation (e.g. furfural, which is formed both from pentoses, with the influence of sulfuric acid, and from carbohydrates, when heated). However, experts predict economic feasibility for the **biorefinery** concept in the near future, which entails converting biomass into important bulk and specialty chemicals. This also applies to plants with a catchment area of around 100 km, taking the respective transportation costs into account. As biomass accumulates on a regular basis and in large amounts, it would present a viable alternative to current ethanol production (as well as production of other important substances such as butanol, ethene, isobutanol, and propanediol) based on food sources.

The second promising substrate is synthesis gas. This gas is a mixture of primarily CO and H_2, with low concentrations of CO_2 and other components such as NH_3, N_2, and H_2S. Synthesis gas is, on the one hand, an important component in the chemical industry; on the other hand, it is also a waste gas, e.g. from steel plants. A number of acetogenic clostridia, e.g. *Clostridium autoethanogenum*, *Clostridium carboxidivorans*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei*, use this gas mixture as their only source of carbon and energy. The typical fermentation product is acetate. By varying the media composition, however, it has been possible to increase the ethanol production to nearly 100 %. Thus, with this process, it has not only been possible to develop an alternative substrate that does not compete with food supplies, but a contribution has also been made to reducing CO and CO_2 pollution in the atmosphere. Demonstration plants are already in operation by the company LanzaTech, and commercial plants are currently being built in China and Belgium, scheduled to start operation in 2018. Biomass might also be used for synthesis gas production, which involves a combination of both alternative substrates. Ethanol is not the only substance that can be produced with these methods. Scientists have also been successful in engineering strains of *C. ljungdahlii* to produce butanol, and natural **2,3-butanediol** production has been verified in *C. autoethanogenum*.

4.3 1,3-Propanediol Synthesis by *Escherichia coli*

4.3.1 Application

1,3-Propanediol is a nontoxic, colorless liquid used to produce the plastic **polytrimethylene terepthalate** (PTT). The manufacturing process involves a polycondensation reaction between 1,3-propanediol and terephthalic acid (Figure 4.3). The polymer PTT is very durable, both mechanically and chemically, and is resistant against stains as well. It feels like wool to the touch and is therefore used in the textile, carpet, and automobile upholstery industries. Until around 2006, 1,3-propanediol was exclusively produced by chemical means. Since then, a biotechnical procedure has been developed, which can be

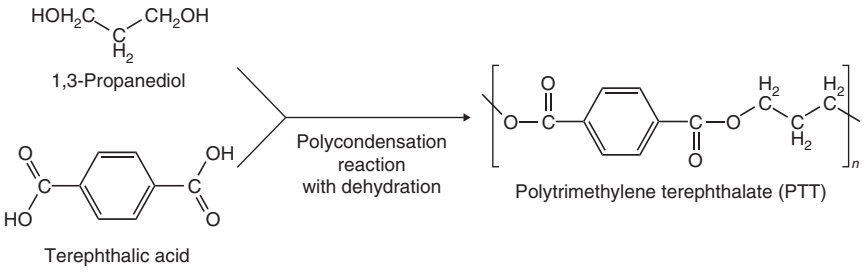


Figure 4.3 Synthesis of polytrimethylene terephthalate from 1,3-propanediol and terephthalic acid.

considered a milestone in the area of targeted strain engineering. The company DuPont manufactures PTT with biologically produced 1,3-propanediol under the brand name Sorona®.

4.3.2 Metabolic Pathways and Regulation

As early as the nineteenth century, 1,3-propanediol was identified as a microbial metabolic end product. Many anaerobic bacteria that ferment glycerol produce 1,3-propanediol in the process. These include the species *Citrobacter*, *Clostridium*, *Enterobacter*, *Klebsiella*, and *Lactobacillus*. The fermentation takes place in an oxidative and a reductive branch. In the oxidative branch, glycerol is initially oxidized to dihydroxyacetone (resulting in the production of NADH), which is converted to dihydroxyacetone-phosphate by ATP hydrolysis. This is channeled via glyceraldehyde-3-phosphate into the glycolysis pathway and converted to pyruvate. In the process, two ATP and one more NADH are produced. Various fermentation products are then formed from pyruvate, depending on the species. In the easiest case, production of lactate by *Lactobacillus*, one NADH is used (Figure 4.4). In this process, the cell creates a net of one ATP, but still needs to reoxidize one molecule of NADH. This is where the reductive branch comes into play. Here, a further molecule of glycerol is converted to 3-hydroxypropionaldehyde by a glycerol dehydratase in a **vitamin B_{12}-dependent reaction**. The reduction with the remaining NADH results in the formation of a molecule of 1,3-propanediol. There is currently only one example of a vitamin B_{12}-independent glycerol dehydratase, an enzyme from *Clostridium butyricum*. An expression in *Escherichia coli*, however, only resulted in minor amounts of 1,3-propanediol.

4.3.3 Production Strains

There have been many attempts to redesign the natural producers of 1,3-propanediol in order to create better production strains. In the past, the comparably high prices involved in using glycerol as a substrate presented an obstacle for commercial implementation. For this reason, the companies Genencor and DuPont began to use glucose as a substrate and engineered an *E. coli* strain, which was able to convert the glucose into large amounts

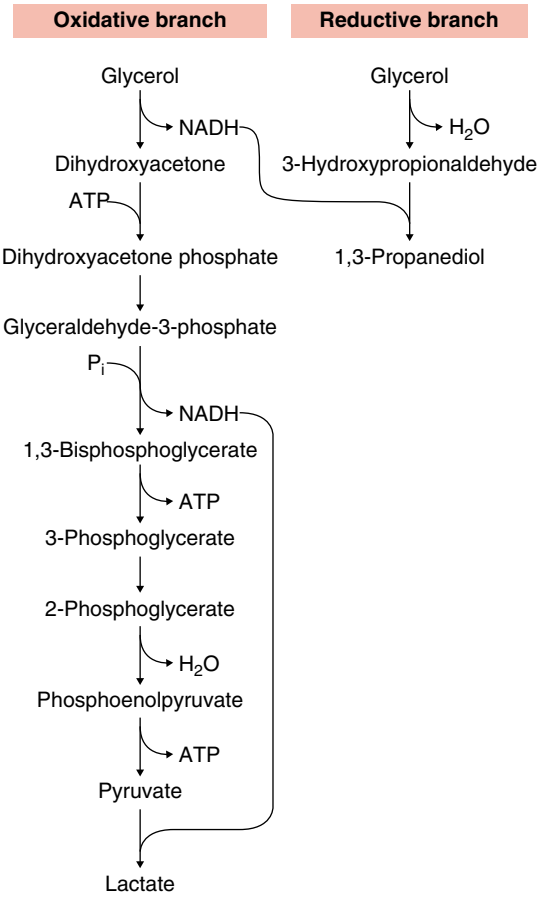


Figure 4.4 Fermentation of glycerol. The fermentation occurs in parallel along an oxidative and a reductive branch. The oxidative branch primarily contains reactions from glycolysis and lactic acid fermentation. 1,3-Propanediol is only produced in the reductive branch.

of 1,3-propanediol. This required more than 30 targeted mutations in the genome, of which only the most important are mentioned here. The natural glucose uptake system of *E. coli* – the phosphoenolpyruvate-phosphotransferase system – was shut down (by inactivation of the gene *ptsG*). Compensation took place via the increased expression of galactose permease (which also transports glucose and is encoded by *galP*) and glucokinase (which leads to the formation of glucose-6-phosphate and is encoded by *glk*). Thus, transport and conversion of glucose were only dependent on ATP at that point, and phosphoenolpyruvate was available in its entirety for the carbon metabolism. A further step was to reduce the expression of glyceraldehyde-3-phosphate dehydrogenase (encoded by *gap*), so that more dihydroxyacetone-phosphate could be formed with triosephosphate isomerase (encoded by *tpi*). This substance was converted by two enzymes stemming from *S. cerevisiae*, glycerol-3-phosphate dehydrogenase (encoded by *DAR1*) and glycerol-3-phosphate phosphatase (encoded by *GPP2*), into glycerol-3-phosphate and then into glycerol. Glycerol decomposition was prevented by inactivating the *E. coli*'s own glycerol dehydrogenase gene (*gldA*). The further conversion of glycerol into 3-hydroxypropionaldehyde was

accomplished by using glycerol dehydratase (encoded by *dhaB1*, *dhaB2*, and *dhaB3*), which stems from *Klebsiella pneumoniae*, as well as its reactivation factors (necessary for the continuous process of radical reaction and encoded by *dhaBX* and *orfX*). The final step of producing 1,3-propanediol required the help of an *E. coli* protein (encoded by *yhdD*) with previously unknown function, which exhibits NADPH-dependent dehydrogenase activity. All of these steps are presented schematically in Figure 4.5.

4.3.4 Production Processes

The company DuPont Tate & Lyle BioProducts, a joint venture of the two companies appearing in the name, has been operating a plant in Loudon (United States)

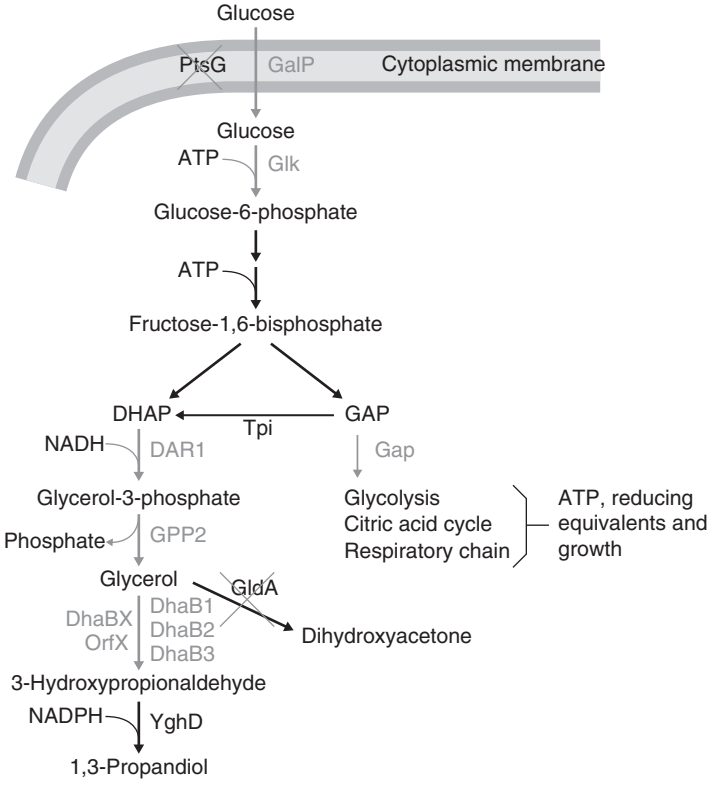


Figure 4.5 Metabolic engineering of an *Escherichia coli* strain to produce 1,3-propanediol from glucose. Grey arrows mark altered reactions, catalyzed by new or modified enzymes (corresponding protein designations in grey). The two grey X marks inactivation of the respective genes. GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone-phosphate. Protein symbols: PtsG, enzyme IICB of the phosphotransferase system; Gal, galactose permease, which also transports glucose; Glk, glucokinase; Tpi, triosephosphate isomerase; Gap, glyceraldehyde-3-phosphate dehydrogenase; DAR1, glycerol-3-phosphate dehydrogenase from *Saccharomyces cerevisiae*; GPP2, glycerol-3-phosphate phosphatase from *S. cerevisiae*; GldA, glycerol dehydrogenase; DhaB1–3, glycerol dehydratase complex from *Klebsiella pneumoniae*; DhaBX, OrfX, reactivation factors of the glycerol dehydratase complex from *K. pneumoniae*; YghD, NADPH-dependent dehydrogenase.

since 2007 for biotechnical production of 1,3-propanediol. This process uses an *E. coli* strain that was engineered according to the abovementioned principles. The feedstock for this fermentation is glucose from corn starch. The plant's capacity is around 63 500 t/yr, which is about half of the annual worldwide production of 1,3-propanediol (a total of roughly 140 500 t/yr in 2013). As the 1,3-propanediol is produced from renewable resources (glucose from corn), DuPont markets the resulting polymer PTT as a renewably sourced plastic, although the terephthalic acid is a product of organic chemical processing from *R*-xylene.

4.4 Butanol and Isobutanol Synthesis by Clostridia and Yeast

4.4.1 History of Acetone–Butanol–Ethanol (ABE) Fermentation by *Clostridium acetobutylicum* and *C. beijerinckii*

At the beginning of the twentieth century, there was an increasing demand for rubber. The raw material for producing rubber was only available from rubber trees (*Hevea brasiliensis*), which were native only to South America. New research projects were thus initiated, with the aim of developing artificial rubber. The English company Strange & Graham recruited scientists Fernbach and Schoen from the Institut Pasteur in Paris and Perkins and **Weizmann** from the University of Manchester. Their basic plan was to use fermentation processes to produce either isoamyl alcohol or butanol, which would then be chemically processed into isoprene or butadiene – the precursors for artificial rubber. Fernbach and Strange submitted two patents in 1911 describing the production of alcohols from mixed cultures. The analytical methods at that time were not yet that advanced; promising cultures were identified by sniffing the vessels. The problem was that a mixture of butanol and acetone smells similar to isoamyl alcohol. This project was not pursued any further as, at this point, large supplies of natural, pure rubber were brought onto the market. Englishman Henry Wickham smuggled *Hevea* seeds out of Brazil in 1876 and started plantations in the former colony of Ceylon (today Sri Lanka), the products of which entered the market in 1909. Weizmann, however, continued to pursue his research independently and was eventually successful in isolating a bacterium (as a pure culture) that produced high concentrations of butanol and acetone – later named ***Clostridium acetobutylicum***.

In 1914, the World War I erupted. It quickly became evident that Great Britain had ammunition problems. In November 1914, the German East Asia Squadron, under the command of Vice Admiral Maximilian Graf von Spee, sank the English battle cruisers Good Hope and Monmouth, which were under the command of Admiral Sir Christopher Cradock, near Coronel off the coast of Chile. The outcome of the battle was significantly influenced by the fact that the English ammunition was of poor quality and that their grenades did not have an adequate range. In order to produce ammunition, the critical component at that time was acetone. This substance was produced from calcium acetate, which was primarily produced in the former double-monarchy Austria-Hungary, which meant that the English did not have access to it. Weizmann's *C. acetobutylicum* proved to be a suitable substitute for acetone production, using starch as a substrate

(from potatoes, grains, and even chestnuts). In addition to factories in England, further plants were built in Canada and, after they had entered into the war, in the United States. Weizmann refused any personal honors, but made clear that he was in favor of a Jewish state in Palestine. There is no doubt that his achievements significantly contributed to the issuance of the Balfour Declaration in 1917, advocating a national home for the Jewish people. The state of Israel emerged, and Weizmann became its first president. Not many other bacteria have made such a significant political impact.

Following the cease fire in 1918, biotechnical production of acetone and butanol was discontinued. The coproduced butanol was stored in large tanks, as there was no use for it during the war. In 1920, however, the United States introduced the prohibition. This meant that not only was ethanol not being produced but amyl alcohol was no longer available either. Amyl alcohol was used to produce amyl acetate, an ideal solvent for fast-drying paints. At the same time, Henry Ford dramatically stepped up automobile production with his introduction of the assembly belt, and he was therefore dependent on large amounts of paint for his vehicles. Butanol, as the source material for butylesters, provided the solution to this problem. As a consequence, the abandoned fermentation plants were reactivated and additional facilities were erected as well. ABE fermentation is one of the largest biotechnical processes that has ever been conducted in the world, second only to ethanol production. The most significant plant was in Peoria (United States), with 96 fermenters, each having a volume of 189 000 l. Roughly, two thirds of the world's supply of butanol and 10% of the acetone supply were provided by means of fermentation. The decline began around 1950, as the applied substrates (molasses) continued to increase in price while chemical synthesis from crude oil became less and less expensive. Only politically isolated countries, or countries with a small supply of raw materials, such as South Africa (Chapter 1) and China, continued to stick to the traditional ABE fermentation for several decades.

4.4.2 Application

Butanol is primarily used to produce butylacrylate- and -methacrylate esters, butylacetate, butylamines, butylglycol, and amino resins. In 2013, the most important components were butylacrylate, with a market share of 38%, butylacetate with 24% and glycolether with 15%. Further applications include use as solvents in paint, extracting agents for drugs and naturally occurring substances (e.g. alkaloids, antibiotics, hormones, camphor, and vitamins), additives in cleaning agents, eluents for chromatographic procedures, and additives in polishes. Butanol also serves as a raw material for various cosmetic products, hydraulic and brake fluids, lubricants, flotation chemicals, and products for leather and paper processing.

A large portion of the manufactured isobutanol is used as a solvent in the painting industry. Additional applications include use as a basic material for further chemical syntheses, as an extracting agent, as an additive for de-icing agents, cleaning agents and polishes, and as an eluent for chromatographic procedures.

Experts predict a large market for butanol and isobutanol as biofuel in the future. In comparison to ethanol, these two substances have characteristics much more similar to those of gasoline (Table 4.2). Butanol and isobutanol are much less hygroscopic, which means they can already be mixed with gasoline in the refinery and existing pipelines can then be used to transport the fuel. The risk of corrosion is significantly reduced as well. Furthermore, butanol and isobutanol have a higher energy content (i.e. more kilometers can be driven per liter of fuel) and a lower steam pressure (safer handling).

4.4.3 Metabolic Pathways and Regulation

The fermentation pathway for glucose is presented schematically in Figure 4.6. It is important to point out, however, that bacterial growth is divided into two phases. In the first phase, the **acidogenic phase**, the predominant products are the acids butyrate and acetate (at a ratio of approximately 2 : 1). Other products include CO_2, H_2, and some ethanol. In the subsequent phase, the **solventogenic phase**, the acids are, for the most part, resorbed and converted into butanol and acetone (Figure 4.7). The butanol:acetone:ethanol ratio at the end of the fermentation is roughly 6 : 3 : 1. The fermentation products that are only produced in small amounts or under certain conditions are acetoin and lactate. Acetoin is produced from two molecules of pyruvate (via acetolactate), and lactate is formed from a molecule of pyruvate. Some strains of *Clostridium beijerinckii* can also reduce acetone to isopropanol.

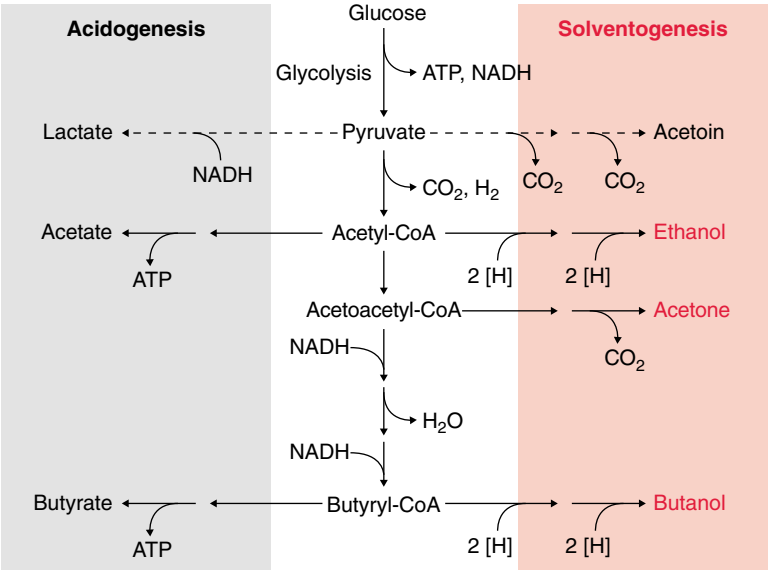


Figure 4.6 Metabolic pathway of acetone–butanol–ethanol fermentation. The reactions during glycolysis and formation of acetoacetyl-CoA are not displayed in stoichiometric terms. Acidogenesis takes place during the exponential growth phase and solventogenesis during the transition to the stationary growth phase. The resulting solvents are depicted in red.

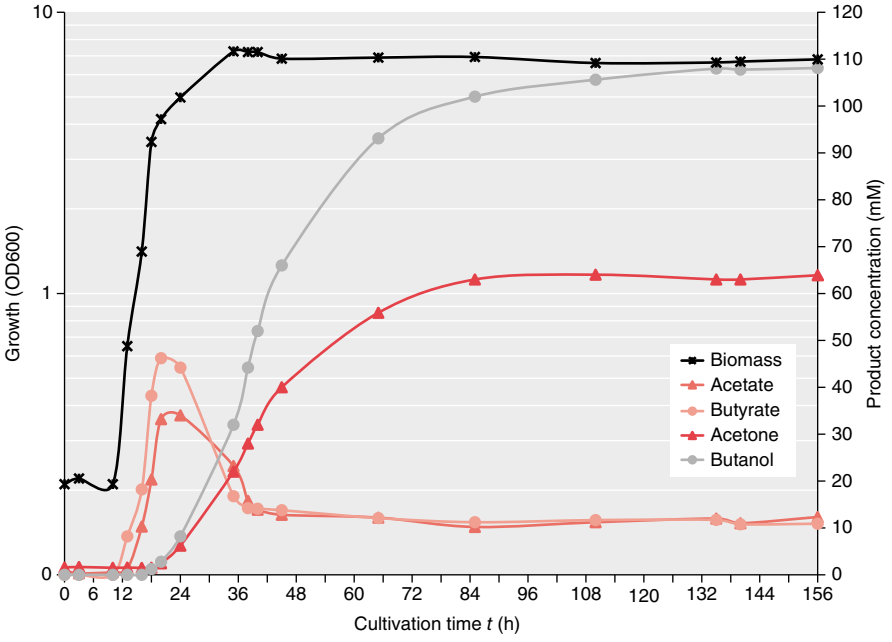


Figure 4.7 Formation of products during acetone–butanol–ethanol fermentation. The graph depicts the fermentation progression of a batch culture in a complex medium. The increase in biomass was followed by optical density (OD at 600 nm). Gas chromatographic analysis was used to measure the products acetate, acetone, butanol, and butyrate. Source: Courtesy of Thiemo Standfest, University of Ulm.

Converting acids into neutral products during the second growth phase provides the bacteria with a physiological advantage. Anaerobic microorganisms cannot, in general, conduct pH homeostasis. Their intracellular pH decreases in accordance with the external pH and is roughly one unit more alkaline. If more acids are produced, the external pH level decreases. The sourer the environment is, the greater the number of acid anions that are converted to undissociated free fatty acids. These can diffuse into the cell cytoplasm through the cytoplasmic membrane and then dissociate into acid anion and proton because of the higher intracellular pH level. This leads to a collapse of the proton gradient across the membrane and thus to cell death. Converting the acids into neutral products prohibits this effect. This, however, is only a temporary solution, as acetone, butanol, and ethanol are also toxic (butanol has by far the greatest toxic effect). Ultimately, *C. acetobutylicum* simply buys itself some time in order to engage in active metabolism longer than other nutrient competitors. That is to say, the long-term survival system of endospore formation is induced at the same time as solvent formation. Such endospores are the most resistant type of cells known today. One report claims successful germination of *Bacillus* spores from salt crystals originating from a layer that is 250 million years old. As soon as environmental conditions are again favorable enough to permit adequate growth, the spores can germinate into vegetative cells, and the cycle begins anew.

It is therefore no surprise that there is a regulatory connection between solvent formation and **sporulation**. The decisive transcription factor for these two

processes is Spo0A, a so-called response regulator, which can be phosphorylated on an aspartate residue and then induce the expression of genes, via histidine kinases (in *C. acetobutylicum*, there are currently three known interaction partners). Butanol is produced during the second, solventogenic phase (Figure 4.7). To this end, there is a strong induction at the level of transcription with the *sol* operon residing on a megaplasmid (contains the genes for bifunctional butyraldehyde/butanol dehydrogenase and acetoacetyl-CoA: butyrate/acetate coenzyme A transferase) as well as both of the chromosomally localized *bdhA* and *bdhB* operons (each encodes a gene for a butanol dehydrogenase). However, the regulatory network is much more complex and not yet fully understood. Further transcription factors are also involved, as is a small regulatory RNA.

In 2011, scientists were successful for the first time with a butanol production in a recombinant strain that even exceeds the concentration achieved by natural producers. This was accomplished by heterologously expressing clostridial genes in *E. coli* and replacing the clostridial butyryl-CoA dehydrogenase with the *trans*-enoyl-CoA reductase from *Treponema denticola*, a Gram-negative spirochete bacterium.

The biotechnical production of isobutanol involves a completely different pathway. Although it also forms naturally in alcoholic fermentation under specific conditions, only trace amounts are produced. A potential use did not become possible until **synthetic biology** was applied. Groundbreaking work in the laboratory of James C. Liao at the University of California in Los Angeles took advantage of the very active amino acid biosynthesis metabolism of *E. coli* and converted the 2-ketoacid intermediates via decarboxylation and reduction into alcohols. In the case of isobutanol, the actual valine biosynthesis pathway is modified (Figure 4.8). The overexpression of *E. coli* enzymes IlvC and IlvD in

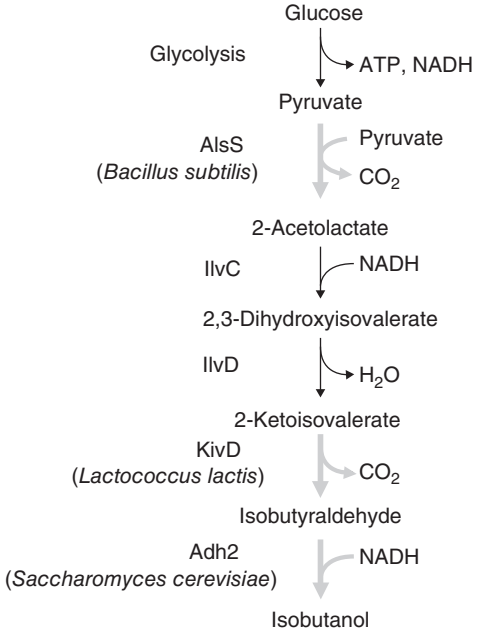


Figure 4.8 Metabolic engineering of isobutanol-producing microorganisms. This diagram depicts a design process using *Escherichia coli* as the host strain. Reactions marked in grey are catalyzed by enzymes that were introduced from foreign microorganisms by means of genetic engineering. AlsS, acetolactate synthase; IlvC, acetohydroxy acid isomeroreductase; IlvD, dihydroxy-acid dehydratase; KivD, 2-ketoisovalerate decarboxylase, Adh2, alcohol dehydrogenase.

combination with the *Bacillus subtilis* enzyme AlsS leads to increased conversion of two molecules of pyruvate into 2-ketoisovalerate, which is then converted into isobutanol via a 2-ketoisovalerate decarboxylase from *Lactococcus lactis* (KivD) and an alcohol dehydrogenase from *S. cerevisiae* (Adh2).

4.4.4 Production Strains

The biotechnical production of butanol is currently being conducted using the classical production strains (*C. acetobutylicum*, *C. beijerinckii*, *Clostridium saccharobutylicum*, and *Clostridium saccharoperbutylacetonicum*). The source substrates are primarily sugar cane, corn, and cassava (manioc). With the latter, the starch is broken down into glucose units via the clostridial own amylases. The strains can also use pentoses. Initially, *C. acetobutylicum* was isolated on starchy media and the other strains on sugary substrates. This is also in part reflected in industrial use. In the laboratory, volumetric productivities of up to 0.9 g butanol/1/h were achieved. For solvents altogether (acetone + butanol + ethanol), 1.5 g/1/h was reached.

In the case of isobutanol, genetically manipulated yeast strains are currently being used. The engineering principle corresponds to the approach described for *E. coli* in Section 4.4.2.

4.4.5 Production Processes

In 2010, approximately 3 million tons of butanol were produced (10–25% of which was isobutanol). The production processes were, for the most part, petrochemical. Fermentation plants for butanol are found in three countries: Brazil, China, and the United States. In the state of Rio de Janeiro in Brazil, a new plant was erected in 2006 in the direct vicinity of a sugar mill and an ethanol fermentation plant (Figure 4.9). This plant's manufacturing process is based on sugar cane and employs saccharolytic, solventogenic, clostridial strains. Precultures are cultivated on two levels to inoculate the eight fermenters, each with a nominal volume of 350 m^3. A production diagram is shown in Figure 4.10.

Figure 4.9 Butanol fermentation plant in Brazil. Source: Courtesy of Prof. Dr. David T. Jones, University of Otago, New Zealand.

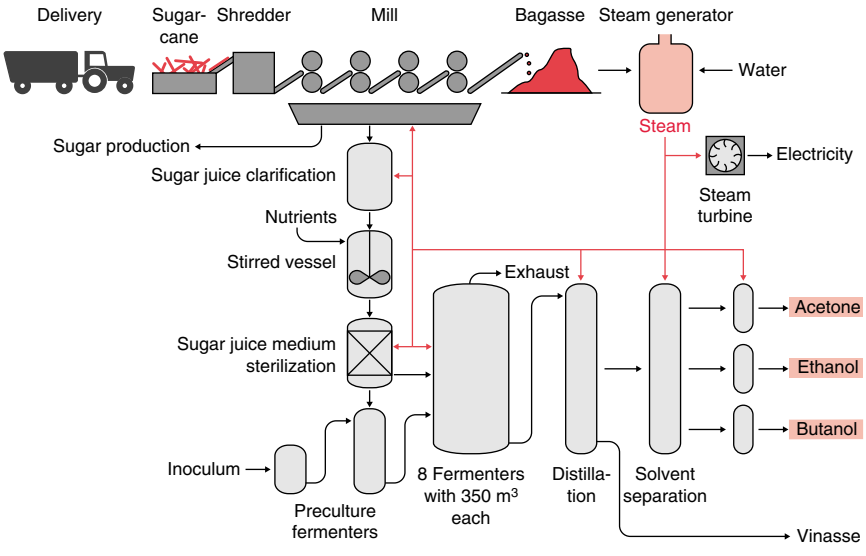


Figure 4.10 Production diagram of the butanol fermentation plant in Brazil. Bagasse is the fibrous waste that results from sugar cane following the grinding process, and which is burned to create steam. Vinasse is the liquid waste resulting from distillation. Source: Courtesy of Prof. Dr. David T. Jones, University of Otago, New Zealand.

In China, there are 13 plants. The three largest ones are Jilin Ji'an Biochemical Co. with a capacity of 150 000 t/yr, Jilin Cathay Industrial Biotechnology Co. (100 000 t/yr), and Jiangsu Lianhai Biological Technology Co. (70 000 t/yr). In 2008, a total of 550 000 tons of butanol was produced in China by means of fermentation. The largest of the aforementioned plants was built in 2007. It has three production lines, with 32 fermenters each, and each fermenter has a volume of 450 m^3. A private power plant and a distillatory complete the facility. The substrate in all plants has largely been corn, and some cassava (manioc). Because of governmental regulations, however, substances that can serve as food supplies can no longer be used and, thus, plants were closed. Instead, more testing is being done on the suitability of vegetable waste material resulting from corn production, and plants are beginning to use it. Lignicell Refining Biotechnologies Ltd., Songyuan, China (formerly Laihe Rockley Bio-chemicals Co. Ltd.), is currently the only commercialized cellulosic biobutanol factory.

The UK-based company Green Biologics retrofitted their commercial scale ethanol facility in Little Falls, MN, USA, for butanol fermentation from corn, using clostridial strains. The plant has a capacity of approximately 63 000 tons and will ramp up production through 2017/2018.

Traditional ABE fermentation is currently operating in the margin of profit (depending on the current oil price), provided that the products go into further processing in the chemical industry. It is not yet economically feasible to use the products as a biofuel. Experts predict, however, that this will change in the near future because of the increasing availability of genetically manipulated strains, alternative substrates, and improved isolation techniques.

The company Gevo has already taken a significant step in this direction. They operate a demonstration facility using recombinant yeast strains (and thus based on ethanol fermentation) for producing **fermentative isobutanol** in St. Joseph, USA, and started a production facility in Luverne, USA (capacity 2243 t in 2016, while simultaneously producing approximately 15–17-fold more ethanol). The large number of companies active in the area of biotechnical butanol/isobutanol production (Table 4.3) demonstrates its great industrial potential.

Table 4.3 Companies involved in biotechnical production of butanol and isobutanol.

Company	Products	Business model
Arbor Fuel Inc. (the United States)	Ethanol, butanol	Developing microorganisms to convert biomass into biofuels
Butalco GmbH (Switzerland) (belonging to Lesaffre group)	Butanol	Developing technologies for production of biofuels and biochemicals, use of pentoses, yeast as production organism
Butamax™ Advanced Biofuels LLC (the United States) (joint venture of BP and DuPont)	Butanol	Developing comprehensive licensing package for retrofitting current ethanol operations
Butrolix, LLC (the United States)	Butanol	Using quorum-sensing peptides to improve butanol fermentation from low-cost feedstock sugars
Celtic Renewables (Scotland, UK)	Butanol, ethanol, acetone, animal feed	Draff and pot ale, by-products of the Scotch whisky industry, are used as substrates in ABE fermentation
Eastman Chemical Company (the United States) (acquired TetraVitae Bioscience, Inc. in 2011)	Butanol	Developing a bio-based production route for butanol
Gevo (the United States)	Isobutanol, ethanol	Carbohydrate-based fermentation using metabolically engineered yeast strains
Green biologics (United Kingdom) (in 2012 merger with ButylFuel Inc., USA)	Butanol, acetone (and their derivatives)	Commercial production of butanol and acetone at US plant, strain development
METabolic EXplorer (France)	1,3-Propandiol, butanol	Developing production strains by metabolic engineering
Phytonix Co. (the United States)	Butanol, pentanol	Engineered cyanobacteria used for alcoholol formation from CO_2

4.4.6 Product Toxicity

As previously mentioned, butanol has a harmful effect on producer cells, as do acetone, ethanol, and isobutanol. In general, an alcohol's toxic effect on bacterial membranes increases significantly with a growing carbon chain. Membrane proteins also become damaged. In the case of butanol, the ATPase of *C. acetobutylicum* appears to be one of the enzymes that is first inactivated. The cells protect themselves from negative influences on the membrane by altering their lipid composition. During solvent production, the ratio of acidic to neutral phospholipids rises and the percentage of alkenylether lipids decreases. These alkenylether lipids are glycerol lipids, which contain a long-chain enol (primarily a C_{19}-cyclopropane side group), connected in ether linkage to the C_1 of glycerol. An external addition of butanol, however, results in a significant increase in saturated fatty acids in comparison to unsaturated fatty acids.

A common approach to strain optimization is to search for **tolerant strains**. The typical procedure for identifying suitably mutated strains is to check the growth in the presence of increasing concentrations of the respective substance. The ideal strain is then the one with the best reported growth at the highest tested concentration. Caution must be exercised with this method, however. Experiments with *C. acetobutylicum* have shown that such a procedure can lead to false conclusions. An external addition of butanol led to a growth inhibition of 50% at concentrations as low as between 100 and 150 mmol/l. Since then, however, strains have been found that can produce more than double the amount of butanol. Thus, a dramatic difference becomes evident when comparing the physiological behavior of cells that produce increasing amounts themselves and those that perceive an external stimulus as stress and react differently to it (such as with the changes in lipid composition). Furthermore, strains with a higher butanol tolerance (isolated following an unspecific mutation) produced a significantly lesser amount of alcohol than the original strain. Selective approaches to lipid changes in the membrane (via overexpression of certain genes) that are aimed at increasing butanol tolerance have so far only resulted in strains with significantly lower productivity.

4.5 Acetone Synthesis by Solventogenic Clostridia

4.5.1 Application

Acetone is utilized in the chemical industry for producing acetone cyanhydrin/methyl methacrylate (to make Plexiglas®), bisphenol, isophorone, methyl isobutyl ketone, and methyl isobutyl carbinol (4-metyl-2-pentanol). It is also used as a solvent, nail polish remover, cleaning agent, and adhesive.

4.5.2 Metabolic Pathways and Regulation

The biotechnical production of acetone involves ABE fermentation. As previously mentioned, the predominant substrates are sugar cane, corn, and cassava

(manioc). Acetone, such as butanol, is produced in the second solventogenic phase (Figure 4.7). In this case, the *sol* operon (contains the genes for a bifunctional butyraldehyde/butanol dehydrogenase and acetoacetyl-CoA: butyrate/acetate coenzyme A transferase) and the *adc* operon (contains the gene for the acetoacetate decarboxylase) are strongly induced at the transcriptional level.

Typically, acetone production in *C. acetobutylicum* is subjected to the same pattern as butanol production. Under certain conditions, however, acetone synthesis is dramatically suppressed (down to a butanol/acetone ratio of 100 : 1). A fermentation of this kind was observed with whey being used as a substrate. Whey is a waste product in the cheese production process, has a high lactose content, and is a comparatively inexpensive feedstock. Acetone production is also suppressed when a substrate mixture consisting of glucose and glycerol is applied (glycerol alone cannot be fermented by *C. acetobutylicum*, a cosubstrate is required). This leads to the so-called **alcohologenic fermentation**, in which the primary products are butanol and ethanol.

The basic regulatory mechanisms are not yet completely understood. Spo0A is involved, as are other transcription factors, which in part belong to new regulator families.

4.5.3 Production Strains

As with butanol, the classical production strains serve for producing acetone: *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum*. Scientists have also been successful in forming acetone with a synthetic operon in other bacteria, such as *E. coli*. For this purpose, three genes of *C. acetobutylicum* are combined into a new unit: *ctfA*, *ctfB*, and *adc*. The two genes *ctfA* and *ctfB* encode the two subunits of acetoacetyl-CoA: butyrate/acetate coenzyme A transferase, which converts acetoacetyl-CoA into acetoacetate and the corresponding CoA-derivative (butyryl-CoA or acetyl-CoA) with the help of either butyrate (preferably) or acetate. Acetoacetate is then converted by acetoacetate decarboxylase, the gene product of *adc*, into acetone and CO_2. In such strains, however, acetone production is dependent on a cosubstrate, to which the CoA moiety can bind (e.g. acetate, which then becomes acetyl-CoA). An approach from the field of synthetic biology has replaced the CoA transferase with a thioesterase from *B. subtilis*. Here, hydrolytic cleavage of coenzyme A (CoA) occurs, and acetoacetate is produced directly. A pathway like this has not yet been observed in nature.

4.5.4 Production Processes

In 2015, approximately 6.1 million tons of acetone was produced, the majority of which was produced by petrochemical means. Ninety-seven percentage of the world's acetone production is a by-product of phenol synthesis. Acetone produced with biotechnical methods stems from the ABE fermentation.

4.6 Outlook

Mobility is without a doubt a supporting pillar in the global economy, and private transportation plays an important role. For many decades to come, we will continue to be dependent on liquid fuels, until perhaps one day, batteries and fuel cells become available across the board for vehicular traffic. However, even then, airplanes will continue to rely on liquid fuels. The Earth's oil reserves, however, are limited, and they currently supply the majority of the raw materials for the chemical industry. As already discussed in Section 4.2.6 on alternative substrates for ethanol fermentation, ethanol from food sources is only an option for countries such as Brazil. For the short or long term, a conversion to lignocellulosic hydrolysates or synthesis gas is desperately needed. Although biofuels can only make a limited contribution to vehicular traffic, they still have the potential to become very important, especially in the area of air traffic. This prediction obviously does not apply to ethanol (risk of icing), but to substances such as butanol and isobutanol as well as the substances produced from them (in WWII, the Royal Air Force and the Japanese Empire already used butanol for airplane fuel).

The success in the field of synthetic biology over the past few years has demonstrated that a series of important bulk components for the chemical industry can be produced affordably and from renewable raw materials. A rapid development most certainly awaits us in this field. The large amounts of glycerol that accumulate during the process of bio diesel production make it possible to imagine 1,3-propanediol production with *C. butyricum* as a potential affordable alternative. Scientists have been successful in producing isobutanol directly from cellulose by *Clostridium cellulolyticum* with the described genetic manipulations. Now, the key is to improve productivity. 2,3-Butanediol is also a very interesting intermediate for producing methyl ethyl ketone (MEK, another term is 2-butanone), which is another important bulk chemical. In 2008 and 2011, it was also possible to produce hexanol with biotechnical means for the first time using different biosynthetic pathways. In these cases as well, the key is to optimize productivity. Developments in this area will continue to emerge throughout the foreseeable future and beyond. Synthetic biology is the decisive key technology for supplying the chemical industry with raw materials as the world's supplies of petroleum abate.

Further Reading

Atsumi, S., Hanai, T., and Liao, J.C. (2008). Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature* 451: 86–89.

Behr, A., Kleyensteiber, A., and Hartge, U. (2010). Alternative Synthesewege zum Ethylen. *Chem. Ing. Tech.* 82: 201–213.

Dürre, P. (2007). Biobutanol: an attractive biofuel. *Biotechnol. J.* 2: 1525–1534.

Geddes, C.C., Nieves, I.U., and Ingram, L.O. (2011). Advances in ethanol production. *Curr. Opin. Biotechnol.* 22: 312–319.

Köpke, M., Mihalcea, C., Bromley, J.C., and Simpson, S.D. (2011). Fermentative production of ethanol from carbon monoxide. *Curr. Opin. Biotechnol.* 22: 320–325.

Nakamura, C.E. and Whited, G.M. (2003). Metabolic engineering for the microbial production of 1,3-propanediol. *Curr. Opin. Biotechnol.* 14: 454–459.

5

Organic Acids

Michael Sauer and Diethard Mattanovich

BOKU University of Natural Resources and Life Sciences, Department of Biotechnology, Muthgasse 18, 1190 Vienna, Austria

CHAPTER MENU

5.1 Introduction

Organic acids are central players within the metabolism of all living cells (see Figure 5.1 for an overview). They are also prominent products accumulated by many microorganisms. The importance of organic acids in the biological world is based on the one hand on the wide variety of chemical reactions directly involving the carboxyl group and on the other hand on the buffering or acidifying capacity of this group, exhibiting the potential to modify the cell's chemical surroundings.

The same reasons that make many organic acids important molecules in the cell render them useful in many other contexts, such as food production or the chemical industry. Indeed, biogenic acids – many of them are of microbial origin – are longtime companions of humankind. First, large-scale productions were food-related fermentations, where the acids have never been obtained in pure form. Examples are fermented vegetables such as sauerkraut or kimchi, which rely on the production of lactic acid by lactic acid bacteria. Another example is vinegar production from sweet juices. First, the sugar is fermented by yeasts, and then the produced ethanol is oxidized by acetic acid bacteria to acetic acid, which gives the characteristic taste and provides the antimicrobial activity, which is sought after.

Industrial Microbiology, First Edition.
Edited by David B. Wilson, Hermann Sahm, Klaus-Peter Stahmann, and Mattheos Koffas.

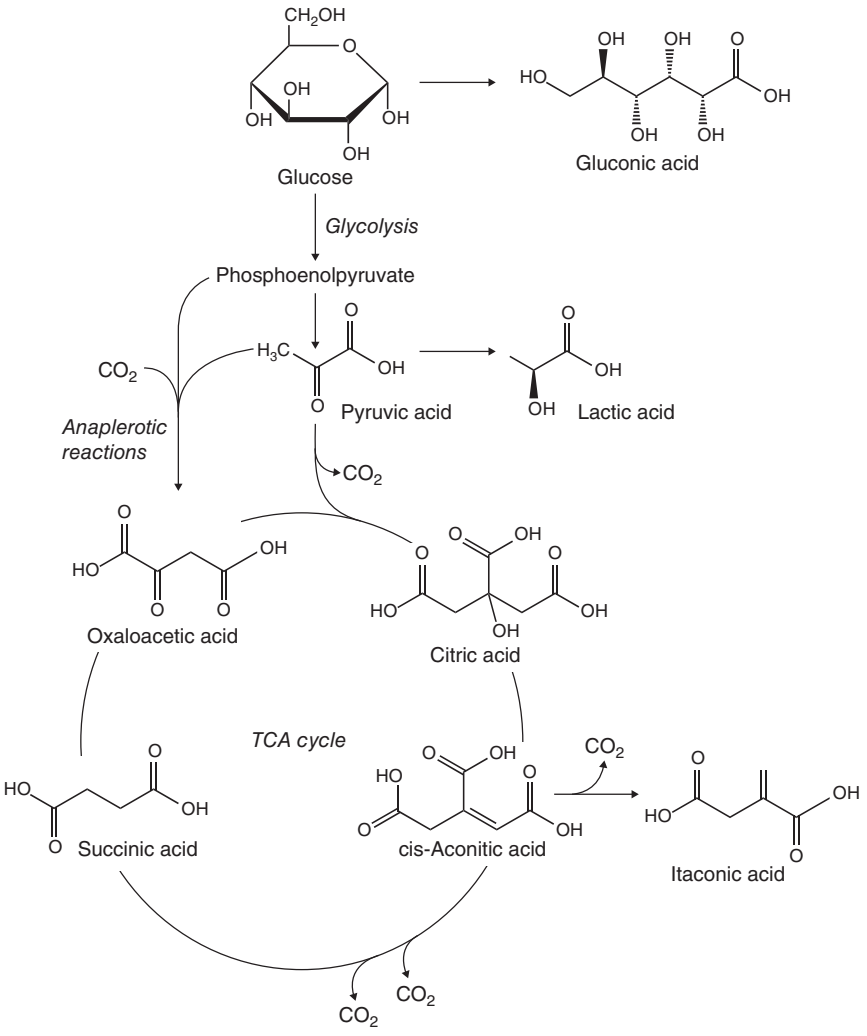


Figure 5.1 Overview of metabolic pathways to industrially relevant organic acids. Glucose is converted via glycolysis to pyruvate, a central metabolic node of primary metabolism. Pyruvate is converted to lactic acid by lactate dehydrogenase or to citric acid and succinic acid via the TCA cycle and anaplerotic reactions (pyruvate and PEP carboxylase). Itaconic acid is a product from decarboxylation of the TCA cycle intermediate cis-aconitate. Alternatively, glucose is also directly oxidized to gluconic acid.

Industrial production processes of microbially produced organic acids in isolated form have been developed from the beginning of the twentieth century onward. The major success story is the production of citric acid with the filamentous fungus *Aspergillus niger*. With current production of about two million tons, citric acid is still one of the major biotech products in the market. Microbial production substituted the extraction from lemons from 1923 based on a seminal paper published by James N. Currie in 1917. Citric acid was – like all

Table 5.1 Overview about market volume of microbially produced organic acids (data obtained 2017).

Organic acid	Market volume (yr^{-1})
Citric acid	2 000 000 t
Lactic acid	750 000 t
Gluconic acid	100 000 t
Succinic acid	30 000–100 000 t[a)]
Itaconic acid	80 000 t

a) The capacity of production is higher than the amount that is seemingly produced. Information about actual production is not available.

of the early microbially produced organic acids – used in the food and beverage industry. Recently, the industrial interest in such products derives not only from nutritional needs but also different acids find more and more diverse applications, such as in cosmetics industry or as polymer precursors in the chemical industry. Table 5.1 summarizes some of the organic acids, which are currently produced by microbial fermentation.

5.2 Citric Acid

Already the name citric acid tells the story from where it has been isolated for the first time – from citrus fruits. The German–Swedish chemist Carl Wilhelm Scheele isolated this three-basic acid from lemon juice in the end of the eighteenth century. Since the beginning of the nineteenth century, citric acid was produced industrially from lemons and found applications in the food and beverages industry. The demand was steadily increasing, but the supply was exclusively based on the Italian citrus industry, leading to high prices. The interest to find other sources of the acid was therefore high. In the beginning of the twentieth century, scientists discovered and described certain filamentous fungi, readily accumulating citric acid when growing on sugar. The first industrial plant that produced citric acid from *A. niger* was opened in Belgium in 1919. True large-scale production commenced in the United States in 1923. Initially, industrial-scale production of citric acid relied on surface fermentations. The fungi were grown on a static medium in trays, forming a mycelial mat. Separation of such a mat from the liquid medium is easy – allowing for an efficient purification of the acid. Another possibility for fungal production processes is solid fermentation – the solid substrate is acting as a carrier and a nutrient source at the same time in this case. However, modern processes for citric acid production with *A. niger* are based on submerged cultivation of the fungus in stirred vessels. Scale-up of such processes to 1000 m^3 is possible. Such scale-up allows for higher productivity and yield and reduces the risk for contamination compared to solid surface fermentation.

5.2.1 Economic Impact and Applications

About 2 000 000 tons of citric acid are produced every year. This makes this compound one of the major biotech products on the market, by volume. The main use of citric acid (70%) is in the food and beverage industry, particularly for soft drinks, but also jams. Citric acid is added as a buffering, flavoring, and preservative agent. Because of its pleasant taste and property of enhancing fruity flavors combined with very low toxicity, it is the most versatile and widely used acidulant. Citric acid is also a chelating agent, efficiently retaining iron – among other metal ions – in aqueous solution. This property is exploited in the pharmaceutical industry, which uses about 20% of the annual production. The same property allows its use in detergents in order to lower the water hardness.

5.2.2 Biochemistry of Citric Acid Accumulation

Major triggers for citric acid production by *A. niger* are a high substrate concentration at the beginning of the process and a high dissolved oxygen concentration throughout the process.

A high substrate concentration is needed to trigger a fast substrate uptake and consequently a high glycolytic flux. The hypothesis is that a high glycolytic flux leads to unfavorably high intracellular concentrations of carboxylic acids, which in turn leads to citric acid excretion. A central enzyme for citric acid production is citrate synthase, which is localized in the mitochondria (see Figure 5.2

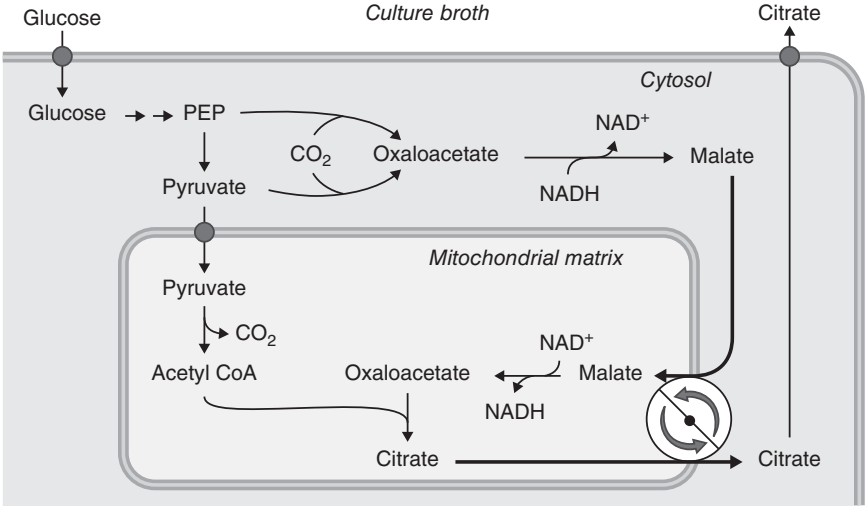


Figure 5.2 Membrane transport processes involved in citric acid formation by *Aspergillus niger*. Glucose is taken up by sugar transporters and converted to pyruvate, which enters mitochondria where it is decarboxylated to acetyl-CoA by pyruvate dehydrogenase. Citrate formation and excretion depletes the TCA cycle, which is refilled by cytosolic formation of oxaloacetate by pyruvate and PEP carboxylases. Oxaloacetate cannot pass the mitochondrial membrane, so it is reduced to malate that is transported into the mitochondrial matrix with an antiporter, transporting equimolar amounts of citrate out of mitochondria into the cytosol. Citrate is transported out of the cells by a transport protein.

for a schematic overview). Although this enzyme is central for the citric acid cycle, the cycle itself clearly plays no role for citric acid production. The main task of the citric acid cycle is to oxidize C_2-units to CO_2 to obtain reduction equivalents to feed into the respiratory chain. Citric acid is one player in the cycle but needs to be retained within the cycle to keep it going. To reroute citric acid to extracellular accumulation, the anaplerotic reaction of pyruvate carboxylase is central, carboxylating pyruvate to oxaloacetate, using one ATP for energy provision. Oxaloacetate is the substrate of citrate synthase, which catalyzes the formation of citrate from oxaloacetate and acetyl-CoA. Acetyl-CoA is also produced from pyruvate by decarboxylation. Therefore, one carbon dioxide molecule is lost on this branch of the pathway, but another carbon dioxide is fixed on the other branch, resulting in a closed carbon balance, forming one molecule of citric acid from one molecule of glucose, via two molecules of pyruvate. Overall, the formation of citric acid from glucose is an oxidative process leading to an excess of electrons in the form of NADH. This is the basis for the high dissolved oxygen requirement of the process. Under normal conditions, this NADH is used to feed the respiratory chain for ATP provision or it is used for anabolic reactions for cellular growth. However, under citric acid production conditions, the fungi hardly grow. They get rid of the excess NADH by an efficient, but in a sense biochemically unproductive, reoxidation of the glycolytically produced NADH by an alternative oxidase. This enzyme catalyzes the reduction of oxygen to water without coupling to ATP production. Instead of ATP, heat is produced requiring efficient cooling of the bioreactors. This excess heat is quite massive – approximately 670 kJ/mol citric acid (3500 kJ/kg) – and can pose a technical problem for citric acid producers. Given a volumetric productivity of 2 g/l/h, 3500 MJ/h need to be removed from a 500 m^3 bioreactor, which is a challenging task, especially during summer. An unwanted drop in oxygen tension during the cultivation would force the microorganisms to shift from the alternative oxidase – which requires very much oxygen – to the main (cytochrome) respiratory pathway, which requires less oxygen. Excess ATP would then trigger other carbon-related reactions than citric acid production, leading to a failed process.

There is one more important point regarding the biochemistry of this process: oxaloacetate is formed in the cytosol of the cell, while citrate synthase, the enzyme metabolizing it, is located in the mitochondria. Curiously, there is no transport protein, capable of oxaloacetate uptake into the mitochondria. The transport protein used is an antiporter, exchanging citrate with malate. That is: malate is imported and citrate exported. To obtain malate, oxaloacetate has to be reduced in the cytosol and malate is reoxidized in the mitochondria. This antiport system is an important player for our understanding, why the fungus overproduces citric acid so efficiently. The transport can be regarded as a revolving door. Excess citrate from the mitochondria needs to be exported, but for each citrate molecule, one malate enters the mitochondria, which in turn is converted into citrate and so on.

Curiously, the precise transport mechanism, which allows the export of citric acid from the cytosol out of the cell (therefore against the concentration gradient), has not been described in the scientific literature until now.

5.2.3 Industrial Production by the Filamentous Fungus *Aspergillus niger*

Modern industrial processes for citric acid production with *A. niger* are submerged batch cultures lasting for five to six days in a scale from 100 to 500 m^3 (up to 1000 m^3). Figure 5.3 shows a typical production scheme. The carbon sources are sucrose (molasses) from sugar beet or sugar cane, or glucose from starch hydrolysate. The final titer reaches 150–200 g/l, the yield approaches 95%, and the volumetric productivity can exceed 2 g/l/h.

The cultures are inoculated with conidia, which have been obtained on solid media. To trigger the production, a high substrate concentration is essential. At the same time, phosphate and nitrogen must be limiting the growth of the microorganisms. Furthermore, the concentration of metal trace elements, particularly iron and manganese, must be low. The presence or absence of such elements strongly affects cellular morphology. The fungi grow pellet-like in submerged culture. However, the pellets can be big and fluffy or very small and compact (see Figure 5.4). Only very small and compact pellets (less than 1 mm) are associated with high citric acid production rates and yields. An excess of trace metal ions results in growth of unproductive filamentous mycelia instead of pellet formation, which finally means the culture is lost, as this condition is irreparable. This poses the need to purify the carbon source for the production

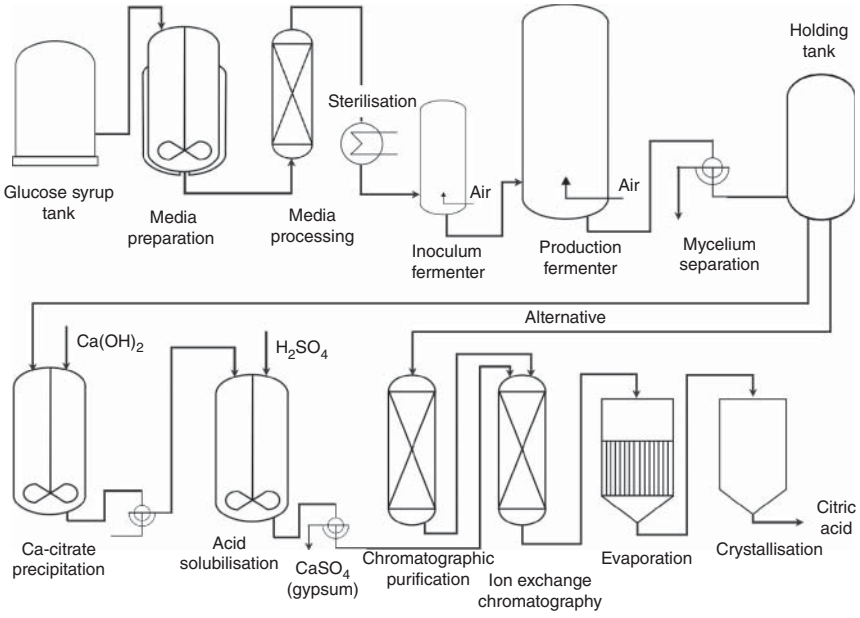


Figure 5.3 Process scheme of citric acid production with *Aspergillus niger*. Media, mostly based on glucose syrup, sucrose, or molasses, are optionally processed by ion chromatography to adjust the concentration of metal ions. After fermentation and mycelium separation, citric acid can be directly purified by chromatographic steps and processed to crystalline citric acid or sodium citrate. Alternatively, a purification step by $Ca(OH)_2$ precipitation and sulfuric acid solubilization may precede the chromatographic steps.

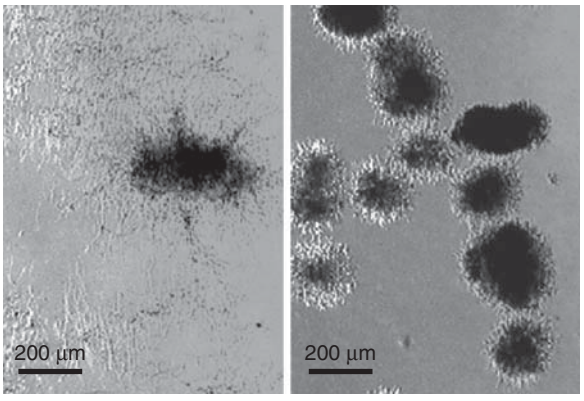


Figure 5.4 Morphology of citric acid producing *Aspergillus niger* in suspension culture. (a) Unproductive mycelium is grown out, forming large, loose aggregates. (b) Productive mycelium forms small, dense pellets.

from these ions, which adds significant costs to the process and is a constant threat, because contamination of the culture medium leads to high financial losses as the whole reactor content must be discarded. A final constraint for productivity is the pH of the culture. A pH value over 6 leads to the accumulation of oxalic acid. At pH of around 5.5, gluconic acid is the predominant product. Citric acid production begins at a pH lower than 3.0 and is optimal below a pH of 2.0.

The strains that are used have been obtained from wild-type strains by constant mutagenesis and selection over decades. Besides the increase in rate and yield for citric acid production, sensitivity to trace metals and ease of sporulation have been in the center of selection. Clearly, every producer has its own strains, which are kept secret inside the company.

Traditionally, citric acid has been purified by crystallization through addition of $Ca(OH)_2$. The crystals are washed and finally the acid is obtained by solubilization with sulfuric acid. This procedure results in large amounts of gypsum, which had to be deposited as a waste product for a long time. Modern production plants deliver a gypsum grade, which can be sold as building material, underlining the necessity to avoid waste accumulation and to gain economic benefit also from side streams of every production process. Newer plants have eliminated the production of gypsum completely by purifying the acid using simulated moving bed chromatography, a technique that has been upscaled recently.

5.2.4 *Yarrowia lipolytica*: A Yeast as an Alternative Production Platform

The yeast *Yarrowia lipolytica* is another microbial cell factory for citric acid described in the literature. It has been shown that this yeast – similar to *A. niger* – naturally accumulates high amounts of metabolic products in the presence of an excess of carbon source when growth is limited by exhaustion of nitrogen. At low pH, this yeast accumulates polyols, but when the pH is kept around 5, the main product is citric acid. The scientific literature reports about process designs reaching more than 150 g/l citric acid. However, the reported yields of 60–70% are significantly lower compared to the current *A. niger* processes.

Advantages of this yeast cell factory include a lower sensitivity to low oxygen concentrations and no peculiar sensitivity to trace metal ions in the medium. Sucrose, a major carbon source for citric acid production by *A. niger* cannot be converted by wild-type *Y. lipolytica* strains because they lack the enzyme invertase, something that is a significant disadvantage. However, the most detrimental fact is that *Y. lipolytica* inevitably accumulates isocitric acid in various amounts, together with citric acid. This lowers the yield, which is a problem. However, most significantly, isocitric acid disturbs the crystallization process of citric acid, thereby hindering purification.

Although *Y. lipolytica* seems to be a useful cell factory and there are certain indications that it is exploited in industry, its real use is difficult to assess.

5.3 Lactic Acid

Lactic acid has been a product for the food industry for a long time. Microbial fermentation of vegetables, such as sauerkraut and kimchi, has been done since ancient times – indeed long before the microbial cause for the fermentation was discovered. In 1780, Carl Wilhelm Scheele isolated lactic acid from soured milk (latin: *lac*). It was Louis Pasteur in the middle of the nineteenth century who discovered the lactic acid bacteria and described their metabolism, which became a cornerstone of our understanding of microbiology. Since 1880, lactic acid has been industrially produced by fermentation in the United States. However, it was only quite recently that this acid gained importance as a building block for polylactic acid (PLA) production. PLA is a bio-based and biodegradable plastic, which promises significant environmental benefits compared to most petroleum-based polymers.

5.3.1 Economic Impact and Applications

The global market for lactic acid is currently about 750 000 t/yr, and it is projected to reach 1.8 million t/yr by 2022. Its traditional use is in the food and beverage industry as mild acidulant. Lactic acid also finds application in the leather and textile industry, as well as in cosmetics and pharmaceutical drugs. Ethyl lactate is being suggested as a green solvent with many applications. However, the most promising application is plastic production by polymerization, which makes lactic acid one of the most promising organic acids for the future. The possibility for polymerization derives from the bifunctionality of the molecule – lactic acid is an alpha-hydroxy acid, which means it bears a hydroxyl and a carboxyl group. The molecules can be polymerized forming ester bonds between these groups.

The new application of polymer production has special requirements to the quality of the acid produced. This is an interesting case of how the final use impacts the production process itself. Lactic acid is a chiral molecule, existing in two forms: L-lactic acid and D-lactic acid. The PLA of high quality can only be obtained from enantiomerically pure lactic acid (L or D). This excludes chemical production of the acid from the beginning, as this always

yields racemic mixtures. However, many naturally producing microorganisms accumulate mixtures of both enantiomers because they have more than one lactate dehydrogenase – the enzyme catalyzing the reduction of pyruvate to lactate and determining the chirality – or the microorganisms express a lactate racemase – an enzyme that catalyzes the interconversion of the D- and the L-form of lactic acid. Furthermore, the lactic acid has to be extremely pure and to be of polymerization grade, but of course, it also has to be cheap – as plastic is a low-value product. This leads to a still ongoing quest to establish new processes for lactic acid production – even if the natural producers are in principle among the most efficient fermenting microorganisms known.

5.3.2 Anaerobic Bacterial Metabolism Generating Lactic Acid

A variety of microorganisms accumulate lactic acid as a major fermentation product. Among them are the lactic acid bacteria (of the order *Lactobacillales*), the bifido bacteria, and certain bacilli, such as *Bacillus coagulans*. Furthermore, a variety of fungi, such a *Rhizopus oryzae*, accumulate lactic acid. Although bifido bacteria have certain importance in the food industry, because of their probiotic properties, only lactic acid bacteria and *B. coagulans* have industrial importance for purified lactic acid production.

The homofermentative pathway for hexose fermentation yields 2 mol lactic acid per mol hexose but only 1 mol ATP. From an industrial point of view, this is a perfect metabolic pathway. The theoretical yield is 100% based on mass. No carbon is lost, and no side products are formed. The microorganism receives only a minimal amount of energy from this pathway. This means that very high flux rates are required to fulfill the actual energy requirements of the microorganism. Consequently, high amounts of glucose are converted into lactic acid in a short time. Table 5.2 shows some exemplary data for such production processes. Homofermentative lactic acid bacteria can only grow on hexoses. Heterofermentative lactic acid bacteria have the ability to use hexoses and pentoses. This is of advantage when lignocellulosic sugars are the carbon source for lactic acid production.

Bacillus coagulans has a glycolysis type of metabolism and can also use pentoses such as xylose, which makes this bacterium attractive for industry.

5.3.3 Lactic Acid Production by Bacteria

Typical lactic acid fermentations are batch cultures of 25–120 m^3, starting with 120–180 g/l sugar (mostly glucose or lactose). These processes typically rely on bacteria belonging to the genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, and *Enterococcus*, which belong to the lactic acid bacteria. They are fastidious microorganisms, which means that they rely on organic nitrogen and require a number of vitamins of the B-group. Addition of complex nutrient ingredients, such as yeast extract, fulfills these requirements but increases the costs and makes the purification more complicated. Lactic acid bacteria are anaerobic, but aerotolerant. Industrially, this is the most desirable characteristic as no expensive measures have to be taken to create an anaerobic environment and

Table 5.2 Substrates, titers, yields, and productivities of LA production by bacteria (literature data).

Production organism	Substrate	Titer (g/l)	Yield (g/g)	Volumetric productivity (g/l/h)
Enterococcus faecalis	Sucrose	140	0.97	5.2
Lactobacillus delbrueckii	Hydrolyzed wheat flour	110	0.91	3.6
Lactococcus lactis	Hydrolyzed wheat flour	110	0.91	2.4
Lactobacillus paracasei	Sweet sorghum	110	0.79	10
Lactobacillus casei	Glucose	80	0.91	5.6
Bacillus coagulans	Lignocellulosic hydrolysate	110	?	5.7

of course aeration is not required. Typically, the fermentations take two to four days, achieving a yield of around 85–95%. Although lactic acid bacteria are low pH tolerant compared to many other microorganisms, from an industrial point of view, they appear sensitive. The pH must be kept between 5.5 and 6 throughout the entire fermentation; otherwise, the conversion rates decline rapidly. Therefore, the broth has to be titrated with base, such as $CaCO_3$, NaOH, or NH_3. This means that after fermentation, the pH has to be lowered for purification – usually by addition of H_2SO_4. Ion exchange and decolorization with active carbon are then followed by distillation to obtain the free acid. Reactive distillation with methanol is another method of choice. The methanol can be recycled, when the free acid is obtained. When PLA production is the aim, another reactive distillation can be employed, yielding dilactides, which are the actual basis for the polymerization reaction.

Lactic acid production with *B. coagulans* follows similar techniques. Electrodialysis is in this case the choice for lactic acid purification – integrated into the fermentation process. (See Section 5.7 for more details on purification of organic acids.)

5.3.4 Lactic Acid Production by Yeasts

Purification is a major cost factor for bulk chemical production by fermentation. In order to keep the purification costs as low as possible, the fermentation process should result in a broth with high titer, as few as possible contaminants (particularly avoiding those with similar chemical properties as the substance, which shall be produced), and the pH should not require any further adaptation. All of this speaks against lactic acid bacteria as production hosts for lactic acid. Because of their requirement of complex media components, the fermentation broth contains many impurities that hamper purification. Because of their demand for high pH, the pH of the broth needs to be lowered before purification, which adds costs for the acid and the base and finally for wasting or recycling of the obtained salt. Although all of this is not very important for food and pharmaceutical uses of lactic acid, it becomes crucial when PLA is the desired end product. Therefore,

Table 5.3 Titers, yields, and productivities of LA production by yeasts.

Production organism	Substrate	Enantiomer	Final pH	Titer (g/l)	Yield (g/g)	Volumetric productivity (g/l/h)
Candida krusei	Glucose	L	< 3	c. 130	?	c. 3.0
Saccharomyces cerevisiae	Glucose	D	3.5	82.6	0.83	3.5
Pichia pastoris	Glycerol	L	5	< 30	0.7	0.146
S. cerevisiae	Xylose	L	4–6	40	0.8	0.03
Scheffersomyces stipitis	Xylose	L	5.5	58	0.58	0.39

yeasts have attracted significant attention as possible lactic acid production hosts. Many yeast species can grow on simple mineral media, and many of them efficiently ferment a variety of sugars also at low pH values. However, most yeast strains do not produce any lactic acid naturally. The typical fermentation end product is ethanol. Metabolic engineering allowed to construct yeast strains efficiently producing lactic acid. The process of Natureworks in the United States, the currently largest producer of PLA, is based on a proprietary yeast strain, which is an engineered *Candida krusei* strain. They produce about 100 000 t/yr of lactic acid from corn starch-derived glucose. The process yields more than 120 g/l of lactic acid in about 36 hours at a pH of 3 (see Table 5.3).

5.4 Gluconic Acid

Gluconic acid is a mild organic acid naturally present in many plants and fruits. It is produced from glucose by a site-specific oxidation. The C1 aldehyde of glucose is oxidized to a carboxyl group. Although this reaction can also be achieved by chemical means, low yields and selectivity strongly favor an enzymatically catalyzed reaction. Microbial processes for gluconic acid production are approved by the Food and Drug Administration (FDA). A variety of microorganisms have been described to accumulate this acid: fungal species such as *A. niger* or *Penicillium luteum* or bacterial species of the genera *Gluconobacter*, *Pseudomonas*, or *Acetobacter* among others. Nowadays, industrial production processes rely on *A. niger* or *Gluconobacter*. Interestingly, the biochemical pathways for the conversion of glucose to gluconic acid are quite distinct in fungal *versus* bacterial species.

5.4.1 Economic Impact and Applications

Isolated gluconic acid and derivatives thereof have an annual market volume of around 100 000 t/yr. Table 5.4 gives an overview of the various uses of this acid and its derivatives. Free gluconic acid and gluconic acid δ-lactone serve mainly in the food industry as mild acidulant. They produce and improve a mild sour taste and have the capability to form complexes with possible traces of heavy

Table 5.4 Use of gluconic acid and derivatives.

Compound	Use
Gluconic acid	Used in the food industry, particularly the milk industry, also for pretreatment of metals before galvanization.
Glucono-δ-lactone	Used as an acidifier in the food industry (e.g. in baking powder, bread, and sausages, also for tofu and cheese making.)
Sodium gluconate	Complexing agent for many divalent ions. Used as a detergent and rust remover. Used in textile and paper industries to keep iron from precipitation. Also used as a cement additive for the modulation of the cement hardening time.
Calcium gluconate	Complex of calcium and gluconate – used for pharmaceutical and veterinary provision of calcium.
Iron(II) gluconate	Complex of iron and gluconate – used as a nutritional supplement to provide iron, also used in fertilizers.

metals. Other forms of gluconic acid are used in the construction, textile, and pharmaceutical industries, where gluconic acid in its various forms is useful because it forms water-soluble complexes with a variety of divalent and trivalent metal ions. The actual main product is sodium gluconate, which makes up about 80% of the gluconic acid market.

5.4.2 Extracellular Biotransformation of Glucose to Gluconic Acid by *Aspergillus niger*

Similar to citric acid production, high glucose concentration, high oxygen availability, and growth limitation by low nitrogen and phosphate availability trigger gluconic acid production by *A. niger*. The difference is the pH value, which must be strictly maintained around 5.5 to maintain the formation of gluconic acid. Then, this process is essentially an enzymatic conversion process taking place outside of the cell. No transport into or out of the cell and no interference with the cellular metabolism take place. This makes this process extraordinarily efficient in terms of rate, yield, and purity of the product. The key enzyme is glucose oxidase, a glycosylated, cell wall-localized, homodimeric flavoprotein. Glucose is oxidized by transfer of hydrogen to Flavin adenine dinucleotide (FAD). The FAD is recycled by the formation of H_2O_2 from O_2. Hydrogen peroxide is toxic and has to be removed efficiently by catalase, which is also produced by the fungal cells. The direct product of this oxidation is gluconic acid δ-lactone, which hydrolyzes spontaneously in aqueous environment.

A typical process starts with 110–250 g/l glucose, high oxygen provision, low nitrogen, and phosphate availability. The pH of the culture has to be kept between 4.5 and 6.5, with $CaCO_3$ or Na_2CO_3/NaOH, depending on the desired product. Fermentation times vary between 24 and 60 hours, which is very short for a fungal process. The obtained yield regularly reaches 95%, but up to 98% have been reported. As this process is so efficient, it is not really surprising that strain improvement did not play a significant role until now.

Crystallization and ion exchange chromatography are among the most important steps for purification of gluconic acid.

5.4.3 Production of Gluconic Acid by Bacteria

Bacteria rely on different enzyme systems than fungi for gluconic acid production. *Gluconobacter oxydans* oxidizes glucose in the periplasmic space by a membrane-bound glucose dehydrogenase. This enzyme uses pyrroloquinoline quinone (PQQ) as the coenzyme. Naturally, the formed gluconic acid is further oxidized to 2-keto-D-gluconate and 2,5-diketo-D-gluconate by the membrane-bound enzymes gluconate-dehydrogenase and 2-keto-D-gluconate dehydrogenase. However, under production conditions, these enzymes are inhibited and gluconate is the major accumulated extracellular product.

It has been hypothesized that this special metabolism of *G. oxydans* evolved because of its incomplete tricarboxylic acid (TCA)-cycle. The absence of the TCA cycle prevents the complete oxidation of the carbon source. Incomplete oxidation of the carbon source by several membrane-bound dehydrogenases allows to channel electrons into the respiratory chain. *G. oxydans* is therefore capable of oxidizing various compounds (such as sugars and alcohols) and it accumulates accordingly various products in the medium.

How far bacterial processes play a role in industry is not clear.

5.5 Succinic Acid

Succinic acid was first described in 1546, when it was isolated from distilling amber (latin: *succinum*). It is an intermediate of the TCA cycle and therefore present in most living organisms. However, it is also one of the products of the mixed acid fermentation of various bacteria. The biosynthetic pathway may take an oxidative route or a reductive route, resulting in different theoretical yields. See Figure 5.5 for an overview of the pathways. The fermentative biosynthesis of succinic acid includes CO_2 fixation, which results in a theoretical yield of 2 mol per mol glucose. However, this pathway does not provide any excess ATP and the redox balance is not closed. To close the redox balance, by-products must be formed, which would compromise the yield again and which would make the purification more difficult. The oxidative way for succinic acid production, directing the carbon flux into the glyoxylate shunt, makes this pathway energetically more favorable but reduces the theoretical yield to only 1 mol succinic acid per mol glucose. Ideally, the oxidative pathway is combined with the reductive pathway, closing the redox balance and providing a positive energy balance. Such a combined pathway obtains a theoretical succinic acid yield on glucose of 1.71 mol/mol.

Succinic acid is a natural product of a variety of bacteria. Many have been isolated from the rumen of different species. In fact, succinate is an important metabolic intermediate in the rumen, where several bacteria obtain energy by decarboxylating succinate to propionate, which in turn serves as a nutrient for the ruminant. Among the described succinic acid producing rumen bacteria are *Actinobacillus succinogenes*, *Anaerobiospirillum succiniproducens*, *Bacteroides fragilis*, and *Mannheimia succiniproducens*.

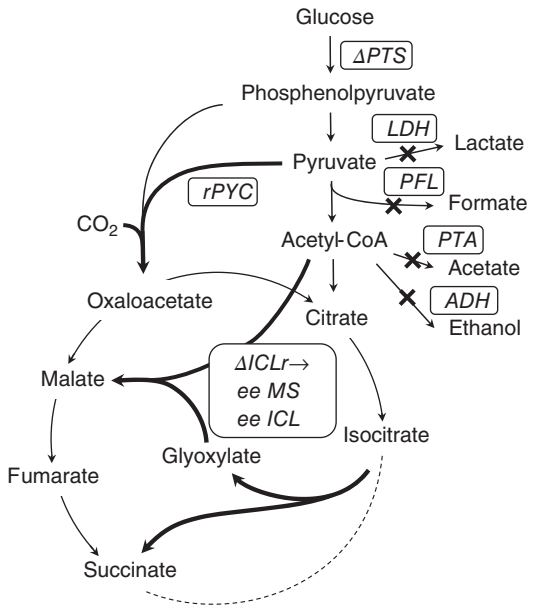


Figure 5.5 Metabolic engineering of succinate overproduction in *Escherichia coli*. Inactivation of the phosphotransferase system (ΔPTS) makes glucose uptake independent of phosphoenolpyruvate. Overexpression of a recombinant pyruvate carboxylase (rPYC) increases flux into the anaerobic branch of the TCA cycle. Deletion of the glyoxylate cycle repressor (ΔICLr) enhances expression of isocitrate lyase (ICL) and malate synthase (MS) genes to enable constitutive glyoxylate cycle flux. Deletion of the genes encoding lactate dehydrogenase (LDH), pyruvate formate lyase (PFL), phosphotransacetylase (PTA), and alcohol dehydrogenase (ADH) inhibit by-product formation.

5.5.1 Economic Impact and Applications

Succinic acid is a new product among microbial organic acids. In fact, it is entering the market only slowly now, with so far mixed success. The market for succinic acid is currently about 30 000 t/y, but a major fraction of this is produced petrochemically. It is used in detergents and surfactants, and as an ion chelator. Furthermore, the food industry uses succinic acid as an acidulant, flavor, and antimicrobial agent. The significant interest in bio-based succinic acid is its possible use as a base chemical. Essentially, it could replace petroleum-derived maleic anhydride, which has currently a market volume of 1 700 000 t/yr. Figure 5.6 shows an overview of chemicals obtainable from succinic acid. However, this replacement is only possible when the price of bio-based succinic acid becomes as low as the one of maleic anhydride. Such a low price is conceivable in future, but not obtainable at the time of writing.

5.5.2 Pilot Plants for Anaerobic or Aerobic Microbes

Succinic acid is one of the few products for which new industrial processes with new microbial cell factories have been established. A number of companies disclosed the successful startup of industrial production of biogenic succinic acid. Interestingly, very different technologies are put into action for this purpose.

BioAmber Inc. operates a succinic acid production plant in Sarnia, Canada, with a nameplate capacity of 30 000 t/yr. A second plant is planned in North America that will produce 70 000 tons of succinic acid per year. The company started off with a pilot plant in France, based on recombinant *Escherichia coli* as cell factory. However, the sensitivity of the bacterium to low pH rendered this

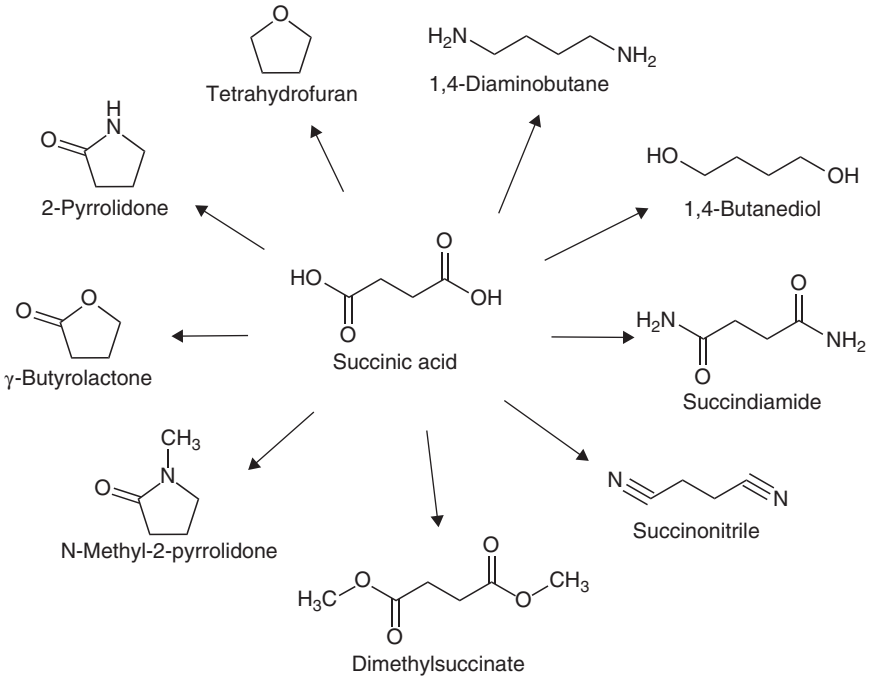


Figure 5.6 Various substances that can be derived from succinic acid by chemical conversion.

process unfeasible and the decision was taken to switch to a recombinant yeast (*C. krusei*) as the production microorganism.

Myriant in Louisiana, USA, is running a demonstration plant with an annual capacity of 13 500 tons using unrefined sugar as the feedstock. Their process is based on a recombinant *E. coli*.

Reverdia was formed through a partnership of DSM with Roquette. The production microorganism is a recombinant low-pH tolerant baker's yeast, using a combination of reductive and oxidative TCA. They opened a demonstration plant in Italy capable of producing 10 000 t/yr. The metabolic engineering strategy followed by DSM is the overexpression of a phosphoenolpyruvate carboxykinase, which generates oxaloacetate from pyruvate and concomitantly ATP. Additionally, the genes encoding malate dehydrogenase and fumarase are overexpressed, enhancing the complete pathway from oxaloacetate to succinate. Decreasing the activity of alcohol dehydrogenase, glycerol-3-P-dehydrogenase, and succinate dehydrogenase leads to a further increase in metabolic flux to the desired product and a decrease of by-product formation.

Succinity was formed by BASF joining forces with Purac. They operate a demonstration plant in Spain with a capacity of 10 000 t/yr. Biocatalyst is *Basfia succiniproducens*, a ruminal bacterium and natural producer of succinic acid from glycerol and glucose.

In summary, the production capacity appears to exceed the current market volume significantly. A possible explanation is that the price of biogenic succinic acid is still too high to fully compete with petroleum-derived maleic anhydride.

5.6 Itaconic Acid

Itaconic acid is a C_5 di-acid, which is obtained from decarboxylation of cis-aconitic acid by cis-aconitic acid decarboxylase. In 1836, S. Baup described the isolation of a new organic acid and named it citricic acid. He obtained it as a by-product of the pyrolysis of citric acid. Two years later, this acid obtained its current name itaconic acid, which was proposed as an anagram of aconitic acid, its metabolic precursor. The filamentous fungi *Aspergillus itaconicus* and *Aspergillus terreus* have been identified as natural producers of this acid, and recently, *Ustilago maydis* has been suggested as cell factory for itaconic acid.

5.6.1 Economic Impact and Applications

Around 80 000 t/yr of itaconic acid are produced, entirely by microbial fermentation. Itaconic acid can be used together with other compounds for lacquer, resin, and fiber production. These are currently the main applications. However, chemically, itaconic acid could be a source for methyl methacrylate, which is the basis for Plexiglas™ production. This could open an entirely new and huge market (more than three million tons of methyl methacrylate are produced annually), provided itaconic acid can be produced at a sufficiently low price.

5.6.2 Decarboxylation as a Driver in Itaconic Acid Accumulation

The key enzyme for itaconic acid production is cis-aconitate decarboxylase. A decarboxylation reaction results in formation of carbon dioxide, which is a gas and as such leaves the reaction easily. Such reactions are strong drivers of metabolic pathways because they are usually nonreversible, that is, every molecule of cis-aconitic acid is "caught" irreversibly by the enzyme and remains as product.

5.6.3 Production Process by *Aspergillus terreus*

Industrial production of itaconic acid is entirely based on strains of *A. terreus*. The process itself is similar to the one of citric acid production with *A. niger*. The fungi are grown in pellet form in a submerged batch culture. Starch hydrolysate or sucrose from sugarcane or sugar beet is used as the carbon source. Important for efficient production are a high sugar substrate concentration of >12% (w/v), continuous and high oxygen supply, low manganese concentration < 2–4 µg/l, and phosphate limitation. The pH value must be kept around 3 to achieve a high titer of itaconic acid (up to 146 g/l have been reported).

5.6.4 Metabolic Engineering for Itaconic Acid Production

Cultures of filamentous fungi are generally more sensitive than bacterial or yeast cultures, particularly because of the sensitivity to trace metal ions, which leads to costs for media preparation and the risk of failure of the entire culture. There is an urge, therefore, to obtain other cell factories, which are less sensitive. Cis-aconitic acid is a cellular metabolite, which is present in most microorganisms and only

one enzyme is necessary to convert cis-aconitate into itaconic acid. Consequently, a wide variety of microorganisms has been suggested as recombinant itaconic acid producers, among them *E. coli*, *S. cerevisiae*, *Y. lipolytica*, *Sugiyamaella lignohabitans*, *Synechocystis* sp., and *Corynebacterium glutamicum*.

The ease of this approach and the promise to use a reaction that is a driver for metabolic pathways was deceptive. Most of the recombinant strains produced itaconic acid only in the 100 mg/l to 5 g/l scale. Most advanced have been the approaches for metabolic engineering of *E. coli*. Thorough metabolic modeling and rational strain and process engineering led to the following promising setup: four genes have been deleted, abolishing acetate production, eliminating the glyoxylate shunt (which is a sink of isocitrate), and eliminating the conversion of phosphoenolpyruvate to pyruvate, thereby forcing the metabolic flux into the anaplerotic reaction of pyruvate carboxylase (see Figure 5.7 for an overview). Additionally, the endogenous isocitrate dehydrogenase was placed under a temperature-sensitive promoter. This allowed to switch the TCA cycle on and off depending on the temperature. This strain can be grown at 37 °C at a fast growth rate. Then, in a second stage, the temperature is lowered to 30 °C, turning off the isocitrate dehydrogenase promoter, abolishing respiration and growth. Under

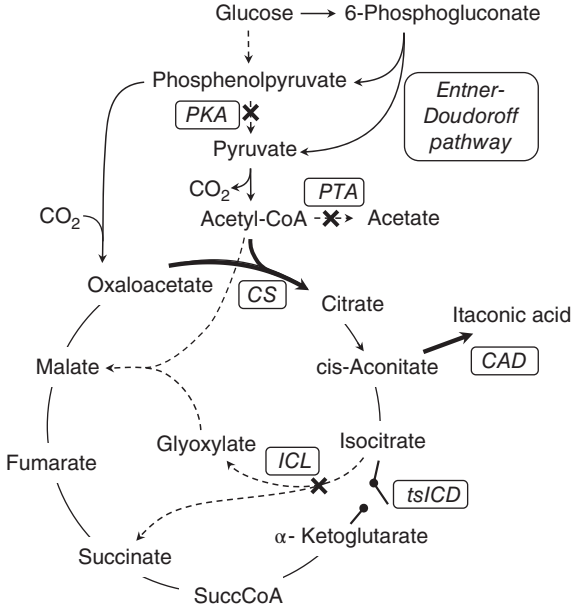


Figure 5.7 Metabolic engineering for enhanced itaconic acid production in *Escherichia coli*. First, cis-aconitate decarboxylase (CAD) of *Aspergillus terreus* and citrate synthase (CS) of *Corynebacterium glutamicum* were overexpressed (bold arrows). Pyruvate kinase (PKA) was deleted to force the flux through the Entner–Doudoroff pathway, leading to equimolar supply of phosphoenolpyruvate and pyruvate. PTA was deleted to avoid acetate formation. To avoid carbon loss in the TCA cycle, isocitrate lyase (ICL) was deleted to disable the glyoxylate shunt, and isocitrate decarboxylase was set under control of a temperature-sensitive promoter (tsICD), so that the TCA cycle flux can be enabled at permissive temperature for growth and blocked by increasing the temperature for itaconic acid production.

these conditions, citrate is shuttled into itaconic acid production with a peak productivity of 0.86 g/l/h and an itaconic acid titer of 47 g/l. Currently, this strain cannot compete with *A. terreus*, but through rational strain engineering, there is little doubt that production will shift to new cell factories in the near future.

5.7 Downstream Options for Organic Acids

Before any organic acid (or any other fermentation product) can be used, it must be separated from the other components of the fermentation broth. The first step is typically the removal of the cells by various filtration techniques (micro/ultrafiltration, flocculation with filter aid) or centrifugation.

The next point to address is the pH of the fermentation broth. If a low pH fermentation was feasible, then no adjustment would be required. However, if the production organisms do not tolerate a pH value that is significantly below the pK_a value of the acid, the pH must be lowered to obtain the free acid before purification. A widely used method of controlling pH in industrial fermentations is the addition of lime ($Ca(OH)_2$). Other bases, such as NH_3 or NaOH, are alternatives. To lower the pH when required, the cheapest and most widely used acid is sulfuric acid. When a Ca-containing base has been used, gypsum will precipitate and has to be removed.

Electrodialysis is a modern method, which allows the purification of the organic acid without prior pH adjustment (see Figure 5.8). In brief, electrodialysis involves using an electrical charge to migrate charged species across alternating ion-specific membranes. This results in salt concentration and selection. The obtained concentrated salt stream (containing the desired organic acid ion and a counter ion, e.g. sodium lactate) is then subjected to water-splitting electrodialysis. This technique splits water into H^+ and OH^-, which migrates

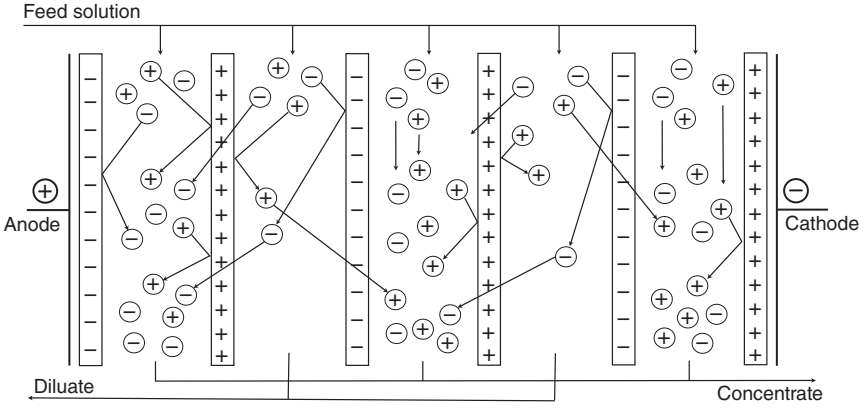


Figure 5.8 Principle of electrodialysis. An electrodialysis chamber consists of individual cells separated by ion-selective membranes. During flow through the cells, ions are attracted by the electrodes. As they can pass only one membrane, ions are concentrated in the odd numbered cells while even numbered cells are depleted of ions forming the diluate. In practice, electrodialysis chambers consist of multiple cells in an odd number.

again across membranes, yielding the organic acid and the base that was used during fermentation (e.g. sodium hydroxide). Thus, the fermentation neutralizing agent is regenerated, acid addition is not required, and there is little salt waste production. However, capital and operating (electricity) costs of this technique are high, particularly membrane fouling might lead to repeating costs for replacement.

Further methods combining acidification and purification are **solid resin adsorption and solvent extraction**. However, both methods include the regeneration of resin or solvent, which again generates salt waste. When adsorbent or extractant is used as the fermentation neutralizing agent, this typically occurs at very low titers – to minimize the toxicity. This demands in turn continuous processes. For large-scale fermentations in industrial reality, this limits the realistic feasibility because many recycle and continuous operations bear the constant risk of contaminations.

5.8 Perspectives

Classical products, such as citric acid, will remain major biotech products. Processes are constantly optimized, as the gains on a unit level are very low. The market price for citric acid is only two to three times higher than the market price of sugar. This means on the one hand that only large-scale production can be economic (economy of scale) and on the other that even minor process improvements can have a big impact on overall economic success.

In the end of the day, this is how the bulk chemical market generally works and this is where all organic acid production industries will have to arrive. Looking, for example, at succinic acid, the price margins are very similar to citric acid. Maleic anhydride, the petroleum-derived counterpart of bio-based succinic acid, has a price that is also only two to three times higher than that of glucose. Process costs must therefore be extremely low for economic viability. A significantly increased petroleum price (which is improbable in the near future) could change the picture. However, it has to be understood that the sugar price is directly connected to the petroleum price, as much energy is needed for agriculture and processing of the sugar plants. Increases of the petroleum price lead to increases of the sugar price, which potentially keeps the margins for the bio-based product small, also in times of high petroleum prices. One important consideration is therefore, which raw materials will be the basis of the processes in future. Streams currently regarded as waste streams or lignocellulosic biomass are promising candidates. Some considerations about lignocellulose-based biorefineries can be found below.

Policy is the final player in this game. The market will always be regulated by price. No "green premium" exists. Although environmental concerns are increasing and there is an urge to greener industries, the final decision is always taken by the price. The majority of consumers in the supermarket will always opt for the lower cost choice. The only measure to counteract this is taxation or subsidies. Subsidies can be used as a measure to allow market entrance of a product. However, after a while, every product must be economically viable on its own.

Unlimited times of subsidies are generally not sustainable. If a production route shall be discontinued, taxation could be used to force industry to replace existing processes. However, such measures are highly controversial and a business plan is better when it is not based on policy measures.

Nevertheless, many projects are ongoing, many ideas are followed to provide to solutions, when bio-based will be required in the future. One such endeavor – targeting the acrylic acid market – is briefly summarized below.

5.8.1 Targeting Acrylic Acid – Microbes Can Replace Chemical Process Engineering

Acrylic acid is a true bulk product, currently derived from petroleum. In 2014, about 5.75 million tons have been produced. It is used for the manufacturing of paints, coatings, paper, textiles, and superabsorbent polyacrylate polymers (for example, for diapers). It appears as a challenging but rewarding goal to replace the petrochemical route for acrylic acid production by a bio-based process. No natural fermentation pathway to acrylic acid is known. However, chemical dehydration of 3-hydroxypropionic acid (3-HP) readily leads to acrylic acid. 3-HP is a positional isomer of lactic acid (the alcohol group is moved from position two to position three). 3-HP is a natural product of the glycerol metabolism of microorganisms expressing a glycerol dehydratase, such as certain lactobacilli or *Klebsiella pneumoniae*. A major hurdle for the development of an efficient microbial production process is the high toxicity of 3-HP, which stops the efficient product accumulation at relatively low concentrations of the acid. There are, however, many attempts to develop such a process, which is reflected in an increasing amount of scientific papers being published about this topic. The approaches involve natural producers and process engineering as well as metabolic engineering of common host strains such as *E. coli*. However, not only academia but also industry is keen to obtain bio-based acrylic acid. Cargill and BASF are among the big companies actively involved in this research.

5.8.2 Lignocellulose-Based Biorefineries

Sugar is a highly refined commodity, which has direct application for human consumption. Organic acid production from sugars is therefore in direct competition with food provision. As long as the acid is also used for food or beverages, it is just an upgrading of one food ingredient to another and therefore clearly acceptable. However, when plastic and solvents are the aim, it becomes questionable to base this on a food resource. Furthermore, sugar is a comparably expensive resource, prone to price fluctuations with petroleum and other food markets. It is therefore desirable to substitute sugar with other carbon sources for large-scale productions for the nonfood market. Lignocellulosic biomass is a promising example for such a carbohydrate source. Two-third to three-quarters of lignocellulose are sugars. The difficulty is to obtain these sugars in a form suitable for microbial conversion. Lignocellulose evolved to give plants longtime stability, so deconstruction is slow and difficult. Generally speaking, the material has first to be chopped or milled into small pieces. This is followed by a physicochemical treatment to

separate lignin from cellulose and hemicellulose and finally the polymers have to be decomposed – chemically or enzymatically – to obtain a sugar solution, ready to go into the fermentation. The entire process chain is long and costly, so that at the moment, most of the existing demo plants are not economically viable on their own. Nevertheless, the technology holds many promises for the future.

One important point to consider in this context is that the use of lignocellulose as carbon source will hardly be economic and sustainable as long as it is seen as a linear process, yielding one product from one substrate. Citric acid production from glucose or starch is a viable linear process. However, already in this simple case, the industrial plant will provide a variety of other products to the market, such as gypsum for construction from the purification, or the dried fungal mycelium as animal feed, just to name two. The lignocellulose-based production plant must be compared to a current petroleum refinery, yielding many products at the same time and valorizing the lignocellulose entirely. Hemicellulose and cellulose comprise a variety of sugars, which might be used separately. Hemicellulose contains a significant amount of acetic acid, which is a product on its own. Lenzing AG, for example, is a wood-based fiber producer, who sells significant amounts of acetic acid to the food industry. Although this acetic acid is not fermentation based, it derives from plants and not from petroleum and is therefore acceptable for the food market. Organic acid production from lignocellulose can therefore be a vital part of a lignocellulose biorefinery, but other products must be added, to obtain a portfolio, which in the end ensures economic success also in a case in which one of the products alone would not be viable.

Further Reading

Ahn, J.H., Jang, Y.S., and Lee, S.Y. (2016). Production of succinic acid by metabolically engineered microorganisms. *Curr. Opin. Biotechnol.* 42: 54–66. https://doi.org/10.1016/j.copbio.2016.02.034.

Berovič, M. and Legiša, M. (2007). Citric acid production. *Biotechnol. Annu. Rev.* 13: 303–343.

Jiang, M., Ma, J., Wu, M. et al. (2017). Progress of succinic acid production from renewable resources: metabolic and fermentative strategies. *Bioresour. Technol.* 245 (Pt. B): 1710–1717. https://doi.org/10.1016/j.biortech.2017.05.209.

Juturu, V. and Wu, J.C. (2016). Microbial production of lactic acid: the latest development. *Crit. Rev. Biotechnol.* 36 (6): 967–977.

Moo-Young, M. (ed.) (2011). *Industrial Biotechnology and Commodity Products, Comprehensive Biotechnology*, 2e, vol. 3. Amsterdam: Elsevier B.V.

Sauer, M., Porro, D., Mattanovich, D., and Branduardi, P. (2010). 16 years research on lactic acid production with yeast – ready for the market? *Biotechnol. Genet. Eng. Rev.* 27: 229–256.

Singh, O.V. and Kumar, R. (2007). Biotechnological production of gluconic acid: future implications. *Appl. Microbiol. Biotechnol.* 75 (4): 713–722.

Steiger, M.G., Wierckx, N., Blank, L.M. et al. (2017). Itaconic acid – an emerging building block. In: *Industrial Biotechnology – Products and Processes* (eds. C. Wittmann and J.C. Liao), 453–472. Weinheim: Wiley-VCH.

Zhao, M., Lu, X., Zong, H. et al. (2018). Itaconic acid production in microorganisms. *Biotechnol. Lett.* https://doi.org/10.1007/s10529-017-2500-5.

6

Amino Acids

Lothar Eggeling

Forschungszentrum Jülich GmbH, IBG-1: Biotechnologie, Wilhelm-Johnen-Straße 1, 52425 Jülich, Germany

CHAPTER MENU

6.1 Introduction

6.1.1 Importance and Areas of Application

Amino acids are the building blocks of proteins and therefore vital for all living beings. However, humans and animals have only limited options to synthesize them: 9 out of the 20 proteinogenic amino acids cannot be made by humans. These essential amino acids are the three branched-chain amino acids L-leucine, L-isoleucine, and L-valine, as well as L-threonine, L-lysine, L-methionine, L-phenylalanine, L-histidine, and L-tryptophan. The body's need for these amino acids is usually covered by the consumption of protein-rich food products. Requirements can, however, vary considerably according to age and state of health. Thus, a child requires three times as much L-lysine as an adult, and in the case of sickness, it may become necessary to systematically supply the body with amino acids. For this reason, amino acids are required for infusion solutions used, for example, to treat chronic inflammatory bowel diseases or during the postoperative phase. Amino acids are also part of special dietic foodstuffs for congenital disorders of amino acid metabolism such as phenylketonuria.

In addition to using amino acids in the pharmaceutical sector, there are numerous other fields of application (Table 6.1). For example, monosodium glutamate is used in the food industry. Monosodium glutamate causes the typical umami taste that – in addition to sweet, salty, sour, and bitter – is one of the five elementary tastes. The word umami comes from Japanese and means

Industrial Microbiology, First Edition.
Edited by David B. Wilson, Hermann Sahm, Klaus-Peter Stahmann, and Mattheos Koffas.

Table 6.1 The amounts of amino acids produced annually and their main uses.

Amino acid	Amount (t/yr)	Main use
L-Glutamate	3 300 000	Umami seasoning
L-Lysine	2 200 000	Animal feed
D,L-Methionine	1 300 000	Animal feed
Glycine	550 000	Herbicides
L-Threonine	520 000	Animal feed
L-Tryptophan	32 000	Animal feed
L-Aspartate	18 000	Sweetener
L-Phenylalanine	18 000	Sweetener
L-Cysteine	7 000	Pharma
L-Cystine	5 000	Pharma
L-Valine	5 000	Animal feed, pharma
L-Arginine	3 000	Pharma
L-Leucine	3 000	Pharma
L-Alanine	2 000	Sweetener
L-Isoleucine	2 000	Pharma

The numbers are estimates for 2015.

"savory," "tasty," "spicy." Furthermore, the amino acids L-alanine and glycine are required for sweetening drinks. In the chemical industry, amino acids are used as building blocks, for example, L-valine to synthesize pesticides, L-phenylalanine and L-aspartate to synthesize the sweetener aspartame, and glycine to synthesize the herbicide glyphosate.

6.1.2 Amino Acids in the Feed Industry

Amino acids are of outstanding economic significance in the feed industry. Adding amino acids to animal feed leads to a balanced composition of amino acids adapted to actual demand. It

- reduces the costs for feed raw materials,
- increases the efficiency of feed utilization, and
- reduces the environmentally harmful excretion of nitrogen.

Wheat as a feedstuff, for example, has a small proportion of L-lysine, so that the other amino acids cannot be used completely (Figure 6.1). Adding L-lysine increases the degree of utilization of the other amino acids. After adding L-lysine, the next amino acid that limits utilization is L-threonine, followed by L-tryptophan. By adding these three amino acids, the nutritional value of wheat is almost doubled, which, in view of increasing meat consumption, means that resources are conserved. When using soybean meal as a source of protein, L-methionine is the first limiting amino acid, so that the demand for it is also relatively great.

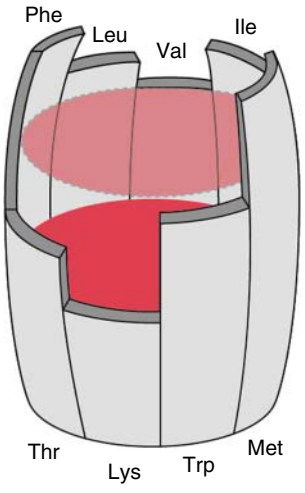


Figure 6.1 The Liebig barrel. The lowest stave illustrates that the nutritional value of wheat as protein source is limited by L-lysine. When L-lysine is added, the next limiting amino acid is L-threonine followed by L-tryptophan. Addition of these three amino acids almost doubles the nutritional value of wheat.

6.1.3 Economic Significance

Owing to rising meat consumption, particularly in Asia and South America, amino acids are increasingly needed as feed additives. This includes L-lysine, D,L-methionine, L-threonine, and L-tryptophan, in particular, the demand which is increasing by 5–7% every year.

The amino acids produced in the greatest quantities are also the cheapest (Figure 6.2) because whenever the demand is great, large numbers of providers compete initially. More efficient fermentation processes are a consequence, reducing costs and therefore resulting in cheaper products. Ultimately, this leads to low prices for large-volume products and the fact that these products are being made by only a few producers. Additionally, the prices for amino acids are subjected to considerable fluctuations as the availability of raw materials for animal feed varies. Thus, soybean meal – which compared to wheat is relatively rich in L-lysine – replaces fermentation-produced L-lysine to some extent in

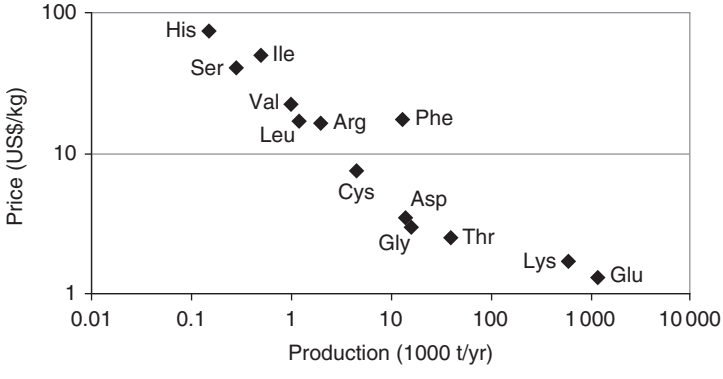


Figure 6.2 Dependence of the prices of the amino acids on the quantities produced.

years with rich soybean harvests, pushing down the price for this amino acid. The reverse applies in the case of poor soybean harvests.

The production costs are determined to a large degree by the source of carbon as well as by the local market. This explains why the large facilities for glutamate production are mainly located in the Far East, where sugar cane and cassava represent the carbon source, as well as being the largest sales market for monosodium glutamate. Around 90% of all monosodium glutamate worldwide is produced in China and the countries in Southeast Asia such as Thailand and Indonesia. The case of L-lysine is different: around one quarter of the global market for this amino acid is in North America, where there is convenient access to maize as a carbon source, resulting in one quarter of L-lysine production capacity also being located there. In almost all cases, the companies producing L-lysine are associated with companies in the cereal industry, and the facilities for enzymatic starch hydrolysis from maize or wheat are directly adjacent to the fermentation plants.

6.2 Production of Amino Acids

With the exception of glycine, D,L-methionine, and L-aspartate, amino acids are produced microbially. Glycine is not chiral and is therefore produced chemically. D,L-Methionine is also produced chemically. In the form of a racemic mixture, it is used in feedstuffs because animals have D-amino acid oxidase and transaminase activities, which means that they can convert D-methionine into L-methionine, the latter being decisive for nutrition. L-Aspartate is obtained enzymatically from fumarate in a whole-cell process. Originally, amino acids were also isolated from protein-rich raw materials such as feathers or hair. However, since this requires aggressive acidic hydrolysis with subsequent reprocessing steps, this method is now used only to a very limited extent. The method of choice is the microbial synthesis of amino acids. It is unbeatable because

- the desired L-enantiomer is the only product,
- the sugar-rich raw material is renewable,
- the process is environmentally friendly and takes place at low temperatures, and
- fermentation facilities are cheaper than chemical plants with respect to both construction and operation.

Corynebacterium glutamicum and *Escherichia coli* strains are used as amino acid producers. The cultivation of *E. coli* has the advantage that its temperature optimum of 37 °C entails lower cooling costs during fermentation than that of *C. glutamicum* with its optimum of 30 °C. This is contrasted by the fact that fermentation of *C. glutamicum* is impervious to bacteriophage infections, which can represent a significant problem for *E. coli* particularly in tropical countries.

6.2.1 Conventional Development of Production Strains

Wild-type isolates of both *E. coli* and *C. glutamicum* only produce the amount of amino acid that they require for growth. This is due to efficient control

mechanisms such as the allosteric control of biosynthetic enzymes through the relevant amino acids, or to the control of transcription initiation through the loading state of the tRNA, or to global regulation mechanisms. For this reason, mutant strains are produced, which synthesize larger amounts of the amino acid. In conventional strain development, the cells are mutagenized in an undirected manner for this purpose, e.g. through UV irradiation. Large-scale screenings then reveal the strain with the best amino acid excretion. For these screenings, amino acid analogs inhibiting the activity of key enzymes are often used. For example, in the same way as L-lysine itself, the lysine analog S-Aminoethyl-L-cysteine inhibits aspartate kinase – the key enzyme of lysine synthesis – but does not permit growth because of the absence of L-lysine (Figures 6.3 and 6.6). Mutants that grow despite the presence of S-Aminoethyl-L-cysteine often excrete L-lysine. Using the isolated mutant as a starting point, the whole procedure is repeated using additional analogs, or according to other selection criteria (Table 6.2), in order to ultimately obtain an industrially suitable strain.

Conventional strain development has its disadvantages, however:

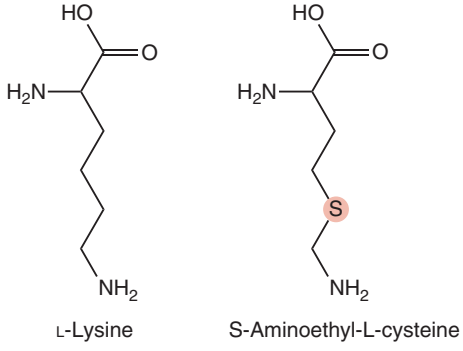


Figure 6.3 The structural similarity between L-lysine and its analog S-Aminoethyl-L-cysteine.

Table 6.2 Genealogy of a conventionally developed L-lysine production strain: the wild type of *Corynebacterium glutamicum* was mutated and, in a first step, the best L-lysine producer was selected from among the AEC-resistant clones.

Strain	Characteristics	Lysine yield (%)
AJ 1511	Wild type	0
AJ 3445	AEC^r	16
AJ 3424	AEC^r Ala^-	33
AJ 3796	AEC^r Ala^- CCL^r	39
AJ 3990	AEC^r Ala^- CCL^r ML^r	43
AJ 1204	AEC^r Ala^- CCL^r ML^r FP^s	50

In five further mutagenesis and selection steps, an increase in product yield was achieved.
AEC^r, resistant to *S*-(β-aminoethyl)-L-cysteine; Ala^-, auxotrophic for L-alanine; CCL^r, resistant to α-chlorocaprolactam; ML^r, resistant to γ-methyl-L-lysine; FP^s, sensitive to β-fluoropyruvate.

- it is very work-intensive because many clones have to be screened,
- it depends on random hits in mutagenesis, and
- mutations accumulate that lead to poor growth.

Poor growth is undesirable because it slows down the production process, making it inefficient. Direct screening for product formation also has its advantages, however. As screening directly targets increased product formation, mechanisms and genes may also be found whose advantageous function may not have been recognized yet. This concerns osmoregulation, for example, or tolerance for pH and oxygen fluctuations during the production process.

Indeed, random mutagenesis and screening for increased product formation have led to the development of strains with excellent product formation characteristics. Undirected mutagenesis has also proved valuable as such approaches result in mutated genes that, for example, no longer code for allosterically regulated biosynthesis enzymes. These are, however, a prerequisite for the advanced development of production strains using recombinant methods.

6.2.2 Advanced Development of Production Strains

Today, molecular recombinant techniques offer the opportunity to introduce mutations into the chromosome in a targeted manner or to overexpress genes. As shown in Figure 6.4, the objective is to increase the entire metabolic flux, including the transport processes, leading to the desired amino acid, to decrease the formation of undesirable by-products, and to overcome global control mechanisms. This, of course, requires knowledge about the relevant molecular mechanisms that need to be investigated. Additionally, molecular techniques also use genes obtained from conventionally obtained strains. Moreover, conventionally obtained strains can also form the basis for achieving improved producers using molecular recombinant techniques.

Global analyses, dubbed "omics," offer further approaches to developing production strains. Thus, the simultaneous determination of as many intracellular metabolites as possible leads to the identification of the metabolome. By comparing the metabolomes of two strains, it can be determined where in the metabolism a metabolite is accumulating. This is an indication that the subsequent step in the synthesis pathway represents a limitation that can then be overcome by means of gene overexpression. A further global analysis determines all the transcripts of a cell, which is known as the transcriptome. This can be helpful, for example, to verify whether a certain expression pattern correlates with a particularly good production process by comparing various fermentations using one production strain. If this is the case, then that makes it possible to use the identified genes for targeted strain optimization. Genome-wide studies on the sequence level, summarized as "genomics," are also used for strain optimization. It is thus possible to obtain complete genome sequences of conventionally produced mutants. Comparing these sequences with the wild-type genome permits mutations to be identified that are important for production. The mutations thus obtained from genome comparisons can then be incorporated in the wild-type genome, so that only those mutations leading to increased product formation are present while those inhibiting growth are absent.

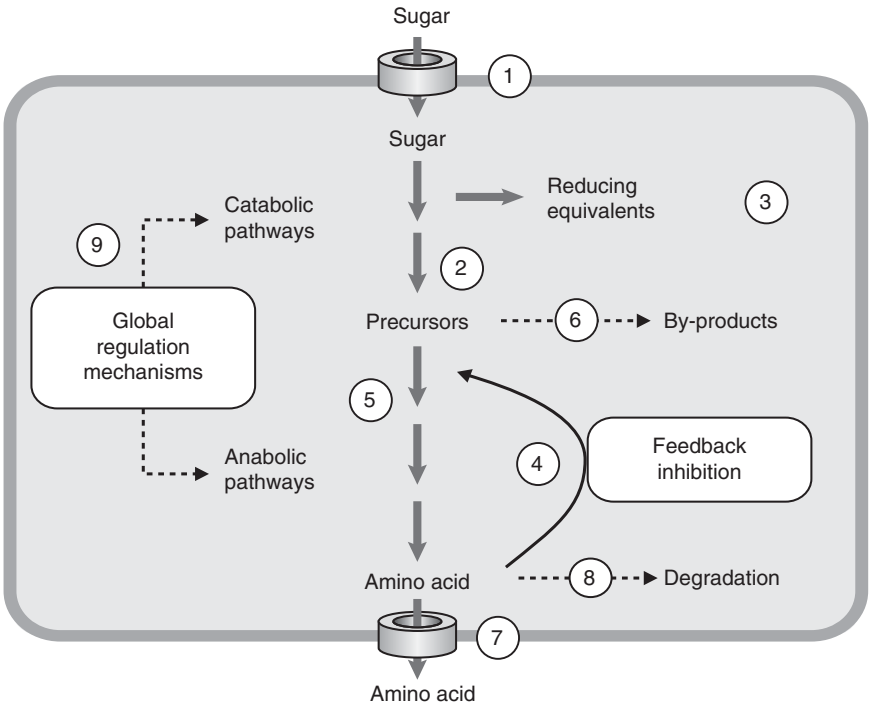


Figure 6.4 Possibilities to improve amino acid synthesis by (1) increasing the sugar uptake, (2) improving precursors supply, (3) improving the provision of reducing equivalents, (4) overcoming feedback inhibition, (5) abolition of limiting synthesis steps, (6) preventing side product formation, (7) improving export, (8) preventing degradation of product, and (9) optimizing global regulatory mechanisms.

6.3 L-Glutamate Synthesis by *Corynebacterium glutamicum*

6.3.1 Synthesis Pathway and Regulation

The Na salt of L-glutamate is a carrier of the specific flavor component "umami," with the molecular recognition of monosodium glutamate being triggered by specific receptors on our tongue. The umami taste is additionally amplified by the ribonucleotides inosine monophosphate and guanosine monophosphate. Although the ribonucleotides are produced by mutants of *Corynebacterium ammoniagenes*, L-glutamate is obtained for the most part from *C. glutamicum*. The discovery of this bacterium in Japan in the middle of the past century, in combination with the observation that it can excrete L-glutamate, was the beginning of the microbial amino acid industry (see Chapter 1), which is now of major economic significance.

Corynebacterium glutamicum metabolizes the absorbed sugar via glycolysis and the pentose phosphate pathway to form phosphoenolpyruvate or pyruvate. Pyruvate is decarboxylated into acetyl-CoA, which – together with oxaloacetate – is then transformed into citrate and introduced into the tricarboxylic acid

cycle (Figure 6.5). Together with ammonium, its intermediate α-ketoglutarate is reductively converted into L-glutamate by glutamate dehydrogenase. The $NADPH + H^+$ needed for this reaction is made available by the previous decarboxylation of isocitrate. L-Glutamate is the central metabolite for providing amino groups for biosynthesis purposes, for example, for transaminase reactions, and is present in the cytosol of *C. glutamicum* in a high concentration of approximately 150 mM. In addition to glutamate dehydrogenase, *C. glutamicum* has glutamine synthetase and glutamine-α-ketoglutarate aminotransferase available as systems for ammonium assimilation. However, labeling experiments have shown that the major proportion of glutamate is formed by glutamate dehydrogenase, which has a high specific activity of 1.5 μmol/min/mg(protein) in *C. glutamicum*. As α-ketoglutarate is removed from the tricarboxylic acid cycle when L-glutamate is formed, oxaloacetate must be provided separately via carboxylation of the C-3 components pyruvate and phosphoenolpyruvate. As it turns out, *C. glutamicum* has two carboxylases for this purpose: pyruvate carboxylase and phosphoenolpyruvate carboxylase (Figure 6.5). For every mol of glucose, 2 mol carbon dioxide are released through pyruvate dehydrogenase and isocitrate dehydrogenase activities, whereby 1 mol is fixed again via the carboxylation of pyruvate or phosphoenolpyruvate. Under nongrowth conditions, a maximum of 1 mol glutamate can therefore be formed for every mol of glucose.

The fact that *C. glutamicum* excretes L-glutamate was discovered by chance: in the middle of the past century, a medium not containing biotin was used to isolate various bacteria. In growth-limiting concentrations of approximately 2 μg biotin/l, *C. glutamicum* excretes L-glutamate while at concentrations above 5 μg/l, excretion no longer occurs. Biotin is a cofactor of acetyl-CoA carboxylase, which is involved in fatty acid synthesis, and decreased acetyl-CoA-carboxylase activity leads to a change in the lipid composition of the cell wall consisting of two lipid layers and peptidoglycan. As we now know, there are many other options for initiating glutamate excretion apart from biotin deprivation, such as adding detergents, increasing the temperature, adding antibiotics such as penicillin or ethambutol, or adding lysozyme. All these triggers of L-glutamate excretion have in common that they interfere with cell wall synthesis and integrity. It thus seemed reasonable to suspect the involvement of a regulatory process in triggering L-glutamate excretion. Although the molecular events are not yet completely understood in detail, recent studies have shown that a regulatory protein does indeed play a key role. This small protein, OdhI, occurs in a phosphorylated or unphosphorylated form (Figure 6.5). The phosphorylation state of OdhI is controlled by protein kinases N (PKN) or a phosphoprotein phosphatase (PPP); these are located in the cytoplasmic membrane. These enzymes apparently detect the state of the cell wall. In the case of a disintegrated cell wall, less unphosphorylated OdhI is present. In this unphosphorylated form, OdhI binds to the OdhA subunit of the ketoglutarate dehydrogenase, reducing the activity of the enzyme to a very low level of residual activity. Thus, α-ketoglutarate is no longer converted into succinyl-CoA but is available to a greater extent for glutamate formation.

The export of glutamate is effected through a mechanosensitive channel, YggB, which has four transmembrane helices. Like comparable channels in *E. coli*, it

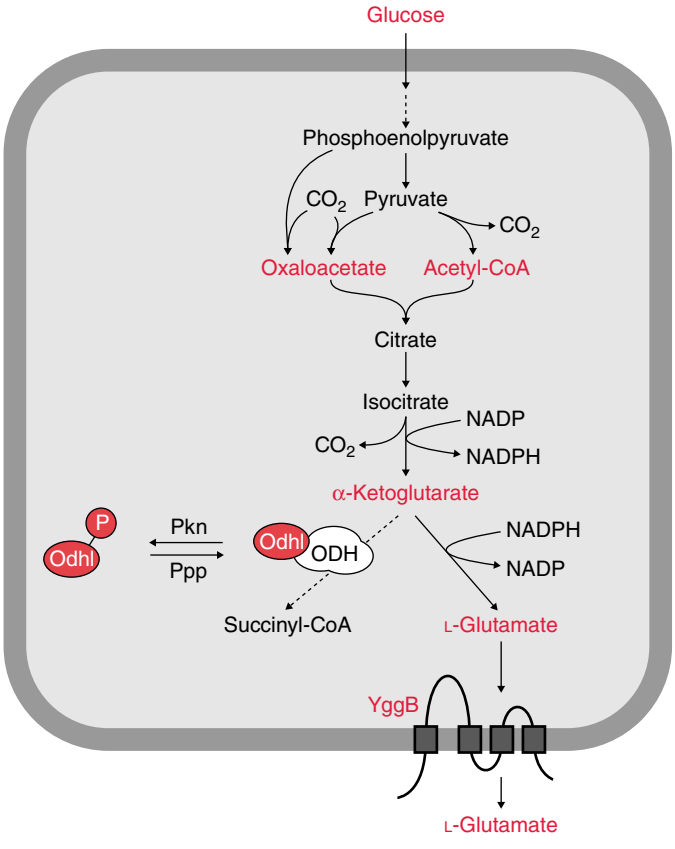


Figure 6.5 Synthesis and excretion of L-glutamate by *Corynebacterium glutamicum*. Inhibition of the oxoglutarate dehydrogenase ODH by the unphosphorylated protein OdhI causes increased L-glutamate synthesis, which becomes excreted by the mechanosensitive channel YggB. The phosphorylation status of OdhI is controlled by the proteins Pkn (protein kinase N) and Ppp (phosphoprotein phosphatase).

presumably serves to maintain the internal cell pressure and osmoregulation. *C. glutamicum* occurs in the soil and therefore has to be able to react to sudden changes of osmotic concentrations. For example, if rain occurs after a dry period, water enters the cell, which could lead to its rupturing if the fast release of osmotically active substances were not possible via mechanosensitive channels. As, according to current knowledge, mechanosensitive channels open and close depending on the membrane tension and lipid environment, the various triggers of glutamate excretion also seem to influence the channel. The deletion of the mechanosensitive channel YggB in *C. glutamicum* substantially decreases glutamate excretion, and the mutants of *C. glutamicum*, which excrete large amounts of glutamate, have mutations in this protein. In summary, a chain of events is triggered in *C. glutamicum* by influencing the cell wall, which fosters both the synthesis of glutamate and its export.

6.3.2 Production Process

In addition to the biotin concentration, the most important process parameters influencing glutamate production are the pH, temperature, and oxygen and ammonium supply. At the beginning of fermentation, the pH in the medium is set to 8.5 using ammonia. As the medium is acidified by glutamate excretion, a base must be added. This is effected during the course of fermentation by continuously adding ammonia. Thus, the pH is constantly maintained at 7.8, while the necessary nitrogen is added simultaneously. In industrial-scale glutamate production, glucose is also added continuously for the most part. For an optimal glutamate yield, sufficient oxygen must also be supplied, as under oxygen-limiting conditions, lactate and succinate are formed in addition to glutamate. In general, process control plays an important role in industrial-scale production, as outlined in Chapter 2 of this book and exemplified for amino acids in Section 6.6.3.

Using *C. glutamicum*, glutamate is produced in stirred bioreactors with a volume of up to 500 m^3 equipped with various measuring and control units. After the growth phase, production is induced, for example, by adding detergents or by increasing the temperature from 32–38 °C. After approximately two days, fermentation is terminated, with a glutamate yield of 60–70% relative to the converted glucose. After separating the cells, the culture supernatant containing the ammonium salt of L-glutamate is transferred into an anion exchanger for further processing. Glutamate binds, and the ammonium released is reused in a further fermentation. Using NaOH, the sodium salt of glutamate elutes from the ion exchanger. It is crystallized, and after further steps such as bleaching and sieving, it is ready for human consumption.

6.4 L-Lysine

6.4.1 Synthesis Pathway and Regulation

L-Lysine is the second most important fermentatively produced amino acid on the global market after L-glutamate. It is used mainly for pig and chicken feed (Table 6.1). The amino acid is produced using *C. glutamicum* and also *E. coli*. Oxaloacetate and pyruvate are components of the carbon skeleton of L-lysine and are provided by the central metabolism. Oxaloacetate is first converted into L-aspartate by means of transamination (Figure 6.6). Aspartate is the substrate of aspartate kinase, which is a very important control point in L-lysine synthesis. The aspartate kinase activity is almost completely inhibited in the wild type of *C. glutamicum* if L-lysine and L-threonine are present at a concentration of approximately 1 mM each. Another important control within the branched synthesis pathway takes place on the aspartate semialdehyde level. On the one hand, aspartate semialdehyde is introduced into the specific L-lysine synthesis pathway together with pyruvate using dihydrodipicolinate synthase activity, and, on the other hand, it is used for the synthesis of L-threonine, L-methionine, and L-isoleucine. At this branch point, the flux into L-lysine synthesis is limited by the dihydrodipicolinate synthase activity. The flux toward L-threonine synthesis

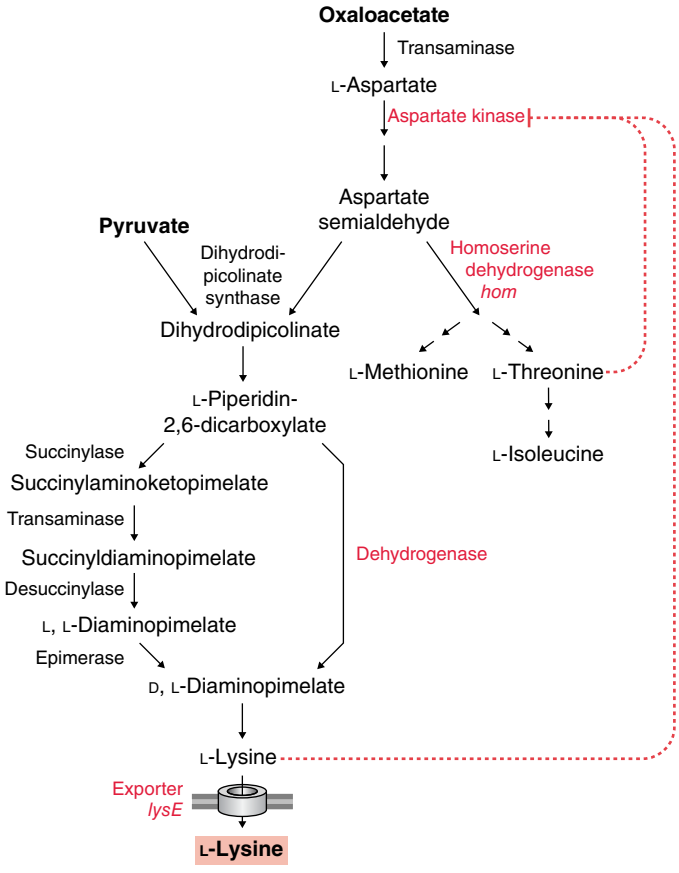


Figure 6.6 L-Lysine synthesis by *Corynebacterium glutamicum*. Within the branched pathway, the key enzyme aspartate kinase is inhibited in its activity by elevated concentrations of L-lysine plus L-threonine (dotted line). The transport of L-lysine from the cytosol through the cytoplasmic membrane is performed by the transporter LysE.

is controlled by inhibiting homoserine dehydrogenase through L-threonine, and, additionally, by repressing the respective *hom* gene through L-methionine. Starting from piperidine dicarboxylate, the second amino group is integrated during the further course of L-lysine synthesis. This is effected either via the dehydrogenase or the transaminase pathway. In the one-step reaction via the dehydrogenase pathway, the amino group is directly derived from ammonia. Via the transaminase pathway in contrast, glutamate is used as an amino donor; the pathway involves a total of four steps and is therefore more complex. These parallel pathways permit *C. glutamicum* to flexibly adapt to different nitrogen availability levels, which is the case in soil, for example, as dehydrogenase has only minor affinity for ammonium. At high ammonium concentrations, lysine is synthesized via the dehydrogenase pathway, whereas at low ammonium concentrations, the more complex transaminase pathway is taken. Meso-diaminopimelate represents a further branch point in the lysine synthesis pathway. It is required to synthesize the

peptidoglycan structure forming the cell wall. A reduction of the MurE activity, which introduces meso-diaminopimelate into peptidoglycan synthesis, causes an increased flux toward L-lysine.

The L-lysine synthesized in the cell is actively excreted by the export protein LysE. As L-lysine has a positive charge, passive diffusion via the cytoplasmic membrane is not possible. With a molecular weight of 25 k, the export protein is relatively small and possesses six transmembrane helices. It is highly likely that the exporter is active as a dimer, as it is known that transporters must have at least 10 transmembrane helices to be functional, and the dimeric structure of proteins related to LysE has been verified. LysE synthesis is controlled by the regulator LysG. Only if the L-lysine concentration inside the cell exceeds 20 mM does this regulator cause the transcription of the exporter gene and in consequence the synthesis of the export protein. As more detailed studies have shown, this system serves to avoid an accumulation of L-lysine in the cell, which may happen when lysine-rich external peptides are available. As many bacteria do, *C. glutamicum* takes up peptides and hydrolyzes them into its amino acid components. If too much L-lysine from such sources is present inside a cell, then high, growth-inhibiting concentrations of more than 1 M L-lysine may occur, as *C. glutamicum* cannot decompose this amino acid. Therefore, under natural conditions, LysE contributes to regulating the cytosolic L-lysine concentration if peptides are exploited. In contrast, other bacteria achieve this by decomposing L-lysine.

6.4.2 Production Strains

Significant L-lysine formation of approximately 20 g/l was achieved many years ago using mutants of *C. glutamicum*, which are auxotrophic for homoserine. In these strains, the intermediate product aspartate semialdehyde can only be converted into L-lysine. If the threonine concentrations added to the medium are low, aspartate kinase is not inhibited in spite of a high lysine concentration. Overproduction of lysine can also be achieved with mutants of *C. glutamicum*, in which aspartate kinase is no longer inhibited. Such mutants can be isolated using the lysine antimetabolite *S*-aminoethyl cysteine (AEC) (Figure 6.3). In the wild type, AEC behaves like lysine, binding to the allosteric center of aspartate kinase, which leads to growth inhibition. In AEC-resistant mutants, however, the allosteric center of aspartate kinase is altered in such a manner that neither AEC nor L-lysine binds to it. Aspartate kinase altered in this way is therefore not subjected to any feedback inhibition and, accordingly, under optimal culture conditions, these mutants of *C. glutamicum* excrete up to 50 g/l L-lysine into the medium.

As already mentioned, strain development has reached a new dimension, thanks to genome analyses of conventionally obtained producers. Genome sequencing of conventionally obtained producers has led to the identification of at least five mutations decisive for L-lysine formation. By integrating these point mutations into the wild-type chromosome, successively increased L-lysine formation can be achieved. Firstly, the nucleotide cytosine in the aspartate kinase gene at position 270 302 of the *C. glutamicum* chromosome, which has a total of 3 309 401 base pairs, was replaced by thymine. The codon altered in this manner

Table 6.3 Construction of an L-lysine producer, starting from the wild type of *C. glutamicum* (WT).

Strain	Additional mutation	Relevant enzyme	Flux control	L-Lysine (g/l)
WT				0
Lys-1	WT + *lysC*-T311I	Aspartate kinase	Initiating reaction	50
Lys-2	Lys-1 + *hom*-V59A	Homoserine dehydrogenase	Branch point	70
Lys-3	Lys-2 + *pyc*-P458S	Pyruvate kinase	Precursor supply	80
Lys-4	Lys-3 + *gnd*-S361P	6P-Gluconate dehydrogenase	NADPH supply	89
Lys-5	Lys-4 + *mqo*-W224-stop	Malate:quinone oxidoreductase	Precursor supply	93

By successive introduction of five point mutations into the chromosome, an optimal flux control and provision of NADPH is achieved.

causes the threonine in position 311 of the aspartate kinase to be replaced by isoleucine (Thr-311-Ile). The enzyme is thus no longer inhibited by L-lysine plus L-threonine. The resulting strain excretes as much as 50 g/l L-lysine (Table 6.3). The additional mutation Val-59-Ala in homoserine dehydrogenase leads to decreased enzyme activity, so that more aspartate semialdehyde is available for L-lysine synthesis, resulting in an increase to 70 g/l L-lysine. Although these mutations concern the L-lysine synthesis pathway itself, further mutations are located in the central metabolism. The mutation Pro-458-Ser of pyruvate kinase found in conventionally obtained producers causes an increase in the oxaloacetate provided. When this mutation is introduced in addition to those found in *lysC* and *hom*, then the strain accumulates 80 g/l L-lysine. A further mutation identified in the conventional producer was the mutation Ser-361-Pro in 6P-gluconate dehydrogenase. This enzyme is located within the pentose phosphate pathway, and the mutation causes a decreased inhibition of the enzyme by elevated $NADPH + H^+$ levels. When this mutation is introduced into the chromosome, the provision of reducing equivalents for biosynthesis is improved, increasing L-lysine formation further. Finally, the inactivation of malate:quinone oxidoreductase causes a further increase. This enzyme catalyzes the quinone-dependent conversion of oxaloacetate into malate, so that by inactivating malate:quinone oxidoreductase, more oxaloacetate is available for L-lysine formation. As can be seen, providing the precursors pyruvate and oxaloacetate is important for increasing the formation of L-lysine. It is therefore not surprising that increasing the pyruvate-carboxylase activity by overexpression boosts L-lysine formation, as does the deletion of phospho*enol*pyruvate carboxykinase, which catalyzes the decomposition of oxaloacetate to phospho*enol*pyruvate. Further metabolic engineering and the targeted introduction of mutations facilitates the formation of up to 120 g/l L-lysine at a productivity of 4.0 g/l/h.

6.4.3 Production Process

In the past, molasses were used as the main carbon and energy source for the industrial-scale production of L-lysine. However, molasses from the sugar industry contain waste products in addition to sugar, and its seasonal variations are considerable so that the process is not well reproducible. For this reason, mainly pure glucose or sucrose is used as a substrate today. Ammonium sulfate or aqueous ammonium hydroxide serve as a source of nitrogen. Biotin is also added, as *C. glutamicum* cannot synthesize this coenzyme itself, and glycine betaine for the osmotic stabilization of the cells. Starting from a shake flask, seed cultures are cultivated in bioreactors of increasing size, up to the reactor used for production, which may have a volume of up to 500 m^3. The inoculation density exceeds 0.1 g cell mass per liter as otherwise the lag phase of the process is too long. The strains display a surprisingly high specific production rate, which can achieve 4 g/l/h. Furthermore, they have a high tolerance with respect to hydrostatic pressure differences and shear forces in the fermenter. Thus, in general, it is the technical potential of the reactor system – such as cooling and oxygen supply, for example – which is limiting, rather than that of the production strain. The course of a typical L-lysine fermentation is depicted in Figure 6.7. Once the initial supply of sugar is consumed, more is added continuously and L-lysine accumulates at concentrations far exceeding 170 g/l. By using ammonium sulfate as a nitrogen source, the excreted amino acid is neutralized during the course of the fermentation process, so that L-lysine is present as a sulfate salt. The output of the entire production facility can be further increased by not completely emptying the reactor once the desired final concentration or maximum fill level is reached, but retaining 10–20% of the volume; this can be directly used as the inoculum for a new batch process, eliminating the lag phase. This "repeated fed-batch" process increases the efficiency but requires the production strain used to be stable. Because of the high costs associated with the sugar, the yield is essential for the efficiency of the overall process. It greatly exceeds 55 g L-lysine per 100 g glucose. Theoretically, up to 75 g of L-lysine per 100 g of glucose is possible. Because of the high cost pressure, good reproducibility is desirable so that, in spite of the large volumes and various liquid flows, the overall process is usually subjected to fluctuations of less than 5%.

Two methods have been established for downstream processing (Figure 6.8). Crystalline L-lysine is obtained after separation of the cells by means of ion

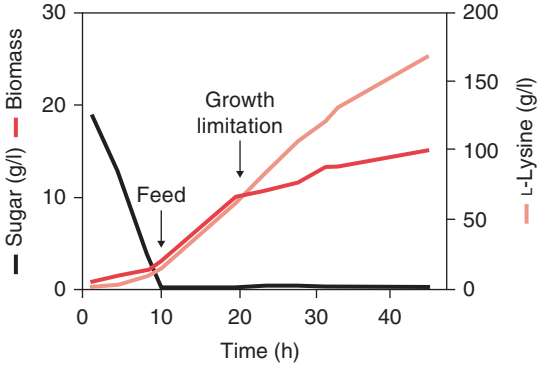


Figure 6.7 Time course of L-lysine and biomass formation in a production plant. After consumption of the initially supplied sugar after 10 hours, new sugar is continuously added.

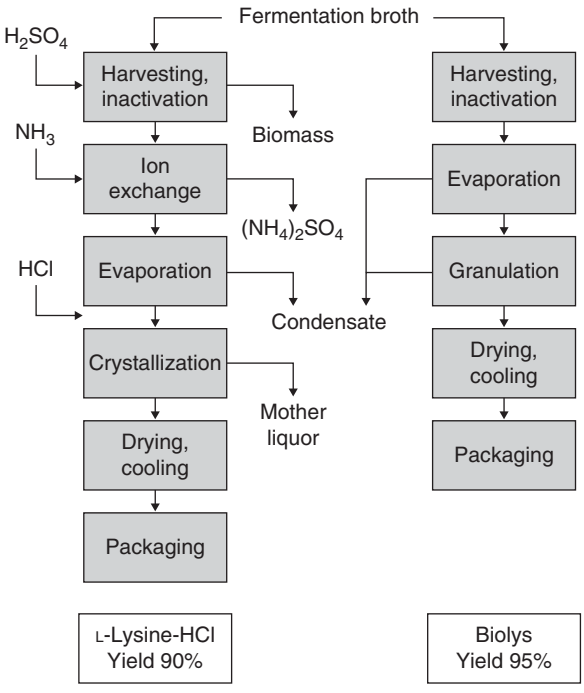


Figure 6.8 Two different work up procedures to derive formulations of L-lysine suitable as feed supplement.

chromatography with subsequent evaporation, crystallization, and packaging. L-Lysine is commercially distributed as L-lysine·HCl salt because it is less hygrosopic than $(L\text{-lysine})_2SO_4$. The competing method involves evaporating the entire fermentation medium, including the cells, by means of spray drying and subsequent granulation and packaging. As *C. glutamicum* is a GRAS organism ("generally recognized as safe"), the organism itself can be used as a feedstuff, representing an additional gain for feed formulation. The process is very cost efficient when it comes to investment costs as, for example, no chromatography columns are necessary, waste products are avoided, and low losses are guaranteed (Figure 6.8). The product thus obtained is commercially distributed under the name Biolys™.

6.5 L-Threonine Synthesis by *Escherichia coli*

6.5.1 Synthesis Pathway and Regulation

L-Threonine is used as a feedstuff additive and is produced by *E. coli*. It is synthesized via a short pathway consisting of only five steps (Figure 6.9). However, the intermediate aspartate semialdehyde is also needed for L-lysine synthesis, and the intermediate homoserine for L-methionine synthesis. Additionally, L-threonine is also a precursor in L-isoleucine synthesis. It appears logical that this synthesis pathway, which is embedded in the synthesis of several amino acids, requires special regulation. In *E. coli*, this is solved by the fact that three isoenzymes with aspartate kinase activity are present, which are each specifically inhibited: one is inhibited by L-threonine, one by L-lysine, and one by

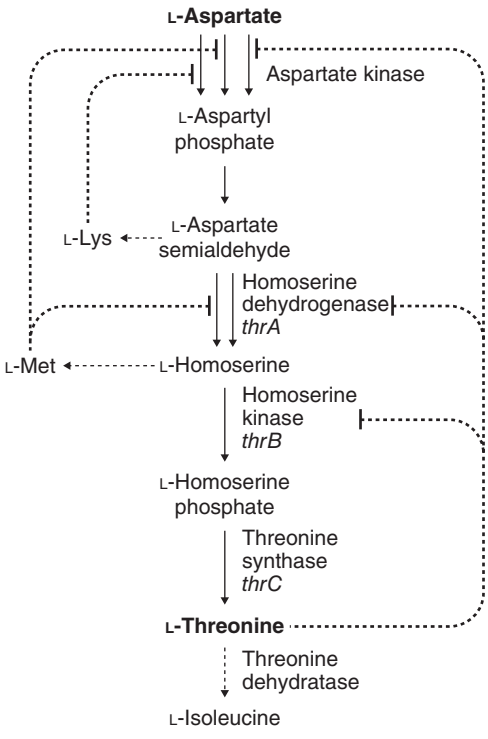


Figure 6.9 L-Threonine synthesis in *Escherichia coli* sharing steps common to the synthesis of L-lysine, L-methionine, and L-isoleucine. Parallel arrows denote isoenzymes, dotted arrows denote feedback inhibition of enzyme activities.

L-methionine. Furthermore, there are two homoserine-dehydrogenase activities, of which one is inhibited by L-threonine, and the other by L-methionine. Additionally, the corresponding genes are arranged in transcription units, so that synthesis depending on amino acid availability is also guaranteed on the level of gene expression. The operon relevant for L-threonine production is *thrABC*, whereby *thrA* codes for a bifunctional enzyme with aspartate kinase and homoserine-dehydrogenase activity, *thrB* for homoserine kinase, and *thrC* for threonine synthase. This operon is subjected to strong transcription control by means of attenuation. The relevant leader peptide located in front of the *thrABC* transcript is Thr-Ile-Thr-Thr-Thr-Ile-Thr-Ile-Thr-Thr. In the cell, the loading state of $tRNA^{Thr}$ and $tRNA^{Ile}$ is detected via the synthesis velocity of this leader peptide. If the tRNAs are not loaded with the appropriate amino acid, then the leader peptide cannot be formed and the transcription of the operon is increased at least 10-fold. In contrast, the transcription is terminated prematurely if too many loaded tRNAs are present.

6.5.2 Production Strains

The keys to producing L-threonine using *E. coli* are (i) the expression of the *thrABC* operon with enzyme activities that can no longer be inhibited, (ii) inhibiting the further conversion of L-threonine into L-isoleucine, (iii) inhibiting the decomposition of L-threonine, and finally (iv) increasing the export of L-threonine.

The gene *thrA* codes for aspartate kinase and homoserine dehydrogenase. The inhibition of these enzyme activities by L-threonine was suppressed. In order to guarantee high expression, the attenuator section of the operon was deleted, preventing the transcription of the *thrABC* operon from being cancelled prematurely. As at high levels of L-threonine synthesis, L-isoleucine was also formed, a mutant with decreased substrate affinity was isolated. This mutant requires L-isoleucine to be added to the medium at low L-threonine concentrations, but at high L-threonine concentrations, it forms just enough L-isoleucine to enable growth. *E. coli* is capable of decomposing L-threonine and forms a threonine dehydrogenase for this purpose. Inactivating the corresponding gene, *tdh*, further improves L-threonine accumulation. A recent further strain development concerns the observation that strains that form large amounts of L-threonine exhibit poorer growth. Mutants were successfully isolated that grow better than the original strain when very high L-threonine concentrations are added. As it turns out, this is due to a base substitution in the promoter section of the gene *rhtA*. This gene codes for an export carrier of the drug/metabolite exporter (DME) family with 10 transmembrane helices. The promoter mutation leads to a near-doubling of the L-threonine export rate, so that high L-threonine concentrations inside the cell are avoided, which are evidently disadvantageous for the cell. The fact that the L-threonine export of *E. coli* is very efficient in comparison to that of *C. glutamicum* is the decisive reason for using *E. coli* strains for the production of L-threonine.

6.5.3 Production Process

The L-threonine producer is cultivated in a simple salt medium using glucose or sucrose as a substrate. After inoculation, more sugar is added continuously once the initial supply is consumed. Under pH control, additional ammonia or ammonium hydroxide is introduced as a source of nitrogen. The feeding strategy is simple compared to that for L-lysine formation as no basic product accumulates and a counterion such as sulfate is not required. After two to three days, fermentation is complete. L-Threonine is now present in the culture medium at concentrations exceeding 100 g/l, so that it already crystallizes to a certain extent. Relative to the sugar used, the yield amounts to approximately 60%. Reprocessing the product is relatively simple because of its low solubility and the small salt load. After separating the cells, the solution containing the L-threonine is evaporated slightly and the crystallization of the amino acid initiated by cooling. Separation and drying of the crystals yields L-threonine of a purity exceeding 90%. Recrystallization is carried out if high-purity L-threonine is needed.

6.6 L-Phenylalanine

6.6.1 Synthesis Pathway and Regulation

Like L-tyrosine and L-tryptophan, L-phenylalanine is an aromatic amino acid. They share a common synthesis pathway up until the stage of chorismate. As can

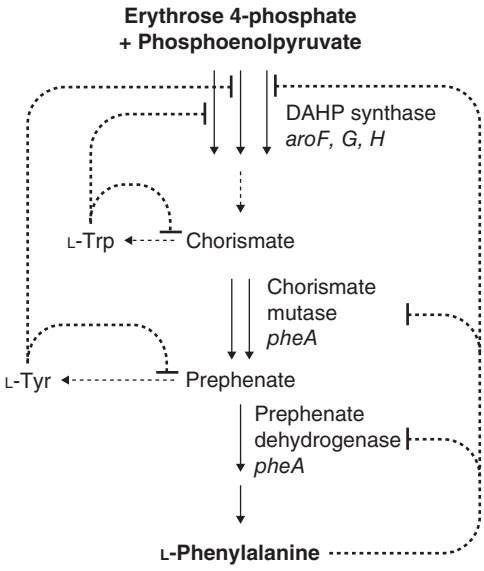


Figure 6.10 Synthesis of the aromatic amino acids in *Escherichia coli*. The key enzyme is the 3-deoxy-D-arabino-heptulosonate-7-phosphate (DHAP) synthase, of which three isoenzymes exist in *E. coli*.

be seen in Figure 6.10, the precursors for aromatic biosynthesis are erythrose 4-phosphate and phosphoenolpyruvate, which are converted in the first step into 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP). Six further enzymes catalyze the conversion of this first intermediate product into chorismate. This intermediate is then converted into L-tryptophan via anthranilate or into L-phenylalanine and L-tyrosine via prephenate.

This aromatic biosynthesis pathway is subject to complex control in *E. coli*. Thus, the bacterium has three DAHP synthase isoenzymes, which are encoded by the genes *aroF*, *aroG*, and *aroH*, with the isoenzyme AroG representing approximately 80% of the entire DAHP synthesis activity. The synthesis and activity of each of these three isoenzymes is regulated by one of the three aromatic amino acids, so that *E. coli* usually does not overproduce these amino acids. Another important step in the regulation of the final phenylalanine synthesis branch is the bifunctional enzyme PheA with chorismate-mutase and prephenate-dehydratase activities, as these activities are inhibited by L-phenylalanine. Additionally, the *pheA* gene expression is also controlled through the loading state of the $tRNA^{Phe}$ by means of attenuation.

6.6.2 Production Strains

Producer strains have feedback-resistant DAHP activity, encoded by *aroG* or *aroF*, and feedback-resistant enzyme activities encoded by *pheA*. Generally, producers are auxotrophic for L-tyrosine. One reason for this is that because of the short pathway – consisting of only two steps from prephenate to L-tyrosine – when large amounts of L-phenylalanine are formed, small amounts of L-tyrosine would also be formed. This is, of course, undesirable. Another reason is that because of the L-tyrosine auxotrophy, the growth can be controlled in a targeted manner by adding L-tyrosine (see 6.6.3). In some *E. coli* strains, a

temperature-sensitive repressor of the λ bacteriophage together with the λP_L promoter, an inducible expression of *pheA* and *aroF* is achieved. Through an increase in temperature, this permits a very high enzyme activity in the production phase, which reduces stability problems due to high enzyme concentrations and activities. This means that the cultivation steps up until the inoculation of the bioreactor can be effected at low expression of the key genes, and only during the actual fermentation process are the genes strongly expressed.

6.6.3 Production Process

As with the other amino acids also for formation of high levels of L-phenylalanine, sophisticated control of the metabolism during fermentation is necessary. There are two reasons for this: on the one hand, the carbon flux must be optimally distributed among the four products of glucose metabolization, i.e. L-phenylalanine, cell mass, acetic acid, and CO_2. On the other hand, the activity of the cells continuously alters during the course of the culture, so that the process parameters have to be constantly adapted during cultivation. Figure 6.11 shows the typical course of L-phenylalanine production. The main problem is that *E. coli* tends to excrete acetic acid in the case of a glucose surplus or lack of oxygen. In order to avoid this, an adaptive sugar-supply strategy was developed, in which data and fluxes on oxygen consumption, sugar concentration, and biomass concentration are recorded online. These data permit the optimal adaptation of the sugar supply during the process. After the initial supply of sugar is consumed in phase 1, the cells enter phase 2, during which sugar is continuously added. As the biomass increases, the rate of added sugar also increases during this phase. Thanks to this adaptive sugar-supply strategy, excessive glucose concentrations are avoided because – as mentioned above – this would lead to the formation of acetic acid. Simultaneously, the glucose concentration should not fall below a certain level as this would

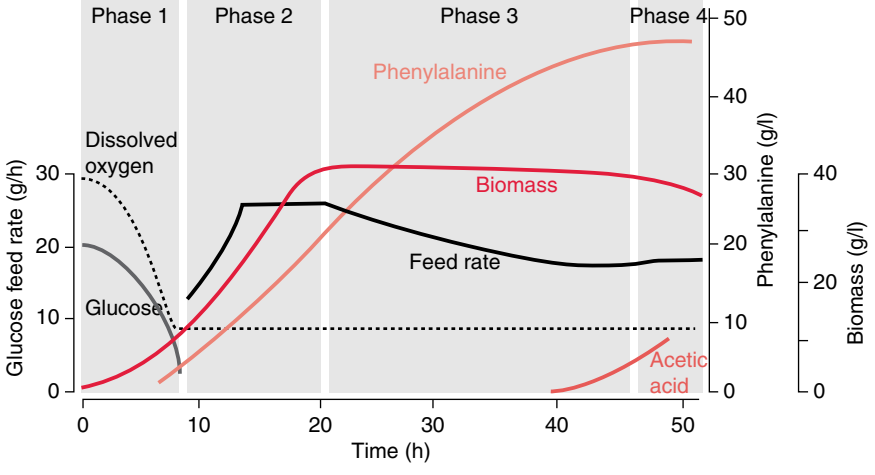


Figure 6.11 Four phases of L-phenylalanine production with *Escherichia coli*. The different phases are characterized by a different metabolic condition of the cell, requiring the appropriate adjustment of oxygen and sugar supply.

lead to high levels of CO_2 formation. Once the initially present L-tyrosine is consumed, the cells enter phase 3. As mentioned, all L-phenylalanine producers are auxotrophic for L-tyrosine. Therefore, the L-tyrosine concentration initially present in the fermentation determines the concentration of the biomass in phase 3, during which glucose is efficiently converted into L-phenylalanine. In phase 3, the metabolic capacity of the cells diminishes slightly, which as a consequence leads to a decreased sugar supply. Because of the further decreasing metabolic activity of the cells, a significant amount of acetic acid is ultimately excreted, with the cells entering phase 4, during which a substantial L-phenylalanine accumulation no longer occurs, so that the process is finally terminated. This example of amino acid production shows that within only 2.5 days, a very high L-phenylalanine concentration can be achieved by means of a sophisticated feeding strategy with adaptive control. Values of 50 g/l L-phenylalanine with a yield of 28% relative to the sugar added have been reported.

6.7 Outlook

The production of L-amino acids is a standard process in microbial biotechnology. Initially, only modest concentrations were achieved and small amounts produced. However, demand is increasing enormously. Global demand amounted to 6.5 megatonnes in 2014 and is expected to reach 10 megatonnes by 2022. It is therefore understandable that production processes must be improved constantly. This leads to exacting requirements, combining molecular, bioinformatics, and process engineering aspects. Thus, optimal use of regenerative raw materials guarantees that amino acids can be obtained in a competitive manner.

The quantitatively most important essential amino acid L-methionine is still produced as D/L-racemate by a chemical process. Patent applications by leading companies suggest that *E. coli* might have the potential to compete on the basis of renewable substrates. Two challenging problems are C1 metabolism, namely methylation of the sulfur, and the reductive metabolism needed for conversion of sulfate. However, for L-cysteine, the second sulfur-containing amino acid, and precursor of L-methionine, this problem had been solved. Until the beginning of the twenty-first century, L-cysteine was isolated exclusively from human hair, which is rich in L-cysteine. The *E. coli* metabolism has been successfully adapted by metabolic engineering, so that approximately one quarter of all L-cysteine produced is obtained microbially. Guaranteeing the availability of sulfur inside the cells was a particular challenge. Sulfate or thiosulfate is used as a source of sulfur during microbial synthesis; 12 genes are involved in their uptake and reduction to sulfide. The production process using *E. coli* involves a feeding strategy where thiosulfate is used as a source of sulfur. High concentrations exceeding 30 g/l of L-cysteine are formed, and under aerobic conditions, it precipitates out in the form of the dimer as cysteine. This is purified and reduced back into L-cysteine by electrolysis.

Further Reading

Becker, J., Zelder, O., Häfner, S. et al. (2011). From zero to hero – design-based systems metabolic engineering of *Corynebacterium glutamicum* for L-lysine production. *Metab. Eng.* 13: 159–168.

Debabov, V.G. (2003). The threonine story. *Adv. Biochem. Eng.* 79: 114–136.

Eggeling, L. (2009). Microbial metabolite export in biotechnology. In: *Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology* (ed. M.C. Flickinger). Wiley & Sons, Inc.

Eggeling, L. and Bott, M. (2015). A giant market and a powerful metabolism: L-lysine provided by *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* 99: 3387–3394.

Ikeda, M., Ohnishi, J., Hayashi, M., and Mitsuhashi, S. (2006). A genome-based approach to create a minimally mutated *Corynebacterium glutamicum* strain for efficient L-lysine production. *J. Ind. Microbiol. Biotechnol.* 33: 610–615.

Konstantinov, K.B., Nishio, N., Seki, T., and Yoshida, T. (1991). Physiologically motivated strategies for control of the fed-batch cultivation of recombinant *Escherichia coli* for phenylalanine production. *J. Ferment. Bioeng.* 71: 350–355.

7

Vitamins, Nucleotides, and Carotenoids

Klaus-Peter Stahmann[1] *and Hans-Peter Hohmann*[2]

[1] *Institut für Biotechnologie, Brandenburgische Technische Universität Cottbus-Senftenberg, Universitätsplatz 1, 01968 Senftenberg, Germany*
[2] *Oberer Baselblick 8, 79540 Lörrach, Germany*

CHAPTER MENU

7.1 Application and Economic Impact

Vitamins are chemically and functionally a group of heterogeneous compounds. Like the essential amino acids, humans and domestic animals lack their biosynthesis pathways so that they have to be taken up with food or feed. Although the daily need for L-methionine is about 1 g, the need for vitamin B_{12} is only 1 μg, 6 orders of magnitude smaller. The reason for that is that vitamins are catalytically active as cofactors in enzymes. As only a low dose is needed for the benefit, e.g. domestic animals that are adequately supplied with vitamins grow faster, a better selling price is possible.

The microbial production of vitamin B_2 shows that chemical production processes, although economically successful for decades, have been replaced by the application of Industrial Microbiology.

The market volumes are very different too. Vitamin C is in the first place, with an annual production of more than 100 000 metric tons [MT]. Last with about 10 MT/yr is vitamin B_{12} produced by *Pseudomonas denitrificans*, but it sells at a much higher price per kilogram.

Vitamins are most frequently produced chemically. Out of 20 vitamins today, only three namely vitamin C, vitamin B_2, and vitamin B_{12}, are produced by microorganisms at an industrial scale.

Industrial Microbiology, First Edition.
Edited by David B. Wilson, Hermann Sahm, Klaus-Peter Stahmann, and Mattheos Koffas.

Table 7.1 Selected industrial commodities produced by bacteria or fungi in 2010.

Products	Microbial production	
	Bacterial or fungal species	**t/yr**
Vitamins		
C	*Gluconobacter oxydans* (plus *Ketogulonicigenium vulgare*)	> 100 000
B_2	*Ashbya gossypii* order *Bacillus subtilis*	< 10 000
B_{12}	*Pseudomonas denitrificans*	> 10
K_2	*Bacillus subtilis*	?
Carotenoids		
β-Carotin	*Blakeslea trispora*	?
Nucleotides		
IMP/GMP	*Bacillus subtilis* order *Corynebacterium ammoniagenes* plus *Escherichia coli*	> 10 000
PUFAs		
Arachidonic acid	*Mortierella alpina*	?

In the multistep chemical processing of vitamin C, either one or two steps are catalyzed by bacteria. For vitamin B_2 and inosine-5′-P, two independent processes exist for each product, each developed for a different microorganism.

Nucleotides, i.e. inosine-5′-phosphate (IMP) and guanosine-5′-phosphate (GMP), function as flavor enhancers in food. Similar to the flavor enhancer, L-glutamate, which is not an essential amino acid but is produced microbially, the successful application of *Bacillus subtilis* for the production of IMP stimulated microbiologists to use the same bacterium for the production of vitamin B_2.

Carotenoids are used as natural dyes. Although the most expensive carotenoid, astaxanthin, is produced chemically or extracted from microalgae, both a chemical and a microbial process is used for β-carotene. The latter, carried out by a fungus, is offered to costumers preferring a biosynthetic pathway over chemical synthesis for foods sold at higher prices.

The compounds discussed in this chapter are not only heterogeneous in chemical structure and physiological function. However, the roles of the microorganisms during their industrial production are very different. On the one hand, they are natural overproducers, e.g. the filamentous fungus *Ashbya gossypii*, and on the other hand, production strains were developed by multiple mutagenesis, e.g. *B. subtilis*. Both routes became economical after genetic engineering allowed modification of genes for specific biosynthetic pathways.

For the production of vitamin C, the plant biosynthesis pathway is not used but a chain of chemical reactions with a whole cell biotransformation was developed. Thus, only a selective oxidation is performed by *Gluconobacter oxydans* (Table 7.1).

Table 7.1 also shows PUFAs (poly unsaturated fatty acids) that are not discussed in detail. They play a role in mother's milk. The biosynthesis of, e.g., arachidonic acid is not yet active in the sucking baby, but the hormone synthesized from this fatty acid is needed for brain development, so arachidonic acid is an additive for

infant food. One route to obtain this C20 fatty acid containing four double bonds is isolation from the mycelium of the ascomycete *Mortierella alpina*.

The production of vitamin K with *B. subtilis* is not discussed as too little is known concerning strain development. Additionally, humans can absorb it from their gut flora. L-Carnitine was omitted, as human anabolism releases sufficient amounts. Finally, niacin is not discussed as an enzyme rather than a microorganism is applied in its production process.

Prices paid for vitamins on the world market range from US$10 per kg, i.e. for vitamin C, to over US$20 for vitamin B_2, up to several thousand dollars for vitamin B_{12}. Prices are highly variable. In the year 2008, prices for vitamin C were unexpectedly high as the amount from Chinese companies that delivered 80% of the world production was reduced. Later prices fell below US$10 per kg. The selling prices for vitamins also depend on their application in feed, food, or pharmaceutical industry. For these industries, vitamins are produced in differing purity and formulations.

7.2 L-Ascorbic Acid (Vitamin C)

7.2.1 Biochemical Significance, Application, and Biosynthesis

About 1550 before Christ, the symptoms of scurvy, i.e. loss of teeth or gum bleeding, was described on an Egyptian papyrus. Scurvy was a severe phenomenon of malnutrition for many centuries especially for seamen. Near the beginning of the twentieth century, it became clear that the reason was an insufficient supply of a nutritional factor that was named vitamin C or L-ascorbic acid. Guinea pigs in addition to humans are one of the few mammals that have to take up L-ascorbic acid from food. This substance was identified after purification from lemon juice in the 1930s; afterward, the chemical structure was solved and confirmed by chemical synthesis. L-Ascorbic acid is a cofactor needed for oxidases involved in the biosynthesis of collagen, L-carnitine, and adrenaline. The stereoisomeric form D-ascorbic acid has no antiscurvy activity. The symptoms of scurvy are caused by a lack of hydroxylation in L-proline and L-lysine residues so that the pro-alpha collagen chains cannot form stable collagen triple helices. As L-ascorbic acid is also active as an efficient scavenger of dangerous radicals that are formed as side products in the mitochondrial respiratory chain, the daily dose recommended for an adult by World Health Organization (WHO) is 60 mg. This dose is significantly higher than that recommended for other vitamins.

The biosynthesis of L-ascorbic acid starts from D-glucose and continues in plants through L-galactono-1,4-lactone. Afterwards, there is an inversion of the carbon skeleton in which C1 of the glucose becomes C6 in the target molecule. In the animal biosynthetic pathway, L-gulono-1,4-lactone is formed through several epimerization reactions. The auxotrophic phenotype in a few mammals results from mutations in the gene encoding L-gulono-1,4-lactone oxidase, which converts L-gulono-1,4-lactone into L-ascorbic acid.

With more than 100 000 MT annually, L-ascorbic acid is quantitatively the most important vitamin. In contrast to many other vitamins, only a minor part (10%) of the industrially produced L-ascorbic acid is used for domestic animal feed. The major part is used for pharmaceutical purposes (50%) and human nutrition

(40%). In oxidation-sensitive nutrients, e.g. fruit juices, an unwanted color change is prevented, an acidic pH is stabilized, and the taste is improved.

7.2.2 Regioselective Oxidation with Bacteria in the Production Process

The industrial production of L-ascorbic acid began with its extraction from fruit. The first chemical production process, established in 1933, was based on L-xylose, but already in 1934, the famous **Reichstein process** was developed. The main idea of that process is that the C2 position of the D-glucose becomes the C5 position in the L-ascorbic acid molecule. Maintaining the stereochemistry the D-hydroxy group at C2 converts to L-hydroxy at C5.

In detail, the Reichstein process starts with D-glucose which is catalytically hydrogenated to D-sorbitol (Figure 7.1). This sugar alcohol is subsequently put under strong acidic condition brought into contact with *Gluconobacter oxidans*, a Gram-positive bacterium originally called *Acetobacter suboxydans*. A pyrroloquinoline quinone (PQQ)-dependent sorbitol dehydrogenase, which oxidizes the C5 hydroxyl group of D-sorbitol with remarkable selectivity, is highly active in *G. oxidans* leading to rapid conversion of D-sorbitol to L-sorbose with a yield above 90%. The process can be run continuously with a substrate concentration above 200 g/l.

The subsequent chemical oxidation of the primary hydroxyl group at C1 of L-sorbose to the carboxylate group after acetone protection of the remaining hydroxyl groups followed by the removal of the protection groups leads to 2-keto L-gulonic acid. L-Gulonic acid is isolated, transferred into an organic solvent, and under acidic or alkaline conditions converted to 1,4-lactone. The 1,4-lactone spontaneously enolizes to L-ascorbic acid.

After establishing the Reichstein process as the industrial standard for the production of L-ascorbic acid, several trials were started to oxidize the primary C1 hydroxyl group by biocatalysis. Indeed, *G. oxydans* is able to perform that reaction, but at a relatively low rate, even after overexpression of the genes encoding a flavin adenine dinucleotide (FAD)-dependent sorbose dehydrogenase and an nicotinamide adenine dinucleotide (NAD)-dependent sorbosone dehydrogenase, which prevented the industrial application of *Gluconobacter*.

Around 1970, *Ketogulonicigenium vulgare* was isolated. This Gram-positive bacterium can oxidize L-sorbose to 2-keto L-gulonic acid via the intermediate L-sorbosone (Figure 7.1). For that oxidation, the bacterium uses several PQQ-dependent or FAD-dependent dehydrogenases the genes of which have been isolated and characterized. Since these dehydrogenases are not very substrate specific they can also oxidize D-sorbitol via D-glucose to D-gluconate. To avoid that side reaction, the biocatalytic oxidation of D-sorbitol to 2-keto L-gulonic acid is performed in two separated steps. As in the Reichstein process, the first oxidation is performed with *G. oxydans* at C2. Subsequently, C1 is oxidized with *K. vulgare*. To become stimulated for optimal productivity, *K. vulgare* needs cocultivation with other bacteria, i.e. *Bacillus megaterium*. A plausible molecular explanation for this well-documented phenomenon has not been provided.

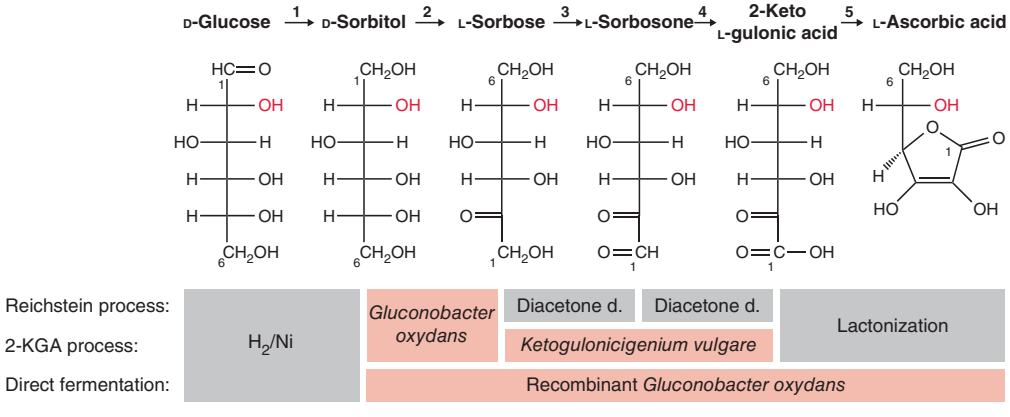


Figure 7.1 All biotransformations in industrial processes for L-ascorbic acid production start with D-sorbitol. In the Reichstein process, only step 2 is microbial. The subsequent oxidations are chemical. Therefore, the hydroxyl groups that should not be oxidized are protected as diacetone-derivatives. The formed 2-keto L-gulonic acid is chemically converted to L-ascorbic acid in step 5. Since the 1990s, steps 3 and 4 are also performed by a microorganism. A future development could be that reaction step 4 is directly leading to a 1, 4-lactone instead of the 2-keto L-gulonic acid. The lactone can spontaneously convert to L-ascorbic acid via keto-enol tautomerization. The OH-group printed in red helps for orientation because the counting inverts after reduction at the original C1 and oxidation at C5.

Because of the formation of a relatively strong organic acid (pK_S of 2-keto L-gulonic acid is 2.5), permanent titration is needed to avoid a pH toxic for the bacteria. With sodium hydroxide as a base, the sodium salt of 2-keto L-gulonic acid, which can accumulate to concentrations above 100 g/l, is isolated from the culture, purified, and as in the Reichstein-Process converted to the end product in an organic solvent. The advantage of the two-step, purely microbial oxidation, the so-called **2-KGA process**, in comparison to the Reichstein process, is the reduced number of process steps and the avoidance of protective groups. The investment costs for the production plant, as well as the operating costs for chemicals and energy consumption, are reduced.

A newer process variant allows formation of 2-keto gulonic acid from D-sorbitol in a single fermenter. This is possible since the specific activity of *G. oxydans* to convert D-sorbitol to L-sorbose is much higher than the specific activity of *K. vulgare* to oxydize L-sorbose to 2-KGA. Provided that the right ratio between the *G. oxydans* and *K. vulgare* in the inoculum is met D-sorbitol is already oxidized to L-sorbose by the former before the latter has the chance to convert D-sorbitol to D-glucose and L-gulose in considerable amounts.

The next step in the development of the industrial production of L-ascorbic acid will probably be a fermentation process directly leading to vitamin C. That would supersede the chemical conversion of 2-keto gulonic acid. After discovery and cloning of genes encoding dehydrogenases converting L-sorbosone directly to L-ascorbic acid, the crucial tool might already be found.

7.3 Riboflavin (Vitamin B_2)

7.3.1 Significance as a Precursor for Coenzymes and as a Pigment

Vitamin B_2 is a yellow (Latin: *flavus*), water-soluble pigment. The chromophore, an isoalloxazine ring, is bound to the sugar alcohol ribitol explaining the trivial name riboflavin (Figure 7.2). The daily need of 2 mg for humans can be covered with a cup of milk. Riboflavin taken up in excess cannot be stored but is directly excreted via the kidney. Therefore, the urine is pending on the vitamin B_2 content of the food, more or less intense in yellow color.

Important for man and domestic animals, riboflavin is the precursor of the coenzymes flavin adenine mononucleotide (FMN) and FAD. Oxidoreductases use both as prosthetic groups. In cryptochromes, proteins needed for synchronization of the circadian clock with the environment, flavo groups are functioning as light receptors.

Elucidation of the chemical structure and chemical synthesis of riboflavin succeeded in the 1930s. Multistage chemical production processes for the vitamin using the raw materials dimethyl aniline, D-glucose, later D-ribose, phenyldiazonium chloride, and barbituric acid were developed which were economically successful until the 1990s. Microbial production processes were developed already in the 1940s but could not stand their ground in competition with chemistry. Economic success was achieved finally in 1990 with *A. gossypii* as production

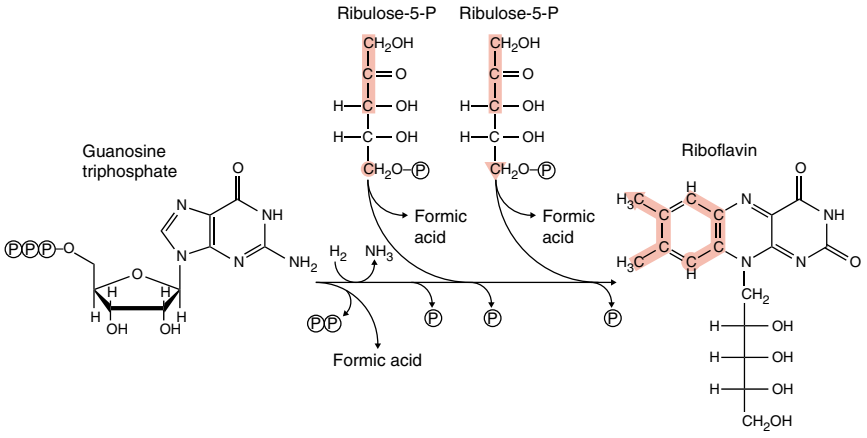


Figure 7.2 Riboflavin is formed from one GTP and two ribulose 5-phosphates. Essentially, its biosynthesis is the same in bacteria and fungi. The coloring ring system origins from the purine of the GTP by opening and conjunction with two carbon-4 compounds (highlighted in red). The latter are formed from two molecules of ribulose 5-phosphate. The complex biosynthetic pathway consisting of seven enzymatic steps is summarized.

microorganism. A little later, a process using *B.subtilis* was achieved. Both processes are now, after more than 25 years of intensive strain and process development, so efficient that they replaced the chemical processes. One reason for their replacement is that the chemical processes are multistage but the microbial processes are single stage. One reason for their replacement is that the chemical processes are multistage but the microbial processes are single stage.

Riboflavin produced in industrial scale is applied for vitamin preparations and for food coloring. Two-thirds of the annual production is used as a feed additive for livestock production. Increasing livestock growth depends on optimization of animal feedstuff and small amounts of essential amino acids and vitamins play an important role.

7.3.2 Biosynthesis by Fungi and Bacteria

In man and domestic animals, riboflavin is needed in catalytic amounts. The same is true for most microorganisms. Therefore, biosynthesis takes place at a low level. An exception is the filamentous fungus *A. gossypii*. The wild type produces up to 100 mg riboflavin per gram biomass under suitable conditions. Probably, such a high dose is needed to protect the *A. gossypii* spores against ultraviolet light. Because of its high productivity, this fungus was used in research to investigate the biosynthetic pathway but riboflavin biosynthesis was also studied in the yeast *Saccharomyces cerevisiae* and in the Gram-positive bacterium *B. subtilis*. The well developed genetics of both mircroorganisms and the availability of riboflavin auxotrophic mutants greatly facilitated the elucidation of the pathway and the isolation of the genes involved.

Biosynthesis of riboflavin starts with two abundant metabolites: guanosine triphosphate (GTP) and ribulose 5′-phosphate (Figure 7.2). These molecules

are not directly coupled but two separate pathways lead after seven enzymatic reactions to the precursor lumazine (not shown in Figure 7.2). Thereby, during condensation of the second ring, a 4-carbon precursor formed from ribulose 5′-phosphate is incorporated. The condensation of the third ring is complex and therefore not explained here. A second molecule of the carbon-4 compound is needed for this step, so that in total, two mol of ribulose 5′-phosphate and one mol of GTP per mol riboflavin are needed.

7.3.3 Production by *Ashbya gossypii*

The filamentous fungus *A. gossypii* was originally isolated by S.F. Ashby as a pathogen of cotton (*Gossypium hirsutum*). On African cotton farms as well as on Cuban citrus fruits, the plant pathogen generated crop losses for many years, but was then pushed back to insignificance. The elimination of agricultural damage caused by *A. gossypii* was probably due to a control of the flying insects needed by the fungus as vectors to get from plant to plant.

Whether the overproduction and secretion of riboflavin serves to attract insects and/or to protect the spores that are nonpigmented but exposed to UV light from the sun is unclear. The regulatory coupling of riboflavin overproduction and sporulation and a negative effect of the second messenger cAMP on both sporulation and riboflavin overproduction are arguments for such a function. Interestingly, cells that generate spores are not overproducing riboflavin, but other cells in the same hyphae do. Overproducing cells show a bright green fluorescence because of the accumulation of riboflavin in the vacuoles. In these cells, the mRNAs levels of the *RIB* genes were shown to increase during the transition from growth to production phase. As long as the fungus is growing, the *RIB3* promoter, controlling expression of a key enzyme in the biosynthetic pathway, has low activity. Probably, exhaustion of complex nutrients, essential for *A. gossypii*, induces differentiation into hyphal cells producing spores or riboflavin (Figure 7.3).

With more than 40 years of development and many rounds of random mutagenesis and subsequent selection, followed by molecular engineering, industrial strains were constructed producing titers of more than 20 g riboflavin per liter. The changes in these industrial strains are not published. It is assumed that several hundred mutations accumulated in such strains. Probably, the number of mutations relevant for an optimal overproduction is 1 order of magnitude lower.

Based on scientific investigations in which the wild-type strain was compared with defined mutants, it was shown that deregulation of precursor supply and biosynthesis as well as elimination of sidetracks diverting the metabolic flux occurred. A few selected examples of changes that were proven to boost productivity are explained in the following paragraphs (Figure 7.4).

For industrial production of riboflavin with *A. gossypii* plant lipids are used as carbon and energy source, which are dissimilated to acetyl-CoA in the beta-oxidation pathway. In the glyoxylate pathway acetyl CoA is converted to malate, which is oxidized to oxaloacetate, the starting point of gluconeogenesis. Isocitrate lyase, the key enzyme in the glyoxylate pathway, is regulated at the transcriptional level. Inhibitors of that enzyme were found to interfere with

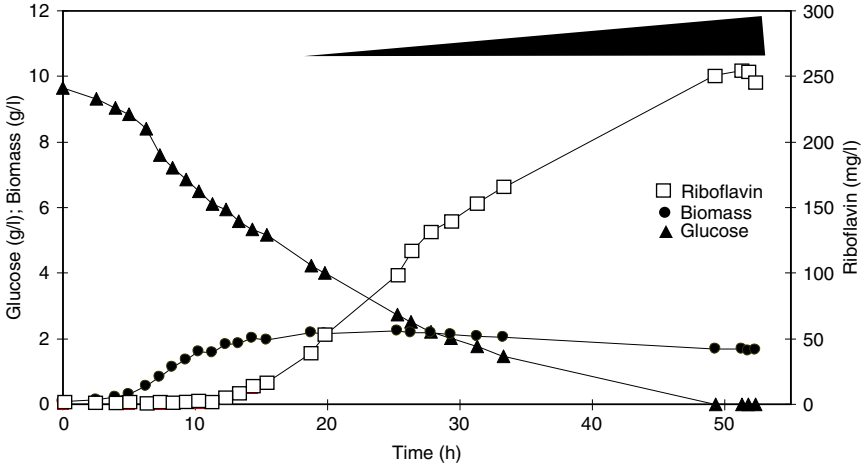


Figure 7.3 The riboflavin titer is increasing during cultivation of the wild-type *Ashbya gossypii* when the rate of biomass formation decreases. A repression by glucose can be excluded as a regulation mechanism. A part of the hyphal cells generate spores. These are forming clumps because of hydrophobic interaction. Therefore, their increase in number is represented as black shim above.

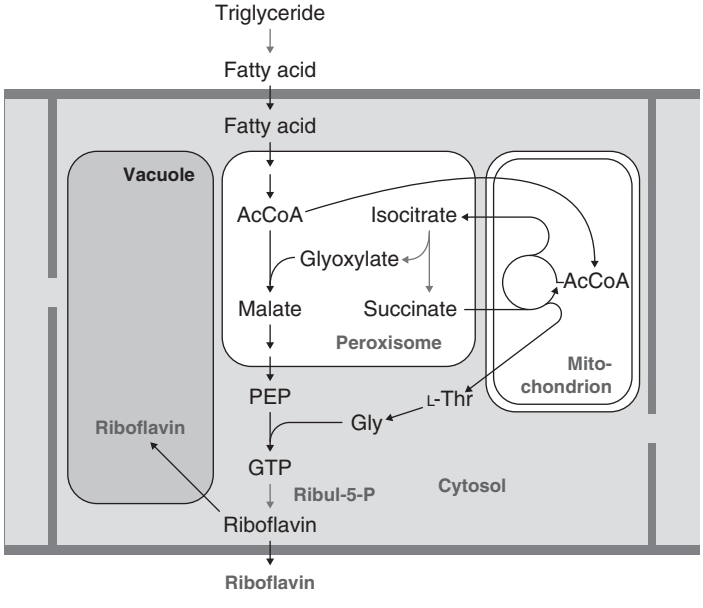


Figure 7.4 Model subcellular localization of the carbon flux in *Ashbya gossypii*. Extracellular triglycerides are hydrolyzed by a lipase. The released fatty acids are degraded in the peroxisomes to acetyl CoA (AcCoA). The formation of isocitrate is carried out in the mitochondria. Isocitrate lyase is liberating the glyoxylate needed for malate synthase in the peroxisome. Gluconeogenesis, purine, and riboflavin biosynthesis are running in the cytosol. About 60% of the vegetative cells in the hyphae are overproducing riboflavin. It is either accumulated in the vacuoles or exported via a specific carrier system in the cytoplasm membrane. Selected reactions controlling the flux via a regulation are printed in gray.

riboflavin overproduction. Increased overproduction of riboflavin in strains resistant to isocitrate lyase inhibitors or overexpressing the enzyme indicate that isocitrate lyase catalyzes a reaction with high flux control.

Purine biosynthesis delivering ATP and the riboflavin precursor GTP is highly regulated. At the transcriptional level adenine represses the expression of *ADE4* encoding the entrance enzyme into the purine pathway. An exchange of the endogenous by a strong constitutive promoter leads to deregulation of ADE4 transcription. Furthermore, the ADE4 encoded enzyme is feedback inhibited by purine nucleotides, which is eliminated upon exchanges at 3 positions of the ADE4 primary sequence. These changes lead to a significant increase in riboflavin production.

By targeted inactivation of genes, encoding enzymes of unwanted side reactions, precursor supply for riboflavin production can be improved. In wild-type *A. gossypii* glycine supply limits riboflavin overproduction. The deletion of SHM2, encoding cytosolic serine hydroxymethyltransferase, leads to a decrease of the flux from glycine to L-serine. Therefore, more glycine is available for GTP biosynthesis.

A direct way to increased riboflavin production capacity is overexpression of the *RIB* genes encoding the riboflavin biosynthetic enzymes. For a constructive overexpression, regulation has to be conserved. Exchange of the promoters by strong and constitutive promoters leads to growth inhibition. However, increasing gene copy number by random or locus specific genomic integration does not interfere with growth. Such integration mutants can be generated by coupling of the target gene with a selectable marker, conveniently an antibiotic resistance gene. After integration the marker gene can be removed from the host strain chromosome by single crossover recombination. Thus, the marker can be reused for further genetic engineering in that strain. Equally important, the final production strain is marker free, which, in case an antibiotic gene was used, is a prerequisite for official authorization.

Industrial scale production by *A. gossypii* is performed in fed-batch runs in stirred vessels with a volume larger than 100 m^3. In the first phase, biomass is produced. The complex medium contains plant lipids like soybean oil and insoluble solid compounds like soybean flour mixed with water. An advantage of this substrate composition is its low osmotic activity. That means, the fungal cells do not need to adapt as they have to at high concentrations of glucose, a common fermentation substrate, by production of compatible solutes. Regulation of the extracellular lipase activity prevents excess free fatty acids in the medium. Even at a concentration of 0.5% free fatty acids downregulate lipase formation so that uncoupling of the repiratory chain is avoided. After the desired biomass concentration is reached, production medium is added. That medium has a low nutrient content, inducing the riboflavin pathway genes, but it does contain precursors that are not sufficiently provided by the fungal metabolism, e.g. glycine. As only a part of the vitamin is exported from the cells into the medium whereas a significant amount is stored in the vacuoles, cell desintegration conveniently by autolysis to release the product is required. Autolysis is a typical fungal response to increased temperatures whereby lytic enzymes, e.g. β-glucanases, are produced

hydrolyzing the hyphal cell walls. The low solubility of riboflavin in water at neutral or weak acidic conditions leads to crystallization of the product allowing product isolation by centrifugation.

7.3.4 Production by *Bacillus subtilis*

In contrast to *A. gossypii*, the Gram-negative bacterium, *B. subtilis*, is not a natural overproducer of riboflavin. Its successful engineering into a competitive production system for riboflavin is a masterstroke of biotechnology. That success should encourage similar approaches for more products. An important argument for *B. subtilis* is its harmlessness shown in the processing of food. For centuries, it has been used in Japan to convert hard to digest soy beans into salubrious Natto.

Riboflavin in *B. subtilis* has probably only a single function. It is the precursor of FMN and FAD. The intracellular concentrations of FMN and FAD are in the micromolar range due to tight regulation of riboflavin biosynthesis at the transcriptional level involving an RFN triggered riboswitch (see Figure 7.5), which is responsive to FMN, but not riboflavin. To overproduce riboflavin the FMN sensing regulatory system has to be inactivated. The natural structural analog roseoflavin, a compound with a chemical structure slightly different from riboflavin, is helpful for this approach. Roseoflavin is produced by *Streptomyces davawensis* and functions as an antibiotic. Like riboflavin, roseoflavin is phosphorylated and adenylated intracellularly to FMN and FAD analogs, which are used as prosthetic groups leading to dysfunctional holoenzymes. Upon plating of a *B. subtilis* population on a growth agar containing roseoflavin, resistant colonies appear after several days of incubation, some of which overproduce and excrete riboflavin. These colonies can be easily identified by the intense yellow color of their incubation medium. One class of this overproducing mutants carry a mutation in the flavokinase/FAD synthase encoding gene leading to an enzyme with drastically reduced activity. The remaining activity supplies enough FMN and FAD to facilitate normal growth of the mutants, but prevent elevated intracellular FMN levels, that would trigger the RFN riboswitch. A second class of mutants with deregulated riboflavin biosynthesis carry mutations in the RFN element preventing the binding of FMN.

Not only regulation but also the structure and function of the enzymes specific for riboflavin biosynthesis in *B. subtilis* were characterized. An example is the bifunctional enzyme encoded by *ribA*. Whereas one domain catalyzes the abovementioned synthesis of a C4 compound from ribulose 5′-phosphate, a second domain opens the purine ring of GTP. The maximal activity of the ribulose-5-phosphate converting enzyme domain is twice as high as the activity of the GTP converting enzyme domain, in line with the stoichiometric requirement of these precursors for riboflavin biosynthesis.

Most interesting is the complex superstructure of lumazine synthase (*ribH*) and riboflavin synthase (*ribE*). In total, 60 lumazine synthase monomers form a perforated icosahedral shell. Inside of that shell, a trimer of riboflavin synthase is present. Such a structure suggests a high concentration of the last riboflavin intermediate lumazine within the lumazine icosahedron. A phenomenon called channeling, postulated for some biosynthetic pathways but difficult to support

by experimental data, seems to be realized by this structure of two sequentially acting enzymes.

Today's industrial strains were generated by a combination of selection after random mutagenesis and molecular engineering. In the first generation strains, the *rib* operon fused *in vitro* with a strong promoter and an antibiotic resistance gene were introduced into the genome of *B. subtilis* by single crossover recombination resulting in iterative sequences, e.g. two *rib* operons flanking the resistance gene. Cultivation of transformed strains at elevated antibiotic concentration selected for bacteria with increased copy number of the resistance gene and the adjacent *rib* operon. The high gene dose together with the strong promoter led to increased transcription of the *rib* genes and finally to increased productivity. In more recent generations of improved strains, the *rib* operon is only present as a single copy in the genome. A strong promoter, e.g. that of the bacteriophage *SPO15*, plus a carefully modified 5′ terminus of the *rib* mRNA leads to a high transcription rate. The latter change prevents early termination of the transcription and increases half-life of the mRNA (Figure 7.5).

The higher the specific riboflavin biosynthesis activity caused by increased *rib* genes expression, the more likely is an insufficient supply of the precursors GTP

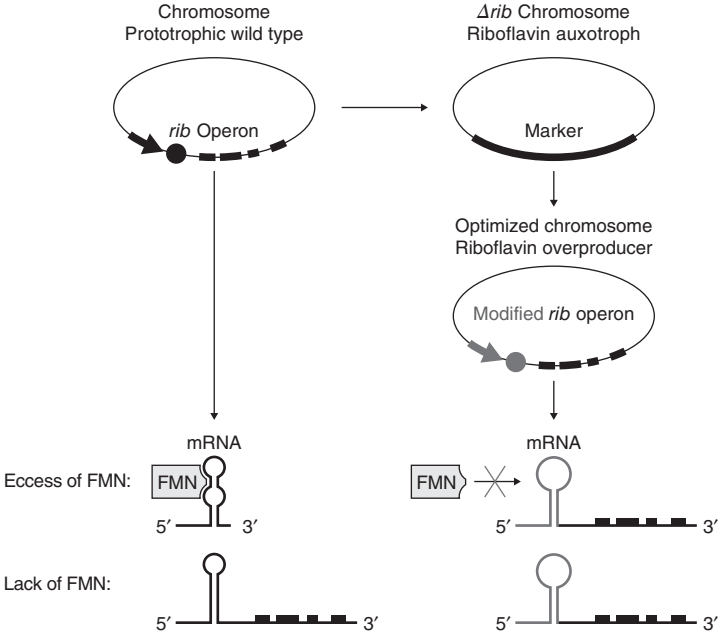


Figure 7.5 Regulation and deregulation of a *rib* operon integrated in the genome of *Bacillus subtilis*. The *rib* operon consists of four genes (black boxes). In the wild type, a sequence (black dot; left) is found upstream of these genes leading to an FMN-binding secondary structure of the transcript (RFN element). If such an interaction occurs, transcription of the rib genes is attenuated (riboswitch). After replacement mutagenesis using transiently a dominant marker, e.g. an antibiotic resistance gene, a modified operon, e.g. under control of the constitutive phage promoter SPO15 (red arrow; right), and with a modified 5′-UTR (red dot) can be reintegrated. The mRNA translated from such a mutation does not bind FMN so that the *rib* genes are transcribed always.

and ribulose 5-phosphate. For GTP biosynthesis, one ribose 5-phosphate and for riboflavin biosynthesis two ribulose 5-phosphates are needed (Figure 7.2). In total, three pentose phosphate molecules for one riboflavin molecule must be supplied by the anabolism. To increase the supply of phosphorylated pentoses, the transketolase in the pentose phosphate pathway was replaced by a mutein reduced in activity by a single amino acid exchange. This leads to accumulation of the sugar phosphates and causes a significant boost in riboflavin production.

B. subtilis has a relatively high maintenance metabolism which is the energy substrate consumption, e.g. glucose, to deliver the energy for maintaining cellular homeostasis. That causes at the high biomass concentrations and low growth rates of industrial fed-batch processes to a high dissimilation of glucose to carbon dioxide. To increase the energy efficiency of the production strain, the cytochrome *bd*-dependent branch of its respiratory chain with low proton translocation efficiency, was inactivated. After successful activation of the more energy-efficient cytochrome *c*-dependent branch, glucose consumption for maintenace purposes declined.

Molasses or starch hydrolysates are used as carbon and energy source for industrial riboflavin production with *B. subtilis*. To reach high product concentrations, a fed-batch feeding regime is applied. In a first phase, excess of substrates allows fast growth. Subsequently, the supply with the carbon source is strictly limited to prevent an unfavorable oxygen limitated metabolism in the production strain.

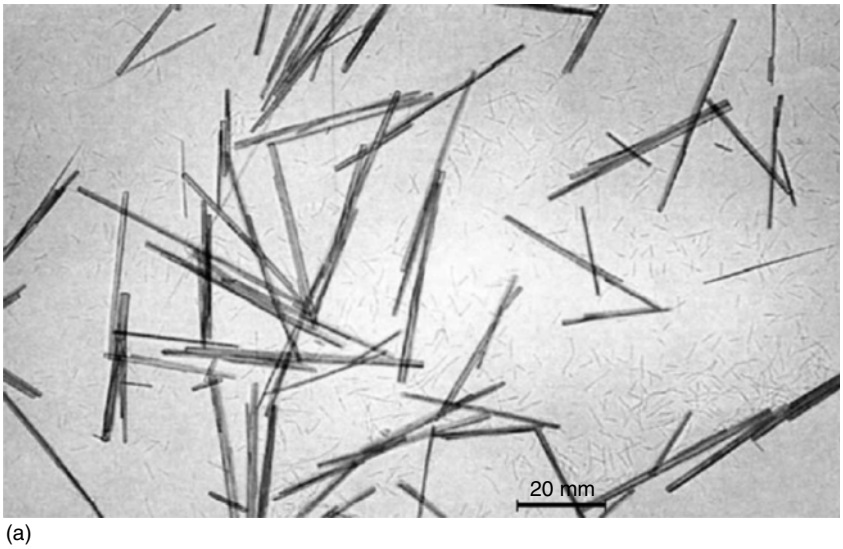
The export of riboflavin into the extracellular space is faster in *B. subtilis* than in *A. gossypii*. A further difference is the faster growth of the bacterium. Possible reasons for that could be less complexity, the better surface/volume relationship because of the smaller cells, and shorter diffusion ways. The bacterial system should beat the fungal system when all theoretical optimizations are realized in the future.

The molecular mechanism of riboflavin excretion is not clear for *B. subtilis* or *A. gossypii*. Understanding transport is not only of scientific interest but also has the potential for further optimization of the industrial process.

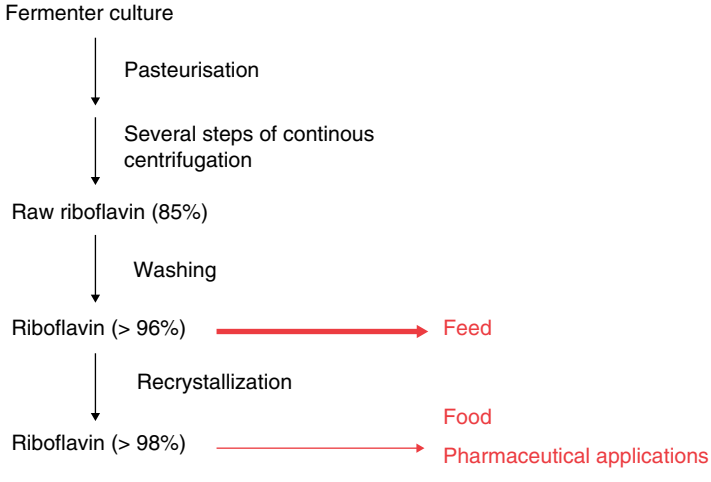
7.3.5 Downstream Processing and Environmental Compatibility

The relatively weak solubility of riboflavin causes crystallization in both the *A. gossypii and B. subtilis* processes. The large size of the crystals in comparison to the smaller *Bacillus* cells is not only impressive but also allows an efficient downstream processing by centrifugation (Figure 7.6).

In a complex study, a conventional chemical production process was compared with two microbial processes (Table 7.2). A 75% reduction of fossil recourses was found for the microbiological process for the same vitamin B_2 product amount than for the chemical process. In addition, emissions into air (−50%) and water (−66%) were reduced. However, the consumption of water is more than double for the microbial processes and the contamination of the process water by ammonia can be up to five times higher than in the chemical process. A positive influence on operating costs (−50%) is caused by the reduction of process steps and cheaper raw materials.



(a)



(b)

Figure 7.6 (a) Microscopic photograph of the fermenter culture with the rod-shaped bacterium *Bacillus subtilis* and the much larger crystal needles of riboflavin. (b) Downstream processing revealing two different product qualities. More than 60% of the industrial riboflavin is applied for the fortification of animal feed.

7.4 Cobalamin (Vitamin B_{12})

7.4.1 Physiological Relevance

During the 1920s, a nutritional factor curing anemia in dogs was identified in liver. Trying to isolate this factor, a compound was identified that prevented the onset of pernicious anemia in humans, untreated the disease is fatal. Initially,

Table 7.2 Microbial riboflavin production replaces conventional chemical process.

Production process	Chemical synthesis 7 steps	*A. gossypii* 1 step	*B. subtilis* 1 step
Energy	100%	94%	66%
CO_2 equivalents	100%	77%	75%
Acidification	100%	32%	50%
NH_4-containing wastewater	100%	401%	551%
Cl-containing wastewater	100%	4%	42%

Up to 2000 chemical syntheses of riboflavin took place in the industrial process. Although Lord S.F. Ashby published the discovery of natural riboflavin overproduction by *Ashbya gossypii* in 1928, it took more than 70 years until a combination of random mutagenesis, genetic engineering, and process development allowed a competitive process. Interestingly, at about the same time, although started 50 years later, a process using *Bacillus subtilis* was developed. An assessment of environmental impacts is given for a few important aspects.

pernicious anemia was cured by administration of large amounts of liver extracts. The factor curing anemic dogs proved to be iron. The compound directed against human pernicious anemia turned out to be a vitamin, designated vitamin B_{12}.

Around 1950 vitamin B_{12} was isolated and its highly complex molecular structure was elucidated. Almost 20 years later, the vitamin, also called cobalamin, was obtained in a brilliant synthetic route involving 70 chemical steps. The cobalamin molecule is a slightly distorted quadratic bipyramid consisting of a 15-membered corrin ring with Co^{3+} as the central atom. The four nitrogen atoms of the corrin ring coordinate with Co^{3+} in an almost planar fashion (Figure 7.7). A dimethylbenzimidazole residue is located in front of the corrin plane occupying a fifth coordination site. A sixth coordination site behind the corrin plane can be occupied by 5′-desoxyadenosine. It is postulated that during enzymatic catalysis involving cobalamin as cofactor, a 5′-deoxyadenosine radical is formed concomitantly by reduction of Co^{3+} to Co^{2+}. During catalysis, the radical abstracts a hydrogen atom from the substrate. Industrially produced cobalamin contains a cyano instead of the 5′-deoxyadenosine moiety. Cyanocobalamin, which is a highly stable molecule, is converted after intake by human or animals into natural cobalamin.

Only two enzymatic reactions in human metabolism are cobalamin dependent: (i) a mutase reaction, which converts L-methyl-malonyl-CoA into succinyl-CoA during the degradation of odd-numbered fatty acids and (ii) a methionine synthase reaction, which transfers a methyl group from tetrahydrofolate to homocysteine during salvage of methionine from *S*-adenosylmethionine.

Sources of cobalamin in the human diet are the intestines of animals like beef liver (c. 60 µg/100 g), fish (c. 8 µg/100 g), or eggs (c. 3 µg/100 g). The RDA (recommended dietary allowance) for adults in the European Union is 2.5 µg/d. Recent studies indicate an inadequate provision of vitamin B_{12} in western industrialized countries despite a sufficient supply of cobalamin-rich food. The elderly and vegans are especially affected.

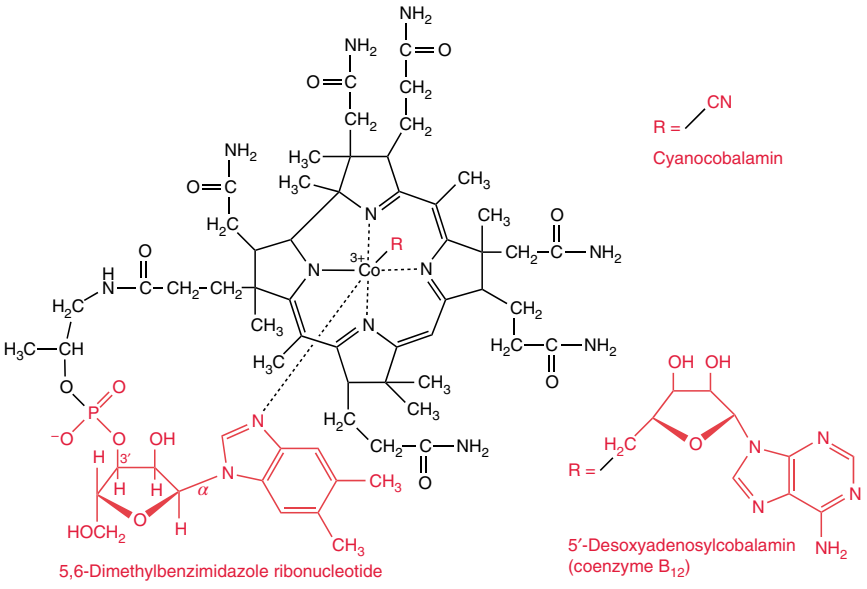


Figure 7.7 The structure of cobalamin is characterized by a corrin ring whose four N atoms coordinate a cobalt ion (Co^{3+}). The corrin ring lies in the drawing plane. From the front binds a N of benzimidazole, while from the back different ligands (R) can bind. The technical product carries a cyano group. As a coenzyme, B_{12} in a mutase, e.g. the 5′-deoxyadenosyl ligand, which can be radicalized at the 5′-C atom while stabilizing a reduced Co^{2+} from the ring.

7.4.2 Biosynthesis

Only bacteria and archaea can synthesize cobalamin *de novo*. In bacteria, two synthetic routes toward cobalamin exist: the aerobic route of, e.g., *P. denitrificans* and the anaerobic route of, e.g., Propionibacteria or *Escherichia coli*. The routes are distinguished by the sequence of corrin ring formation and cobalt insertion.

Cobalamin biosynthesis is highly complex. Starting from succinyl-CoA, an intermediate of the citrate cycle, δ-aminolevulinic acid is synthesized followed by formation of uroporphyrinogen III, a 16-membered ring structure comprising four pyrrol moieties and four bridging methine groups. Uroporphyrinogen III is also used as a precursor for the prosthetic groups of hemoglobin and chlorophyll. Further steps toward cobalamin are formation of the corrin ring before or after cobalt insertion and modification with (i) propanolamine, (ii) desoxyadenosine obtained from ATP, and (iii) dimethylbenzimidazole obtained from riboflavin. More than 20 enzyme reactions are required to convert uroporphyrinogen III to adenosylcobalamin (Figure 7.8).

7.4.3 Production with *Pseudomonas denitrificans*

During the 1950s, cobalamin was extracted from sludge or from *Streptomyces* biomass obtained during antibiotics fermentations. After development of microorganisms as production strains, vitamin B_{12} was obtained in dedicated fermentation processes. The yearly production volume is relatively low, 30 tons

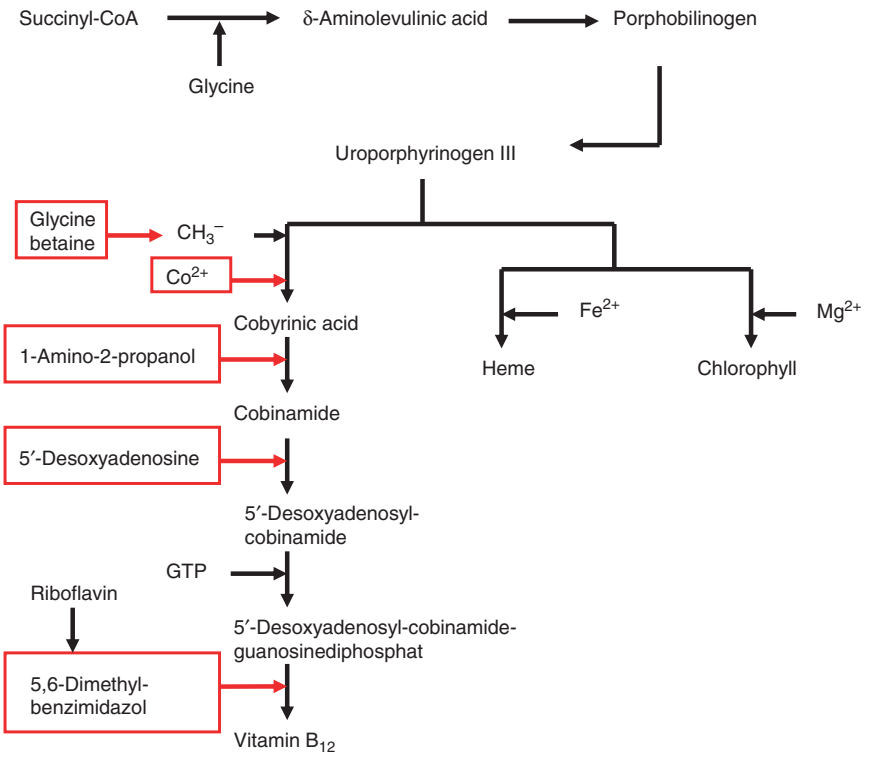


Figure 7.8 Simplified biosynthetic pathway of cobalamin with complex precursors. Precursors whose addition to the medium in *Pseudomonas denitrificans* leads to stimulated production are red. The structurally similar cofactors heme or chlorophyll differ, inter alia, from the cation, which is important for catalysis.

produced per year. About 80% is used for feed and 20% is used for food and pharma applications.

In the past, production strains from the species *Bacilli*, *Methanobacteria*, *Propionibacteria*, or *Pseudomonas* were used. Since the end of the 1990s, only processes based on *P. denitrificans* prevailed. The production strains were obtained by traditional mutagenesis and selection/screening procedures or by molecular genetics.

Around 1990, the aerobic cobalamin biosynthesis pathway was almost completely elucidated. After the cloning of 22 *cob* genes from *P. denitrificans*, production strains were created with plasmid-based enhanced expression of the cobalamin biosynthetic genes. These strains should be superior with regard to productivity (space/time yield) and the yield on the efficiency of raw material conversion.

A carefully designed fermentation process is crucial for high performance in this process. As carbon sources starch hydrolysates and/or sugar beet molasses are used. They are applied in fed-batch mode. Glycine betaine present in sugar beet molasses in significant amounts is an essential cosubstrate. It was proposed that the production stimulating effect of the glycine betaine is due to the many C_1

carbon units generated during assimilation of the molecule, which are required for the eight methylation reactions during cobalamin biosynthesis. However, a mass balance indicates that during the course of a typical vitamin B_{12} fermentation run, the C_1 groups generated by glycine betaine degradation exceed the demand for product synthesis several hundred folds. Furthermore, it has been demonstrated that *P. denitrificans* mutants defective in betaine homocysteine methyltransferase catalyzing the first step of glycine betaine assimilation are still able to overproduce vitamin B_{12}. The formerly used *Propionibacterium*-based processes did not require glycine betaine cofeeding. In summary, the accurate molecular explanation for the glycine betaine effect still awaits elucidation.

The vitamin B_{12} fermentation medium provides, in addition to the carbon substrate, glycine betaine and the other common fermentation substrates cobalt ions, dimethylbenzimidazole, and sometimes 3-amino-2-propanol, which are all components of the cobalamin molecule.

Although in many other fermentation processes sufficient aeration of the cultivation medium is important to avoid oxygen limitation of the production microorganism, the vitamin B_{12} process is carried out under oxygen-limiting (not anaerobic) fermentation conditions conducive to significant higher yields from the key raw materials, the carbon sources and glycine betaine. Under optimal fermentation conditions, around 200 mg/l vitamin B_{12}, predominantly in the form of mainly adenosyl-cobalamin, accumulates in the fermentation medium during a 7 day run. The downstream steps to isolate and purify vitamin B_{12} comprise filtration, cyanide treatment, chromatography, extraction, and crystallization yielding cyano-cobalamin in high purity.

7.5 Purine Nucleotides

7.5.1 Impact as Flavor Enhancer

The sweet or spicy notes of some amino acids and peptides are well recognized. Interestingly, the purine nucleotides IMP and GMP act as flavor enhancers for some amino acids. For instance, the characteristic umami (savory) taste of L-glutamate is threefold enhanced, whereas the sweet taste of sucrose is not affected by purine nucleotides. One of the three known umami taste receptors in the tongue (the T1R1/T1R3 receptor) can interact with both L-glutamate and purine nucleotides, leading to signal enhancement if stimulated by both molecules simultaneously. Via sensory axons, the signal is transmitted to the brain, indicating a rich food source. About 14 000 tons of purine nucleotides are produced per annum and are mainly used in the food industry as a taste enhancer.

7.5.2 Development of Production Strains

Industrial production of purine nucleotides is carried out in a two-step process. First, the nonphosphorylated nucleosides inosine and guanosine are obtained by fermentation, and then in a second step, they are phosphorylated chemically or more recently, biocatalytically. Production microorganisms for the nucleosides

are *Bacilli* or *Corynebacteria* strains. The strains were obtained by selection for their resistance to purine analogs out of a randomly mutagenized population after, for instance, exposure to UV light. Purine analogs are toxic to wild-type bacteria. However, some mutants in the mutagenized population randomly acquired a defect in the regulatory mechanisms of purine biosynthesis. These mechanisms have evolved to adapt the physiological requirements for purine nucleotides to their *de novo* synthesis. Purine regulatory mutants overproduce purine nucleotides resulting in elevated intracellular concentrations and excretion after dephosphorylation as purine nucleosides into the cultivation medium. They compete with the purine analogs present in the selection medium providing protection against the toxic effects of the later.

Presumably, in addition to strains obtained by CSI (classical strain improvement), strains derived by molecular breeding are in industrial use today.

7.5.3 Production of Inosine or Guanosine with Subsequent Phosphorylation

The immediate products of the purine metabolism are the nucleotides, i.e. nucleoside-5′-phosphates. Excretion into the cultivation medium occurs only after dephosphorylation. Traditionally, the nucleosides are isolated, further purified, and phosphorylated with phosphorylchloride ($POCl_3$) as a phosphorylating agent.

A more recent process, developed by a Japanese company, uses whole cell biocatalysis with recombinant *E. coli* expressing a phosphatase/phosphotransferase gene from *Escherichia blattae*. In contrast to most other phosphokinases, the *E. blattae* enzyme is used as cosubstrate and source of energy-rich phosphate not ATP, but relatively cheap and readily available pyrophosphate.

The undesired phosphatase activity of the wild-type enzyme was reduced and the transferase activity was enhanced by enzyme engineering. The optimized phosphotransferase contained amino acid replacements at 11 positions. It was reported that 79% of the inosine in the starting medium was phosphorylated within 24 hours at the 5′-position resulting in above 150 g/l inosine-5′-phosphate final concentration.

In an alternative biocatalytic process, inosine is produced with *Corynebacterium ammoniagenes*. Toward the end of the fermentation run, recombinant *E. coli* overexpressing an inosine kinase gene is added to the fermentation broth (Figure 7.9). ATP produced by *C. ammoniagenes* is taken up by *E. coli* and used as cosubstrate for phosphorylation of inosine, which is taken up by *E. coli* as well. The ADP generated during the kinase reaction in *E. coli* is recycled to ATP by *C. ammoniagenes*. The ATP/ADP exchange between the two strains in the fermenter is facilitated by detergent permeabilization of the bacterial membranes. ATP regeneration occurs by substrate-level phosphorylation, as because of the membrane permeabilization, the establishment of a protein gradient over the membrane and oxidative phosphorylation is not possible. Cheap inorganic phosphate added to the fermenter is the phosphate source in this process.

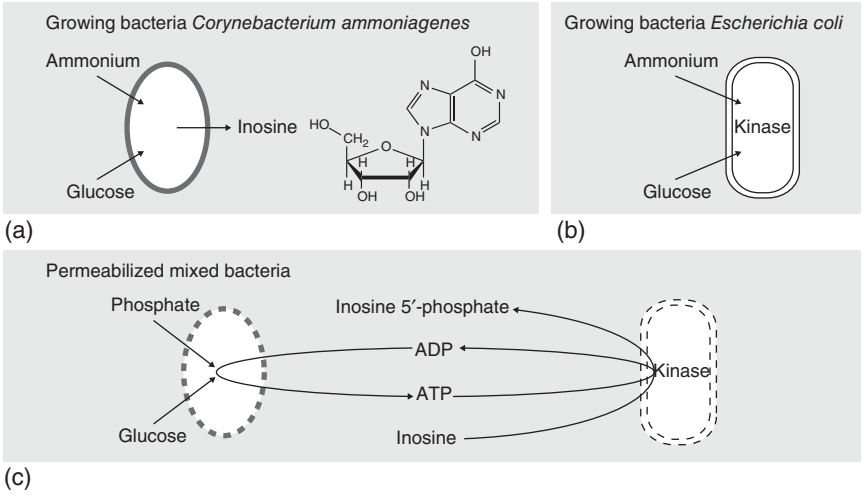


Figure 7.9 Production of inosine with *Corynebacterium ammoniagenes* followed by regioselective phosphorylation by incubation with an *Escherichia coli* strain overexpressing an inosine kinase gene. The regeneration of the required ATP takes place in *Corynebacterium ammoniagenes*. First, both bacteria are grown in parallel (a, b), followed by mixing and permeabilization (c).

7.6 β-Carotene

7.6.1 Physiological Impact and Application

β-Carotene, also called provitamin A, is a C_{40} tetraterpenoid. The red-orange pigment supports light harvesting in photosynthetic active plants and microorganisms. During digestion of carrots and vegetables, β-carotene is split into two C_{20} vitamin A (retinal) molecules by a monooxygenase. Bound to rhodopsin in the retina, vitamin A is crucial for color vision. The compound is an efficient coloration pigment and approved as a food colorant (E160a). As a fat-soluble molecule, vitamin A accumulates in the cell membranes leading to toxic side effects if overdosed.

A chemical synthesis for β-carotene for larger scale production was developed in the 1950s. The first commercial successful application of synthetic β-carotene was in margarine to replace the azo dye butter yellow, which was recognized as carcinogenic and banned.

Today, several hundred tons of β-carotenes are produced per year, mainly by chemical synthesis. However, there is a growing market for food colorants from natural sources. To meet these demands, β-carotene-rich plant extracts, e.g. from carrots, are offered. In addition, two β-carotene-rich microorganisms, the filamentous fungus *Blakeslea trispora* and the marine algae *Dunaliella salina*, are cultivated to obtain the pigment. The fungal process will be discussed here in more detail.

7.6.2 Production with *Blakeslea trispora*

The zygomycete *B. trispora* commences a sexual life cycle if cells of the two different mating types of this fungus come into close contact (heterothallism). The two mating types designated as (+) and (−) are distinguished by their mating locus comprising not more than a few kilo bases. The product of the sexual cycle involving recombination of the genomes of the mating partners are haploid zygospores. Cultivated separately, (+) and (−) strains form a mycelium-like biomass. On solidified nutrient media, they develop asexual spores in sporangia at the tip of stalk hyphae. After mixing the mycelia of the two mating types and cocultivation for a few hours, the biomass adopts an intense reddish color, which is an early visible step of the sexual life cycle of the fungus. The pigment is β-carotene, whose biosynthesis is strongly induced in the (−) mating type. The inducer molecules are trisporic acids. They are synthesized in a concerted manner by both mating partners starting from β-carotene, which is constitutively present in both cell types at low levels. Cultivating the (+) and (−) strains separately, trisporic acid formation is prevented and, hence, β-carotene productivity is low (Figure 7.10).

In the technical *B. trispora* β-carotene production process, the mating partners are propagated in different seed fermenters and after mixing cultivated in a well-aerated main fermenter with vegetable oils, carbohydrates, and corn steep liquor as the main raw materials. For optimal productivity, it is important to limit the nonproductive (+) mycelium in the fermenter to an amount, which is just sufficient for enough trisporic acid formation. More of the (+) mycelium would waste raw materials and since the mycelia of the two mating types cannot be separated after fermentation would reduce the β-carotene content in the final biomass. Industrially used *B. trispora* strains developed from their wild-type progenitor strains during many cycles of mutagenesis and selection accumulate more than 5% β-carotene in the mixed dried mycelia. With over 100 g/l dry biomass formed during a typical fermentation run lasting for more than 100 hours, at least 5 g/l product in broth are obtained. After fermentation, the *B. trispora* biomass is harvested by filtration, dried, and extracted with an organic solvent after cell breakage, e.g. with a hammer mill. After crystallization, β-carotene is obtained at high purity.

7.7 Perspectives

As the identification and cloning of carotenoid biosynthetic genes around 1990, many attempts were made to obtain carotenoid production strains by transforming common host microorganisms such as *E. coli* or *S. cerevisiae* with these genes. Pigmented transformants could be isolated, but their carotenoid content was too low for commercial exploitation. In more recent patent literature, recombinant *Yarrowia lipolytica* strains carrying carotenoid genes from various organisms are described, which produce β-carotene at substantial levels. The high productivity for this carotenoid as well as for the commercially highly attractive xanthophylls canthaxanthin, zeaxanthin, and astaxanthin offers the

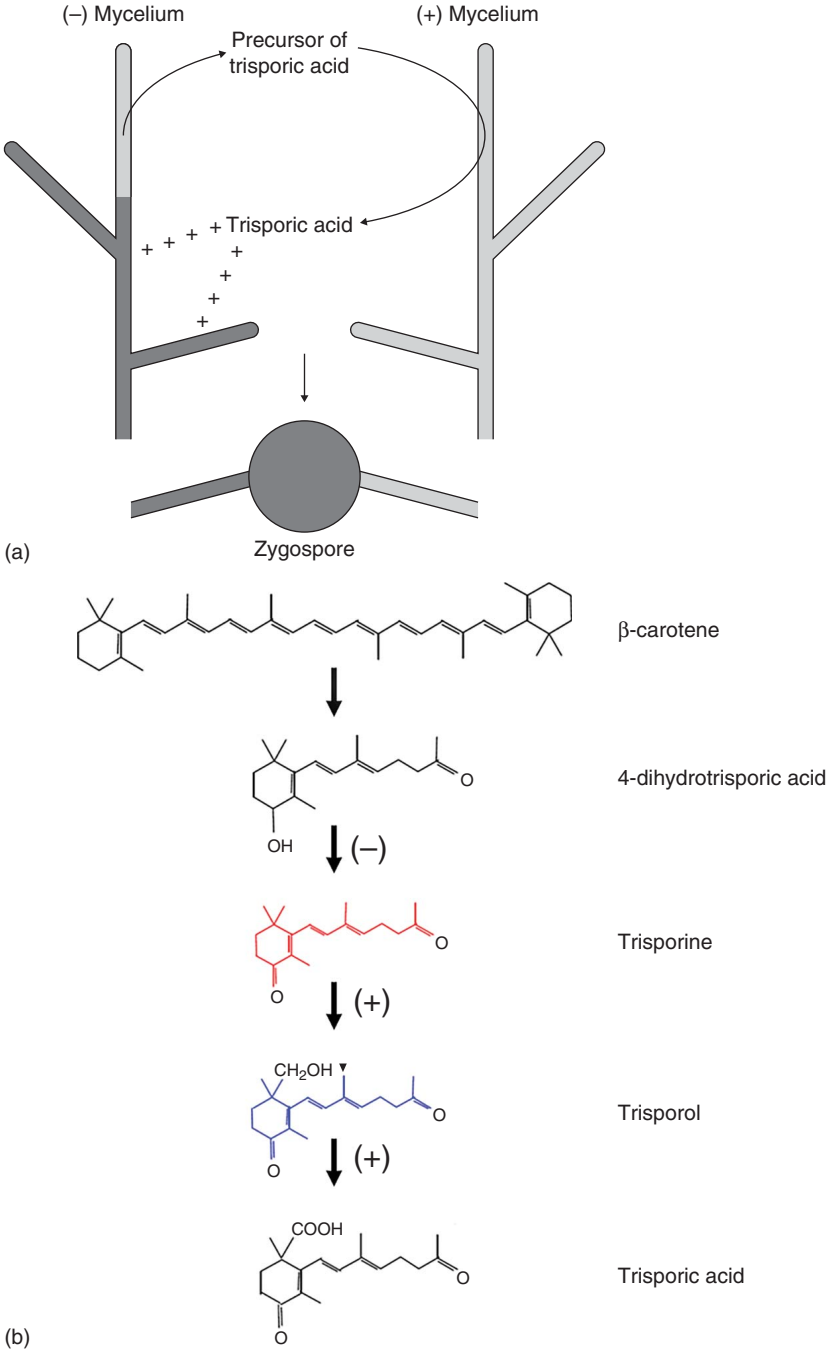


Figure 7.10 (a) Simplified representation of the interaction of the *Blakeslea trispora* (–) and (+) mating types, which is responsible for the formation of β-carotene (red)-pigmented zygospore. (b) The mating type-dependent conversion of β-carotene into trisporic acid is shown.

possibility to develop industrial relevant microbial processes based on this so-called unconventional, oleaginous yeast.

Microbial production processes for other fine chemicals, e.g. pantothenic acid (vitamin B_5) with recombinant *B. subtilis* or *E. coli* or biotin (vitamin B_7 or vitamin H) with recombinant *E. coli*, have reached an advanced level and might become competitive within the foreseeable future.

Further Reading

Bremus, C., Herrmann, U., Bringer-Meyer, S., and Sahm, H. (2006). The use of microorganisms in L-ascorbic acid production. *J. Biotechnol.* 124 (1): 196–205.

Hohmann, H.P. and Stahmann, K.P. (2010). Comprehensive natural products II. *Chem. Biol.* 10: 115–139.

Ishige, T., Honda, K., and Shimizu, S. (2005). Whole organism biocatalysis. *Curr. Opin. Chem. Biol.* 9 (2): 174–180.

Martens, J.H., Barg, H., Warren, M., and Jahn, D. (2002). Microbial production of vitamin B12. *Appl. Microbiol. Biotechnol.* 58 (3): 275–285.

Pappenberger, G. and Hohmann, H.P. (2014, 2014). Industrial production of L-ascorbic acid (vitamin C) and D-isoascorbic acid. *Adv. Biochem. Eng. Biotechnol.* 143: 143–488.

Vereshchagina, O.A. and Tereshina, V.M. (2014). Trisporoids and carotenogenesis in *Blakeslea trispora*. *Microbiology* 83 (5): 438–449.

8

Antibiotics and Pharmacologically Active Compounds

Lei Fang[1], *Guojian Zhang*[1,2,3], *and Blaine A. Pfeifer*[1,2,3]

[1] *University at Buffalo, The State University of New York, Department of Chemical and Biological Engineering, 904 Furnas Hall, Buffalo, NY, 14260-4200, USA*
[2] *Ocean University of China, Key Laboratory of Marine Drugs, Chinese Ministry of Education, School of Medicine and Pharmacy, Qingdao, 266003, People's Republic of China*
[3] *Laboratory for Marine Drugs and Bioproducts of Qingdao National Laboratory for Marine Science and Technology, Qingdao, 266237, People's Republic of China*

CHAPTER MENU

8.1 Microbial Substances Active Against Infectious Disease Agents or Affecting Human Cells

8.1.1 Distribution and Impacts

Small molecule natural products have had a powerful impact on human health and follow-up commercial development. In this chapter, a heavy emphasis will be placed on those small molecules that possess antibiotic activity, a capability that has particularly impacted modern medicine, in addition to alternative therapeutic activities associated with these same types of compounds. A common definition of an antibiotic (derived from "antibiosis," which was first used by Vuillemin in 1889) is a chemical substance produced by a microorganism that can kill or inhibit the growth of other microorganisms. More broadly, an antibiotic may be any small molecule with antimicrobial activity. Microorganisms have developed metabolic pathways devoted to natural products that possess antibiotic activity. Such pathways are referred to as secondary metabolism, distinct from primary metabolism in that the final compounds (secondary metabolites)

Industrial Microbiology, First Edition.
Edited by David B. Wilson, Hermann Sahm, Klaus-Peter Stahmann, and Mattheos Koffas.

Table 8.1 Top 10 antimicrobial agents including microbial production host (and/or synthetic or semisynthetic chemical formation), natural product classification, and US market volume production in 2011.

Semi-synthetic antibiotics/ synthetic chemical	Microorganism	Class	Name	Annual market volume (Sales in tons)
Semi-synthetic	*Penicillium chrysogenum*	β-Lactam (penicillins)	Amoxicillin	1100
Biosynthetic	*Penicillium chrysogenum*	β-Lactam (penicillins)	Penicillin V	130
Semi-synthetic	*Acremonium chrysogenum*	β-Lactam (cephalosporins)	Cephalexin	310
Semi-synthetic	*Acremonium chrysogenum*	β-Lactam (cephalosporins)	Cefdinir	41
Semi-synthetic	*Streptomyces rimosus*	Tetracyclines	Doxycycline	62
Biosynthetic	*Streptomyces rimosus*	Tetracyclines	Tetracycline	45
Semi-synthetic	*Saccharopolyspora erythraea*	Macrolides	Azithromycin	90
Semi-synthetic	*Saccharopolyspora erythraea*	Macrolides	Clarithromycin	50
Biosynthetic	*Saccharopolyspora erythraea*	Macrolides	Erythromycin	26
Chemical synthetic	*None*	Quinolones	Ciprofloxacin	210

do not contribute to the primary growth and maintenance goals of the microbial cell. Many times, the terms "natural products" and "secondary metabolites" are used interchangeably, with the common feature being a small molecule (relative to macromolecules within the cell). The biological features of these compounds include antibiotic properties as a way to engage in a form of chemical warfare with competing microbes for limited nutrients in the environment. Once identified, the scientific community developed a pipeline for natural product production with the goal of redirecting the antibiotic properties toward clinical applications. The goal of this chapter is to give the reader an appreciation for the history, biosynthetic mechanisms, classification examples, engineering development, challenges, and future opportunities for this remarkable class of compounds.

To begin, we present in Table 8.1 top antibiotic compounds across multiple molecular classifications. The antibiotic activity across compound classification is indicated in Figure 8.1. Each classification impacts functions of cellular activity, leading to antibiotic action or bioactivity. Nine out of the ten compounds in Table 8.1 result from microbial biosynthesis (i.e. they are natural products). However, ciprofloxacin is a chemically synthesized small molecule that is also effective as an antibiotic. Thus, the table as a whole represents the broader definition of antibiotic encompassing small molecule compounds capable of

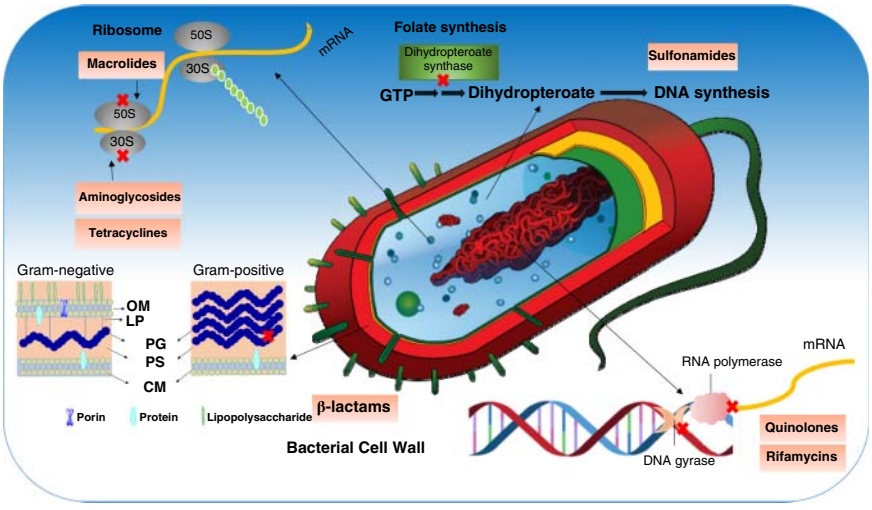
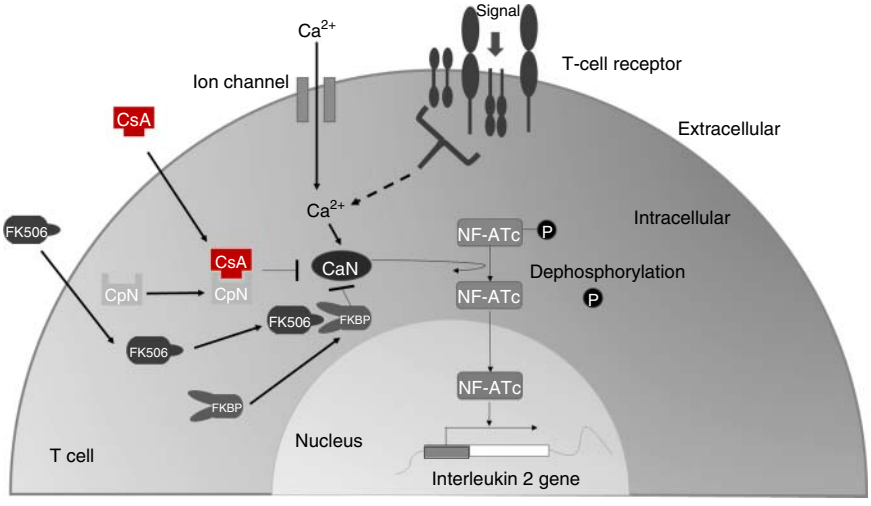


Figure 8.1 Common mechanisms of action of antibiotic compounds. OM: outer membrane, LP: lipoproteine, PG: peptidoglycane, PS: periplasmic space, CM: cytoplasmic membrane.

inhibiting microbial growth. Table 8.1 also highlights differences between natural biosynthetic compounds (resulting directly from microbial sources) and those compounds indicated as "semisynthetic," which means that the naturally biosynthesized compound was further modified through synthetic chemistry. The reasons for additional chemical modification are numerous, spanning improvements in final bioactivity to improved pharmacokinetic properties (i.e. internal anatomical distribution and clearance) once the compound is administered to a patient. A specific example for clarithromycin will be presented more thoroughly in the later section devoted to erythromycin. Finally, although antibiotics like those featured in Table 8.1 have had a profound impact upon human medicine, a similar utility has resulted within animal health maintenance. In particular, veterinary applications ranging from livestock to domestic companion animal health have been an additional outlet for antibiotic products.

The history of antibiotic natural products perhaps is best represented by the penicillin case, where initial antibacterial activity was observed as an abiotic halo surrounding a fungal contaminant (producing the compound) within a lawn of bacteria growing on a solid culture medium. This monumental observation by Alexander Fleming and the follow-up development by Howard Florey and Ernst Boris Chain resulted in the 1945 Nobel Prize, history-shifting events, socioeconomic impact upon national health standards, and countless lives saved.

The first observation of penicillin's activity via the unintentional coculture of fungi and bacteria also provides a backdrop to the native role of natural products. Effectively, these compounds serve as a means of communication between microbial populations. Antibiotic activity is a function of hostile communications because of competition for limited nutrient resources. Figure 8.1 highlights the common targets of antibiotic natural products. Alternatively, a multitude of other



CsA: Cyclosporine A
CpN: Cyclophylin
CaN: Calcineurin
NF-ATc: Cytoplasmic component of the nuclear factor of activated T cells
NF-ATn: Nuclear component of the nuclear factor of activated T cells FK506: Tacrolimus
FKBP: FK506-binding protein

Figure 8.2 Mechanism of action of cyclosporine on a human T cell. Cyclosporine A (CsA) binds with cyclophilin (CpN), disrupting the signaling pathway that leads to functional T-cell activity. The end result is a suppressed immune response.

activities have also been linked to natural products including impact upon mammalian cells (Figure 8.2). The chemical warfare evolved by cellular entities in the environment then became the basis for an industry focused upon harnessing this activity for beneficial purposes.

The penicillin example also provides a blueprint for the development of numerous other natural products. Generally, the scaled development of natural products initially relied on mutagenesis and selection methods that systematically generated higher producing strains of the original producer; this approach was a precursor to the more directed efforts of cellular engineering available today and completed because of typically low initial compound production levels from the native host. Thus, a trend emerged for penicillin and those compounds that followed. First, discovery was based on observed antibiotic (or other therapeutic) activity. Second, once the active compound and producing microorganism were identified, development efforts applied mutagenesis and screening to the native producer. Those mutants producing higher compound titers were taken forward for future strain improvement strategies and production of the final compound. It is important to note that such an approach required the ability to isolate both the natural product and the native producer. If successful, the lack of molecular biology knowledge (especially during the "Golden Age" of natural production discover, c. 1940–1970) forced a more nondirected approach of cellular perturbation

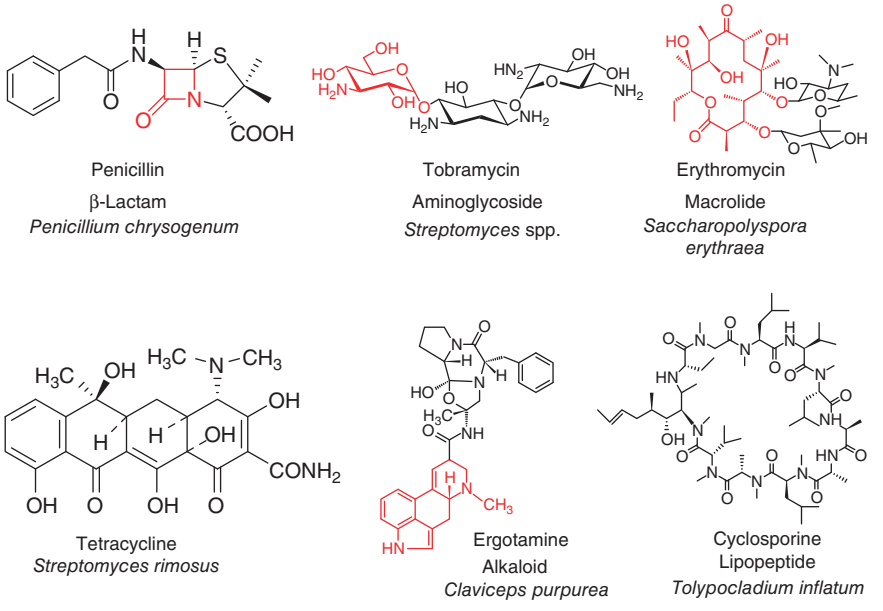


Figure 8.3 Representative antibiotic structures across classifications with production hosts indicated. Representative structure features are indicated: penicillin: β-lactam ring; tobramycin: amino sugar; erythromycin: macrolide; ergotamine: nitrogen content. Both erythromycin and tetracycline are polyketide natural products made through either Type I (modular) or Type II polyketide synthases, respectively.

and screening that, although laborious, resulted in many of the production strains still used today. Newer methods of compound identification and overproduction (highlighted at the end of the chapter) make up much of the current research directions of the field.

8.1.2 Diversity of Antibiotics Produced by Bacteria and Fungi

To that end, the discovery, identification, and production path have led to the therapeutic natural products outlined in Table 8.1 and Figure 8.3. Table 8.2 provides a timeline across several compounds identified during the Golden Age of natural product discovery with the addition of more recent compounds, one of which (azithromycin) is a semisynthetic alteration of erythromycin discovered earlier. β-Lactam antibiotics were the first classification discovered and, as introduced above, showed antibacterial activity. Macrolide antibiotics possess a similar activity profile to β-lactam compounds and are commonly prescribed as substitutes for patients allergic to penicillin. Aminoglycosides offer an alternative activity profile relative to most β-lactam and macrolide compounds and are, thus, used to treat a different spectrum of infectious diseases. Tetracycline compounds have shown activity across a broader array of microbial hosts and, as such, this and similar compounds are referred to as "broad spectrum" antibiotics.

Table 8.2 Antibiotics discovered across decades and natural product classifications.

Decade	Antibiotic	Classification
1920s	Penicillin	β-Lactam
1940s	Tetracycline	Tetracycline
1940s	Cephalexin	β-Lactam (cephalosporins)
1950s	Erythromycin	Macrolide
1960s	Tobramycin	Aminoglycoside
1970s	Imipenem	β-Lactam (carbapenems)
1980s	Daptomycin	Cyclic lipopeptide
1980s	Azithromycin	Macrolide

As will be described in more detail below, the microbes responsible for infectious disease possess either native or mutation-based properties that render antibiotic compounds more or less effective. As such, antibiotic compound activity (as surveyed within Figure 8.2) is directed at appropriate microbial infectious disease targets. As additional examples, streptomycin is commonly used to treat tuberculosis, whereas daptomycin has shown activity toward the same types of bacteria susceptible to penicillins (termed Gram-positive bacteria) with activity against an antibiotic resistant form of *Staphylococcus aureus* (termed methicillin-resistant *Staphylococcus aureus* [MRSA]). The following sections will now go into more detail regarding the classifications of natural products that have emerged around biosynthetic mechanisms, compound structure, and therapeutic function.

8.2 β-Lactams

8.2.1 History, Effect, and Application

β-Lactams represent a historic contributor to antibiotic natural product utility. As briefly introduced above, penicillin provided a blueprint for antibiotic natural product discovery and development. The activity of β-lactams can be traced to the namesake lactam ring and how this disrupts cell wall biosynthesis (Figure 8.1). The necessity of cell wall formation by bacterial microorganisms emphasizes both the specificity of the bioactivity and the broad utility of the compound against bacterial species with (Gram-negative) and without (Gram-positive) outer membranes.

8.2.2 β-Lactam Biosynthesis

β-Lactam biosynthesis will be highlighted through two widely successful examples: penicillin and cephalosporin. The shared biosynthetic mechanisms of both compounds are highlighted, as are their differences (Figures 8.4 and 8.5).

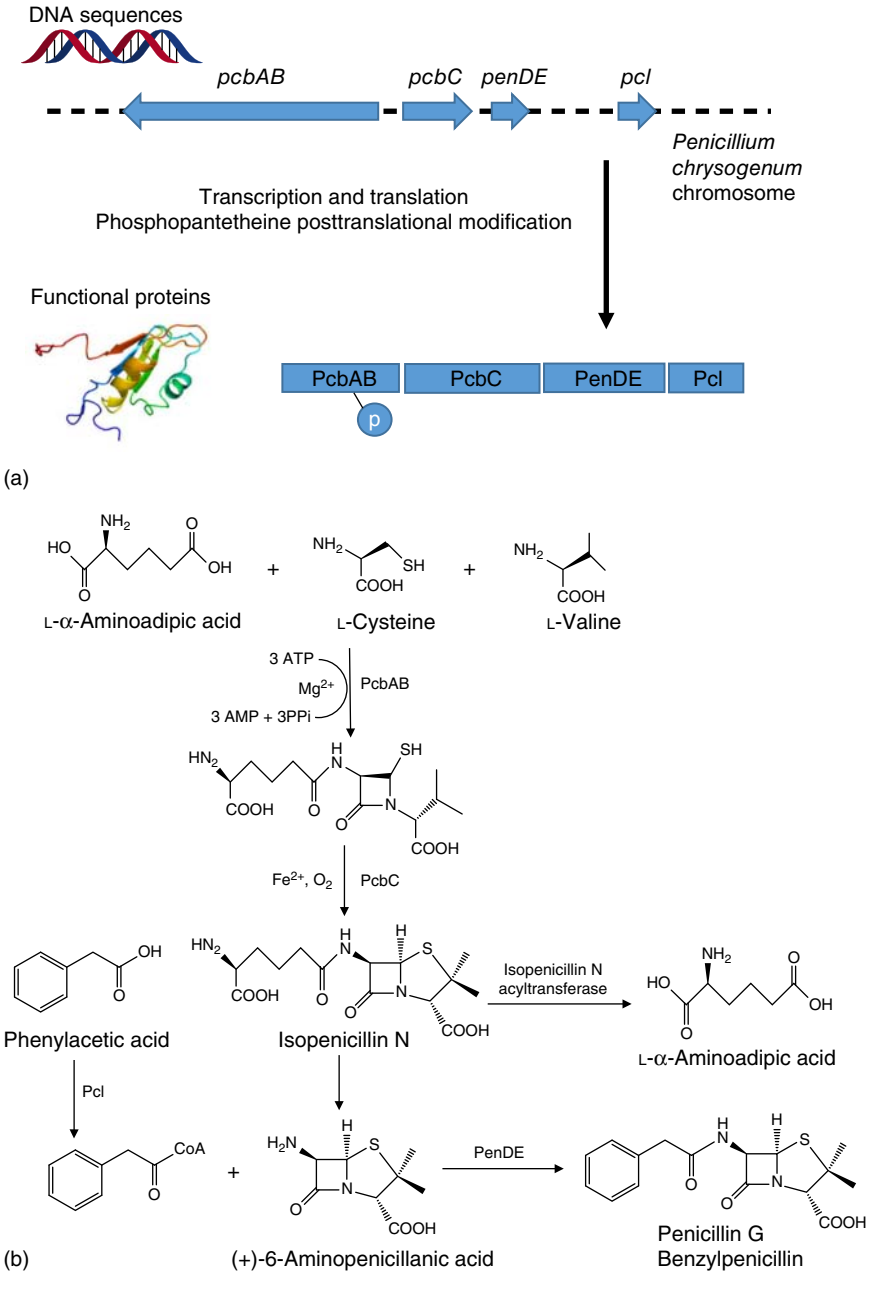


Figure 8.4 Penicillin biosynthetic pathway. Indicated are the steps from gene expression (a; with the circled "p" indicating 4′-phosphopantetheine posttranslational modification of PcbAB to activate the protein for functionality) to biosynthesis (b). The *pcbAB* gene encodes an *N*-(5-amino-5-carboxypentanoyl)-L-cysteinyl-D-valine synthase; *pcbC* encodes isopenicillin-N synthase; *penDE* encodes isopenicillin-N *N*-acyltransferase; *pcl* encodes phenylacetate-CoA ligase.

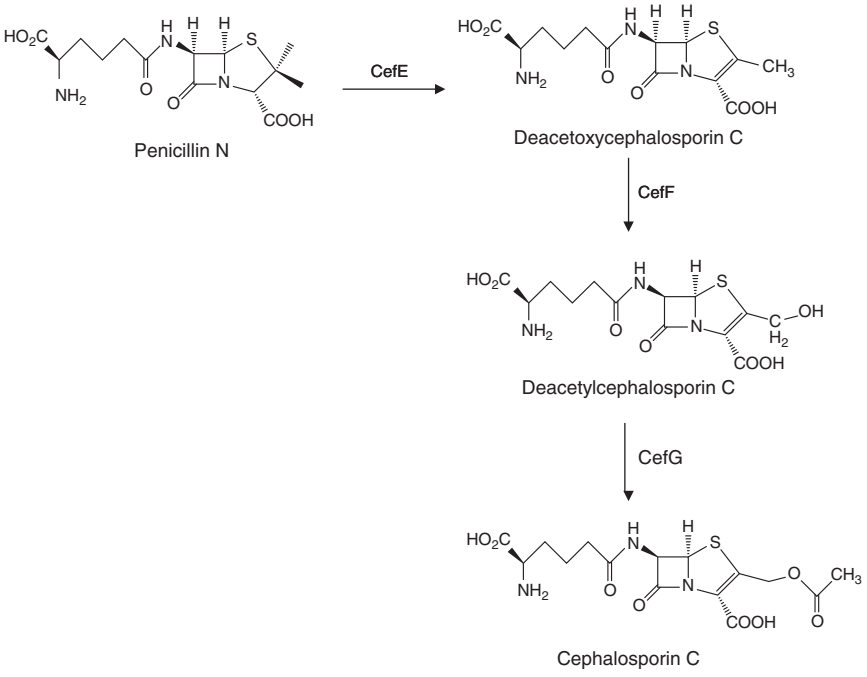


Figure 8.5 Cephalosporin biosynthetic pathway. The isopenicillin N product from Figure 4 is converted to cephalosporin C. PcbAB: *N*-(5-amino-5-carboxypentanoyl)-L-cysteinyl-D-valine synthase; PcbC: isopenicillin-N synthase; CefD: isopenicillin-N epimerase; CefE: deacetoxycephalosporin-C synthase; CefF: deacetoxycephalosporin-C hydroxylase; CefG: deacetylcephalosporin-C acetyltransferase.

Together, these compounds have had a major impact on the antibiotic industry; combined, they represent 15 billion US$ sold per year with amoxicillin (an aminopenicillin with a wider activity spectrum) alone produced at 1100 t/yr.

For the production of penicillin, there were several engineering advances required to satisfy production demands. First, cellular production was improved through the course of an extensive effort to mutagenize and select production strains that sequentially produced more products. These strains were then the basis for large-scale bioreactor operations. Bioreactor operations required aseptic maintenance during the scaling process (to avoid contamination) while minimizing costs associated with power consumption and culture nutrients. Spurring the labor-intensive efforts to improve penicillin production was the concurrent timing of World War II, during which many casualties resulted from infections. Hence, a government–public–private initiative (across Allied countries) was implemented to improve and scale penicillin production for use during the campaign. In a typical 30–1000 gal bioreactor running in fed-batch mode, all the required nutrients (carbon sources, amino acids, etc.) are added to support initial growth of the producing microorganism (i.e. to support primary metabolism). As *Penicillium chrysogenum* is an aerobic mold, a stream of air is also supplied to maintain hyphal growth, and a steady pH of 6.5 is maintained

during culture. Growth is supported over time with the addition of a nutrient stream with cellular metabolism shifting to penicillin production as secondary metabolic pathways are upregulated. Optimization of the process with enhanced production strains has seen a boost in final penicillin titers from 1 to 50 g/l. With high-volume production of penicillin, the accompanying antibiotic price has dropped from $300 per kg in 1953 to $10 per kg now.

8.2.3 Penicillin Production by *Penicillium chrysogenum*

The source microorganism for penicillin is *P. chrysogenum*, first isolated in 1942 (although the original producer identified by Fleming was termed *Penicillium notatum*). The isolation and identification of penicillin then preceded the elucidation of the biosynthetic pathway illustrated in Figure 8.4. The foundation of the biosynthetic pathway is the activity of a type of enzymatic class called nonribosomal peptide synthetases (NRPSs). These and other natural product enzymatic classifications will be introduced together with the highlighted compounds within this chapter. Each biosynthetic classification has spurred its own research fields both in fundamental understanding of the biosynthetic enzymatic mechanism and the application of that knowledge to the production of the original or altered final compounds.

8.2.4 Cephalosporin Production by *Acremonium chrysogenum*

Figure 8.5 presents the biosynthetic pathway for cephalosporin produced from the fungus *Acremonium chrysogenum*. The base nonribosomal peptide nature of this compound mirrors that of penicillin. However, the difference results from isopenicillin N remaining as the key background compound of the final product. Cephalosporin is a commonly used as an alternative antibiotic to penicillin, applied in cases where penicillin resistance is observed during infection. When compared with penicillin, cephalosporin presents a broader spectrum of antibiotic activity, and the compound is more stable to many β-lactamase enzymes (the primary means of bacterial resistance development). Since the first isolation of the natural product, more than 15 semisynthetic cephalosporins were developed in five generations. Cefdinir, listed in Table 8.1, is a third-generation cephalosporin. In a multiple step reaction, the L-α aminoadipic acid side chain is replaced with an amino thiazole moiety.

8.3 Lipopeptides

8.3.1 History, Effect, and Application

Lipopeptides are unique for combining separate chemical moieties into one natural product. Effectively, the compounds contain a base structure derived from nonribosomal peptide biosynthesis. To this structure, a lipid chain is added, imparting cell wall and membrane penetration activities that contribute to antimicrobial activity. The examples outlined below (daptomycin and

cyclosporine) show both antimicrobial and antifungal activity. Hence, these compounds range in activity across prokaryotic and eukaryotic systems.

Daptomycin was discovered by Eli Lilly and Company in the late 1980s, but the development was halted after high doses of daptomycin showed negative effects on skeletal muscle, kidneys, the gastrointestinal tract, and the nervous system. After renewed assessment by Cubist Pharmaceuticals (later acquired by Merck), daptomycin proved effective against Gram-positive bacterial pathogens when a lower concentration of the drug was used. Additionally, daptomycin also demonstrated comparable antimicrobial activity against antibiotic-resistant strains such as MRSA. With all of these traits, the development of daptomycin was resumed after Cubist purchased the patent, and the antibiotic was then approved by the Food and Drug Administration (FDA) in September 2003.

In addition to antifungal activity, other lipopeptides are utilized as immunosuppressants (targeting human T cells; Figure 8.3). For example, cyclosporine is prescribed for patients during organ transplantation to reduce the chance of rejection of the incoming organ by the patient's own immune system. Given this activity against eukaryotic targets, background toxicity must be considered carefully when developing and administering these compounds because their eukaryotic targets will overlap significantly with nontarget human cell types.

8.3.2 Lipopeptide Biosynthesis

As the name indicates, lipopeptides will combine peptide and lipid components. This combination confers the bioactivity properties upon the final compound via the structural differences of both components. The peptide portion is provided by nonribosomal peptide biosynthesis, whereas the lipid derives from fatty acid biosynthesis.

8.3.3 Daptomycin Production by *Streptomyces roseosporus*

In the case of daptomycin (Figure 8.6), production derives from the bacterial *Streptomyces roseosporus* native producer. This host is also the basis for commercial production, which was developed by Cubist Pharmaceuticals. The mechanism of action for daptomycin is the disruption of potassium transport within Gram-positive bacteria (thus depolarizing the cellular structure and causing cell death) by binding to cellular membranes, facilitated by the lipid aspect of the daptomycin compound.

8.3.4 Cyclosporine Production by *Tolypocladium inflatum*

Figure 8.7 outlines the production of a specific cyclosporine from the *Tolypocladium inflatum* fungus. *T. inflatum* acts as an insect pathogen during reproduction, during which cyclosporine is produced as a secondary metabolite to eliminate competitive fungi. This particular compound also happens to possess immunosuppressive bioactivity as a result of a direct interaction with intracellular proteins within immune system lymphocytes, and while potent, the compound is prone to adverse reactions owing to the human cellular

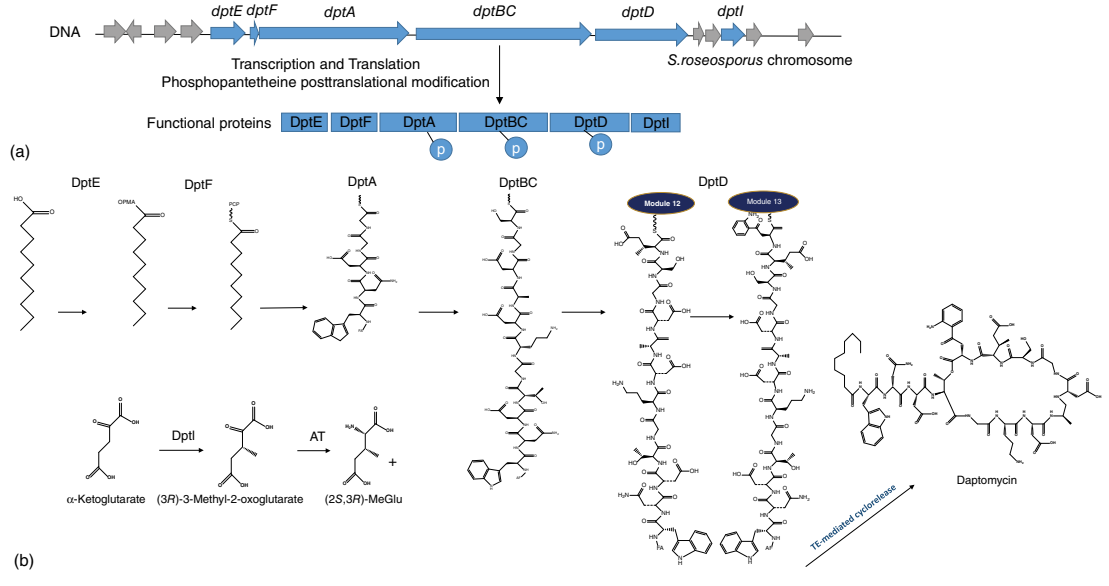


Figure 8.6 Daptomycin biosynthetic pathway. A schematic of the genetic cluster for daptomyc in converted from DNA to protein via transcription and translation (a; including posttranslational modification needed for enzymatic activity) before the enzymatic steps for compound formation (b). The biosynthesis pathway of daptomycin is composed of three NRPSs: DptA, DptBC, and DptD. The fatty acid substrate is generated by enzymes DptE and DptF. AT stands for aminotransferase, DapI is a methyltransferase, and TE stands for thioesterase.

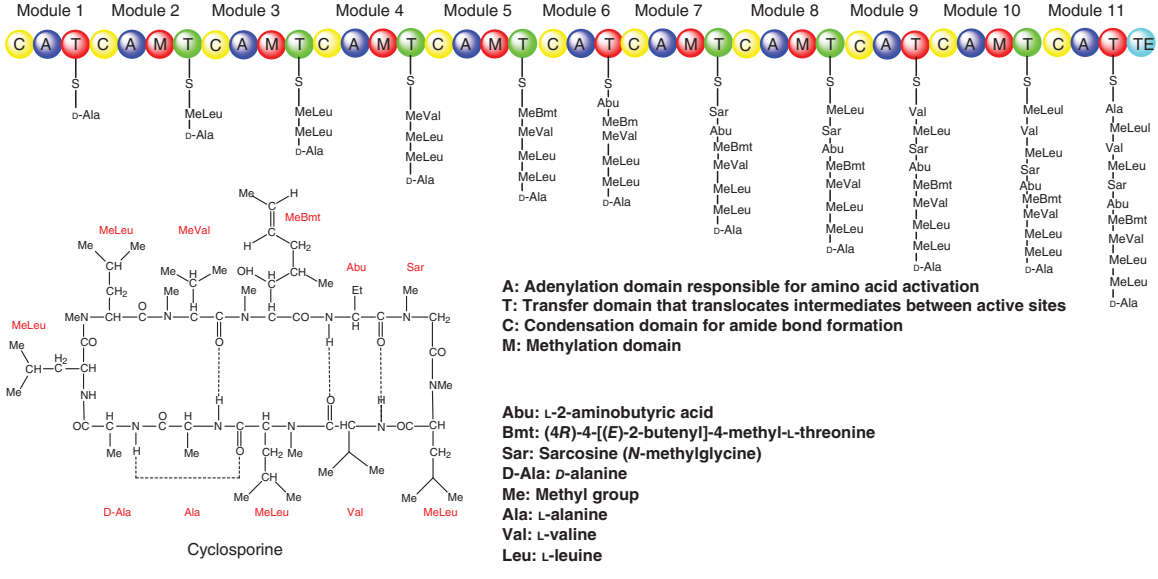


Figure 8.7 Cyclosporine biosynthetic pathway. The biosynthesis pathway contains 11 NRPS modules, each consisting of several domains for loading and processing amino acid substrates.

targets which it affects. In other words, bacterial targets that serve as the basis for antibiotic natural products often result in substantially reduced side effects (comparatively) because of the inherent differences from human cells.

8.4 Macrolides

8.4.1 History, Effect, and Application

Macrolide antibiotics rose to prominence in the 1950s, primarily on the discovery and application of the erythromycin compound. Other bioactivities are also associated with macrolide natural products and these include immunosuppression, motilin agonist (to stimulate gastrointestinal mobility), and antifungal function. Often utilized as an alternative to penicillin, erythromycin was an effective antibiotic with an emphasis on Gram-positive pathogens in respiratory illnesses. Several semisynthetic variants of erythromycin have found renewed or altered antibiotic activity (Table 8.1) conferred by the medicinal chemistry applied to the core structure of the compound.

8.4.2 Macrolide Biosynthesis

Macrolide biosynthesis features a second class of natural product enzymology associated with polyketide synthase catalysts. Sharing many similarities to nonribosomal peptide biosynthesis, polyketides result from the Claisen-like condensation of acyl-CoA units. The process is completed by the dedicated cyclization of the polyketide compound to form a macrolactone core structure. To this structure, many tailoring reactions enable a wide variety of adorning motifs that include hydroxyl, methyl, glycosylation, and keto groups.

8.4.3 Erythromycin Production by *Saccharopolyspora erythraea*

Erythromycin is produced by the soil bacterium *Saccharopolyspora erythraea*. The biosynthetic pathway (Figure 8.8) represents a classical formation of a macrolactone, followed by core structure adornment with several functional groups that confer final bioactivity. The primary target of the erythromycin compound is the peptide transfer center of bacterial ribosomes with the unique planar nature of the erythromycin compound able to disrupt the ribosomal protein synthesis machinery.

The production of erythromycin utilizes strains of *S. erythraea* that, similar to production hosts for penicillin and other natural products, have been mutagenized and engineered to significantly boost cellular production of the erythromycin product. Production medium typically consists of sucrose, corn steep liquor, soybean oil, yeast extract, NaCl, and $CaCO_3$ precipitate with a large-scale bioreactor reaching 300 tons in scale. Culture conditions for the bioreactor include a pH range between 6.6 and 7.2 and an incubation temperature between 33 °C (initially) and 31 °C during the erythromycin production stage. Propanol is added every 24 hours during the production stage as well to supply a precursor

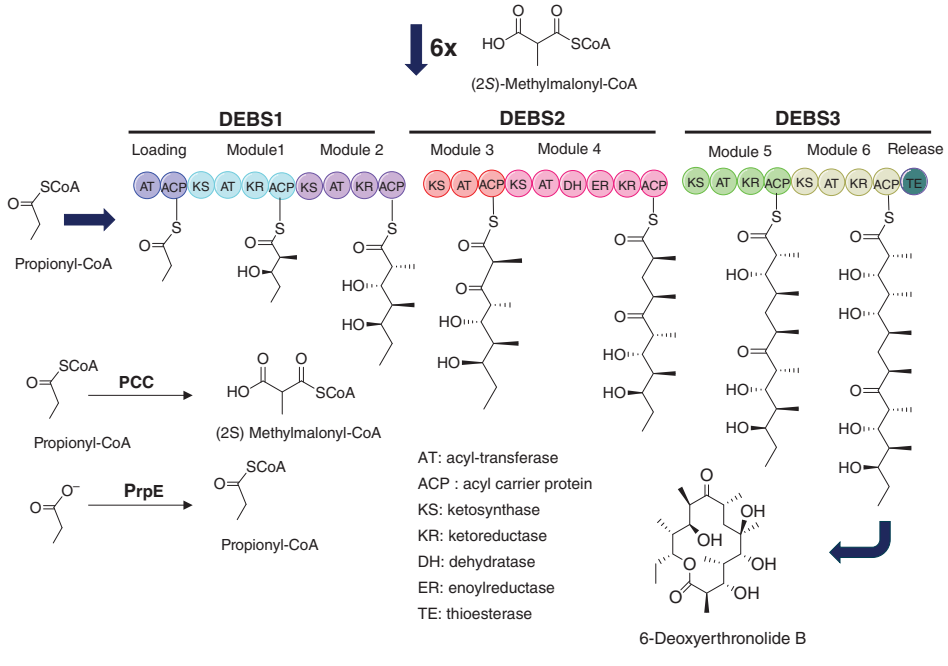


Figure 8.8 Erythromycin biosynthetic pathway. DEBS1,2,3 : 6-deoxyerythronolide B polyketide synthase enzymes (the ACP domains require phosphopantetheinylation); PrpE: propionyl-CoA synthetase; PCC: propionyl-CoA carboxylase; dTDP-D-deosamine biosynthesis includes EryBII/EryBIII/EryBIV/EryBVI/EryBV; dTDP-L-mycarose biosynthesis includes EryCI/EryCII/EryCIV/EryCV/EryCVI; EryF: 6-deoxyerythronolide B hydroxylase; EryK: erythromycin 12 hydroxylase; and EryG: erythromycin 3″-*O*-methyltransferase.

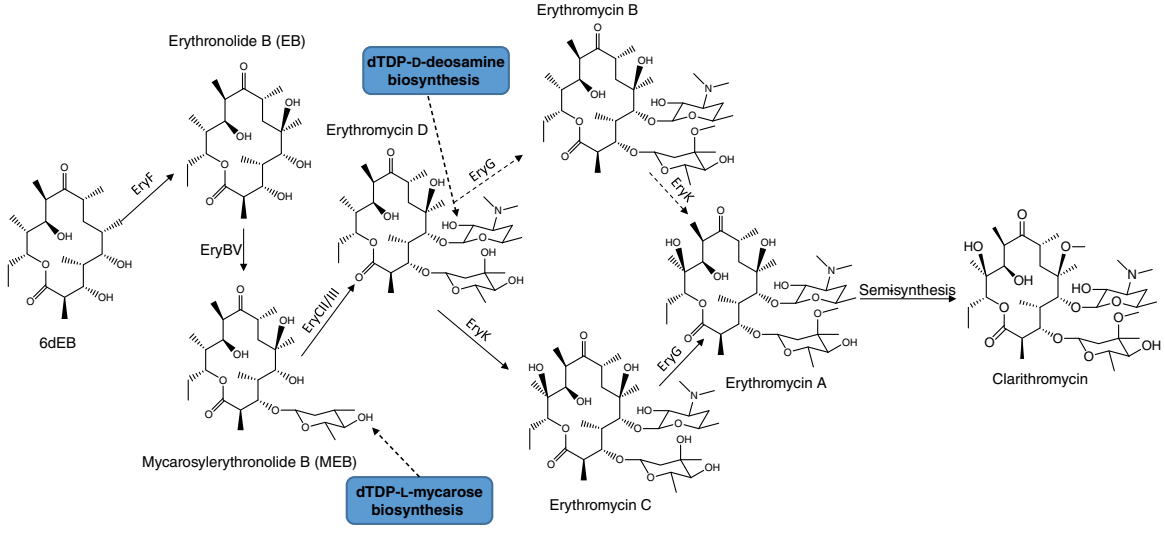


Figure 8.8 (*Continued*)

source for the propionyl-CoA substrate required for erythromycin biosynthesis (Figure 8.8); glucose is also supplied every 6 hours until 12–18 hours before the termination of the culture, which ranges from 3 to 7 days.

For the downstream extraction of erythromycin, a final bioreactor broth is first adjusted basic by sodium hydroxide addition. The resulting solution is then extracted with butyl acetate with the erythromycin compound predominantly partitioned to the organic phase. The organic phase is then re-extracted by pH 5 water, and the pH of the resulting erythromycin-enriched water phase is then elevated to 9.5. Under these conditions, erythromycin will crystallize, allowing easy separation from the water phase. Additional steps are included to dehydrate the final erythromycin product.

Figure 8.8 also includes the structure for clarithromycin, which was introduced in Table 8.1 as a semisynthetic version of erythromycin. The compound is generated through synthetic organic chemistry steps that include protection of the C-9 keto group and methylation of the C-6 hydroxy group by methyl iodide. As indicated in Table 8.1, production of clarithromycin has surpassed that of the original erythromycin compound. The reason for the increased utility of clarithromycin is that the original erythromycin compound is not stable in the low-pH environment of the stomach upon administration. The methylation at C-6 afforded by the clarithromycin compound stabilizes the drug under these acidic conditions and therefore provided extended bioactivity.

8.5 Tetracyclines

8.5.1 History, Effect, and Application

Tetracyclines are another historical group of antibiotics first discovered in the 1940s and produced natively through *Streptomyces rimosus*. The primary mechanism of action is peptide biosynthesis disruption, and the antibiotic properties have extended to both Gram-positive and Gram-negative bacterial strains. The compound has proven effective against difficulty to treat pathogens including bacterial species capable of intracellular localization (such as *Brucella* species), spirochaetal infections (such as syphilis and Lyme disease), and rarer diseases such as anthrax and plague. However, there are also a plethora of side effects that have been observed with tetracycline use that include adverse bone development. This is due to a rapid absorption into bone tissue. Alternatively, this same property has been used as a marker of bone growth because of the natural fluorescence of the tetracycline compound.

8.5.2 Tetracycline Biosynthesis

Tetracycline biosynthesis results from the action of polyketide synthases that act in a separate mechanism than those associated with erythromycin formation. Namely, a modular or Type I form of polyketide synthase is associated with erythromycin, whereas a Type II system is associated with tetracycline biosynthesis. The Type II biosynthetic mechanism is more common in bacterial species and is iterative in nature (meaning that the same set of enzymes successively build

upon a growing polyketide chain through repetitive addition of acyl-CoA extender units); hence, there is a typical symmetry in the final polyketide product before molecular diversification with various tailoring enzymes.

8.5.3 Tetracycline Production by *Streptomyces rimosus*

The Type II biosynthetic process is highlighted in Figure 8.9. Here, individual enzymes (that would be covalently tethered in the biosynthesis of modular polyketide compounds such as erythromycin) combine acyl-CoA compounds to form the core structure of the final compound. Various tailoring enzymes (such as cyclases, aromatases, and methylases) then further modify and produce the final compound. The compound is produced at scale through bioreactor processes reliant on the native *S. rimosus* producer. The *Streptomyces* genus, in particular, is responsible for a large number of complex natural products (such as polyketides and nonribosomal peptides), and certain species of this microorganism are consistently presented throughout the remaining examples of compounds produced within the context of this chapter.

8.6 Aminoglycosides

8.6.1 History, Effect, and Application

As the name suggests, aminoglycosides are a covalently linked series of sugars with one or more amino-modified units. Examples include kanamycin, streptomycin, neomycin, gentamycin, and tobramycin, and native production is common to both *Streptomyces* species (spp.) and *Micromonospora* spp. The mechanism of action associated with most aminoglycosides is bacterial protein synthesis disruption, and they are particularly active against Gram-negative bacteria. For example, streptomycin was the first effective antibiotic used to treat tuberculosis. In addition, a combination therapy with β-lactam antibiotics (penicillin) allows for broadened activity against Gram-positive pathogens (such as streptococcal infections).

However, aminoglycosides also possess unwanted toxicity effects that include inner ear (affecting hearing and balance) and kidney damage. As such, despite their important activity against Gram-negative pathogenic microbes, they are often not prescribed as the first option for infections. As alternatives, isonicotinylhydrazide, rifampin (a polyketide compound), carbapenems (a β-lactam), cephalosporins, and chloramphenicol are also used for infections caused by Gram-negative microbes.

8.6.2 Aminoglycoside Biosynthesis

Biosynthesis of aminoglycosides proceeds through the systematic amino functionalization of hexose units during successive condensation of the resulting amino sugars. Thus, supporting biosyntheses are the native sugar pathways of the producing microorganism with dedicated gene clusters providing enzymatic machinery for sugar combinations and the amino modifications.

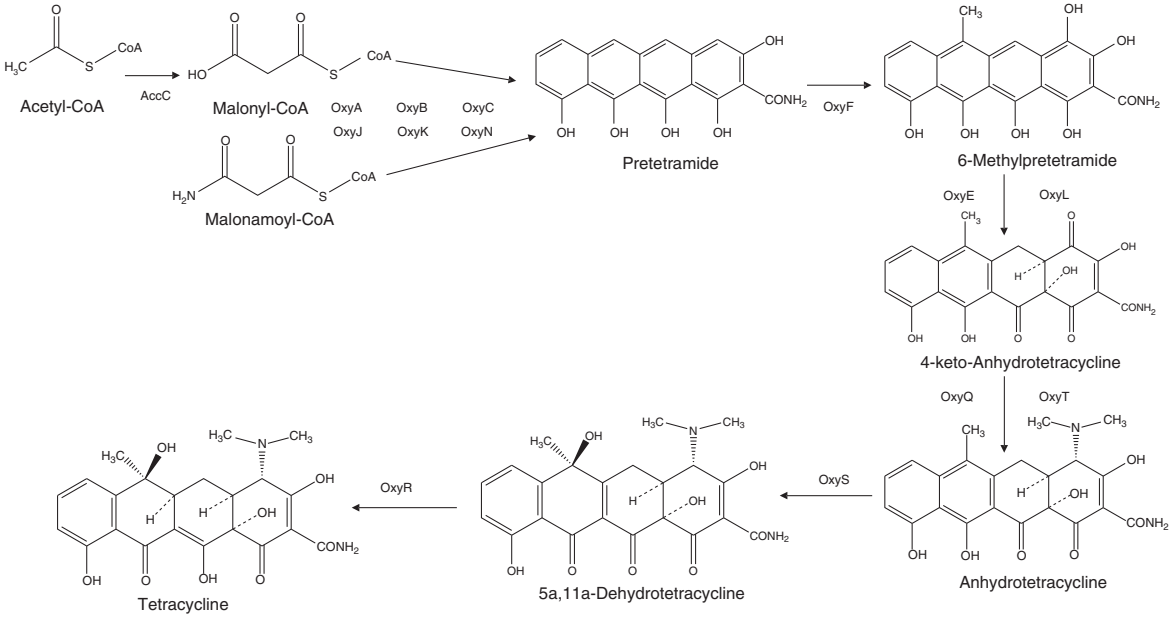


Figure 8.9 Tetracycline biosynthetic pathway. AccC: acetyl-CoA carboxylase; OxyA/OxyB/OxyC/OxyJ: Type II polyketide synthase; OxyK: bifunctional cyclase/aromatase; OxyN: cyclase; OxyF: C-methyltransferase; OxyE: monooxygenase; OxyL: NADPH-dependent dioxygenase; OxyQ: aminotransferase; OxyT: *N,N*-dimethyltransferase; OxyS: anhydrotetracycline monooxygenase; OxyR: 5a,11a-dehydrotetracycline reductase.

8.6.3 Tobramycin Production by *Streptomyces tenebrarius*

To demonstrate aminoglycoside biosynthesis, the tobramycin pathway is provided in Figure 8.10. Here, a hydroxyl removal step is required in addition to the sugar amino modifications. The final compound has a significant structural overlap with the kanamycin and gentamycin compounds.

8.7 Claviceps Alkaloids

8.7.1 History, Effect, and Application

Claviceps is a genus of fungi capable of producing alkaloid natural products. A primary example includes ergotamine derived from *Claviceps purpurea*, which is used as a treatment for migraine headaches. Interestingly, ergotamine was discovered and marketed in the early period of the 1900s, before antibiotic compounds began their rise to medicinal prominence. However, ergotamine side effects include deleterious impacts upon blood circulation and neurotransmission, including burning sensations in the limbs, hallucinations, nausea, and convulsions.

8.7.2 Alkaloid Biosynthesis

Alkaloids feature nitrogen as a primary element during biosynthesis, which typically involves amino acids as base components for product formation. The inclusion of amino acids introduces the mechanism of nonribosomal peptide biosynthesis as a means of building to final compound structure. Various tailoring reactions will then support specific structural features associated with certain final compounds.

8.7.3 Ergotamine Production by *Claviceps purpurea*

Biosynthesis of the ergotamine is presented in Figure 8.11. Here, nonribosomal peptide biosynthesis is involved with the core formation of the compound. However, the steps in generating the base compound include a tryptophan that has been prenylated; thus, the isoprenoid biosynthetic steps responsible for a separate classification of natural products (with specific examples including the antimalarial compound artemisinin and the anticancer compound taxol) are involved with the generation of this compound. Additional tailoring reactions then give yield to the final compound.

8.8 Perspectives

8.8.1 Antibiotic Resistance

The discovery and development of natural products for antibiotic application has had a tremendously positive impact on human health. However, the application of antibiotics will inevitably lead to resistance development by target cellular populations. This issue is becoming more urgent as an increasing number of human pathogens are showing resistance to once effective antibiotics (Table 8.3).

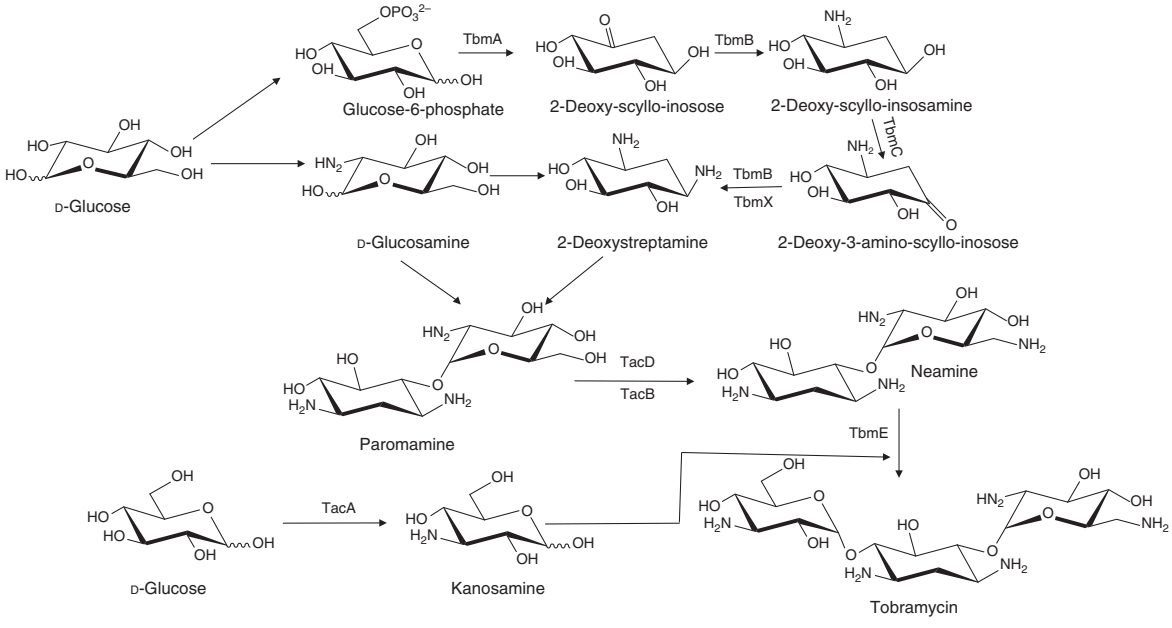


Figure 8.10 Tobramycin biosynthetic pathway. TbmA: 2-deoxy-scyllo-inosose synthase; TbmB: L-glutamine:scyllo-inosose aminotransferase; TmbX; L-glutamine:scyllo-inosose aminotransferase; TbmC: dehydrogenase; TacD: dehydrogenase; TacB: dehydrogenase; TacA: carbamoyltransferase; TbmE: tobramycin synthase.

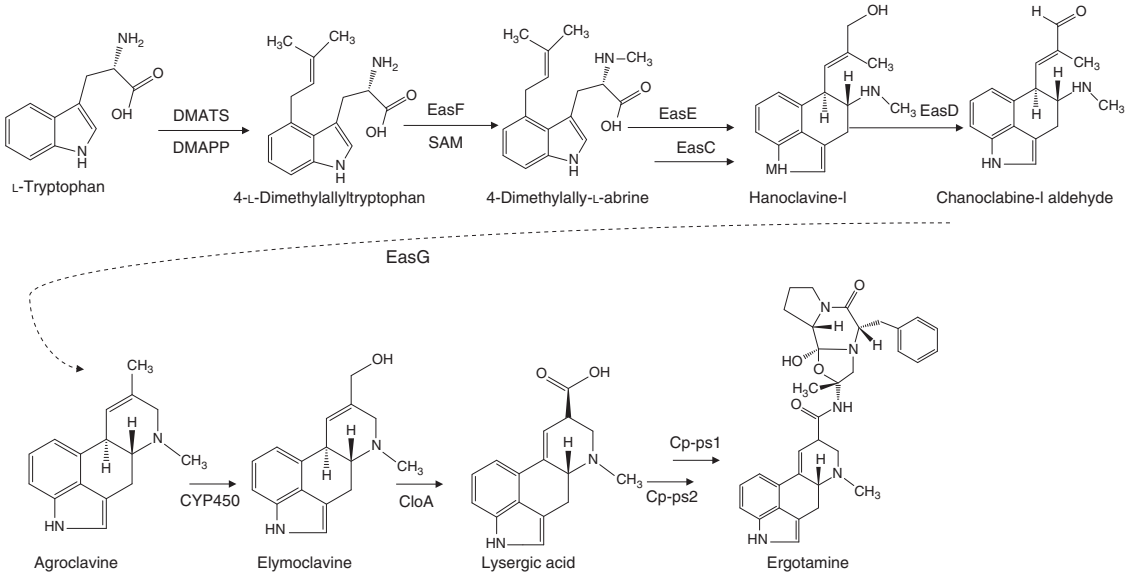


Figure 8.11 Ergotamine biosynthetic pathway. DMAPP: dimethylallyl diphosphate; DMATS: prenyltransferase 4-dimethylallyltryptophan synthase; EasF: 4-dimethylallyltryptophan *N*-methyltransferase; EasE: the FAD-dependent oxidoreductase; EasC: catalase; EasD: short-chain dehydrogenase/reductase (SDR); CYP450: cytochrome P-450 monooxygenases; CloA: clavine oxidase; SAM: *S*-adenosylmethionine; Eas: agroclavine synthase; Cp-ps2/Cp-ps1: D-lysergyl-peptide synthetase.

Table 8.3 Top antibiotic resistant bacterial strains.

Top antibiotic resistant strains	Degree
Clostridium difficile	Urgent
Carbapenem-resistant *Enterobacteriaceae* (CRE)	Urgent
Drug-resistant *Neisseria gonorrhoeae*	Urgent
Multidrug-resistant *Acinetobacter*	Serious
Drug-resistant *Campylobacter*	Serious
Fluconazole-resistant *Candida* (a fungus)	Serious
Extended spectrum β-lactamase-producing *Enterobacteriaceae* (ESBLs)	Serious
Vancomycin-resistant *Enterococci* (VRE)	Serious
Multidrug-resistant *Pseudomonas aeruginosa*	Serious
Drug-resistant *nontyphoidal Salmonella*	Serious
Drug-resistant *Salmonella typhimurium*	Serious
Drug-resistant *Shigella*	Serious
Methicillin-resistant *Staphylococcus aureus* (MRSA)	Serious
Drug-resistant *Streptococcus pneumoniae*	Serious
Drug-resistant tuberculosis	Serious
Vancomycin-resistant *Staphylococcus aureus* (VRSA)	Concerning
Erythromycin-resistant Group A *Streptococcus*	Concerning
Clindamycin-resistant Group B *Streptococcus*	Concerning

Over time, this concern is expected to become a significant health issue within the twenty-first century.

Similar to the cellular targets associated with antibiotic effectiveness, Figure 8.12 provides several common means of antibiotic resistance. As both targets and resistance mechanisms become better understood, better approaches can be adopted in developing new antibiotic strategies. One of the ultimate goals in this regard is to identify a potent antibiotic compound that limits or altogether eliminates the development of resistance mechanisms.

8.8.2 New Research Model for Compound Identification

Table 8.4 highlights the reduction in research efforts by large pharmaceutical companies as it relates to natural products. Part of this trend is the cost of drug development and how this process provides an economic impediment to producing any new drug. Another aspect is the curative (as opposed to prolonged) administration of antibiotic compounds and, therefore, the lack of greater revenue streams over time (which further exacerbates the issue of costs associated with drug development initially). Thus, research efforts, including those devoted to new natural product discovery, have been largely left to academic laboratories, larger-scale research centers (typically academic or government), and biotechnology start-up companies. An emerging model of discovery and development is

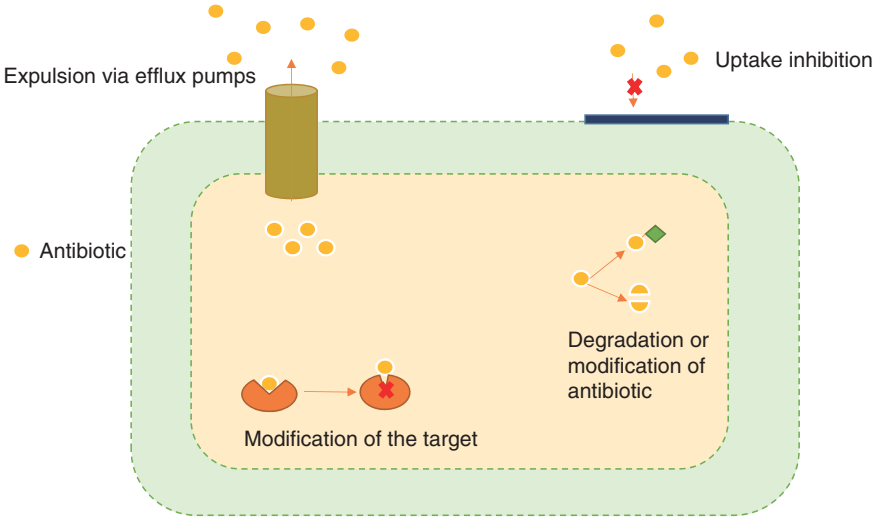


Figure 8.12 Primary antibiotic resistance mechanisms.

Table 8.4 Reasons for reduction in the industrial development of antibiotic natural products.

Reasons for reduction in companies devoted to natural product development
1. Significant economic investment
2. Less profitable than chronic disease prescription medicines
3. Relatively low sales price for antibiotics
4. Low usage of new antibiotics due to advised restraint
5. Inevitable drug resistances
Major pharmaceutical companies downsizing antibiotic research
Pfizer Inc.
Johnson & Johnson
Eli Lilly & Co.
AstraZeneca
Sanofi S.A.

to utilize smaller research institutions for compound identification followed by larger entities with greater infrastructure then leading efforts to scale production of the new compound.

8.8.3 Future Opportunities

Dealing with emerging antibiotic resistance is a significant future challenge. As such, new means of antibiotic natural product discovery and development have been at the forefront of current research efforts. Prime cellular targets that

Table 8.5 Novel cellular targets that might limit resistance development and examples of antibiotic compounds for these targets.

Cellular targets to limit resistance development	Antibiotic examples
Efflux proteins	Omadacycline
Antibiotic modifying enzymes	Plazomicin
Restore antibiotic binding affinity to ribosome	Telithromycin
β-Lactamase inhibitors	Siderophore-containing β-lactam
	Clavulanic acid Sulbactam Tazobactam Avibactam Relebactam Boronic acids
Transcription factors	ZHAWOC1035/1132

may limit resistance development are presented in Table 8.5. The key goal then becomes finding the new compounds for these and other pathogenic targets.

There are several ongoing approaches to identifying new antibiotic natural products as summarized in Table 8.6. The traditional discovery process involved

Table 8.6 New discovery options including those utilizing pathway engineering, deregulation, and new activity screens.

Approach	Sub-category
Principal component analysis	High throughput screening
Imaging MS	High throughput screening
Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)	High throughput screening
Colorimetric assay	High throughput screening
SMURF (Secondary Metabolite Unknown Regions Finder)	Bioinformatics tools
antiSMASH (Antibiotics & Secondary Metabolite Analysis Shell)	Bioinformatics tools
Kinase knockout to activate silent gene clusters	Native host expression
Knock in promoter to activate silent gene clusters	Native host expression
RecET direct cloning	Heterologous host expression
DNA assembler	Heterologous host expression
Combinational biosynthetic approaches	Heterologous host expression
MetaVelvet-SL	Shotgun metagenomics
CopyRighter	Marker gene metagenomics

isolating microbial sources from different environments (with the soil being a particularly fruitful location for antibiotic-producing hosts) and testing extracts from these microbial sources against tester bacterial strains. However, today, this same approach often leads to replication of those same compounds discovered and commercialized previously. A simple update to this approach would be to screen against those pathogens posing the most current risks (such as antibiotic resistance) to identify completely new compounds in addition to altering the screening process through new technology updates that increase detection capability and throughout.

Alternatively, an approach termed metagenomics seeks to capture the genomic material from the microbial samples collected from the environment. The collected genomic content can then be packaged into expression vectors and transferred to surrogate host systems that would allow simplified screening owing to the ease of working with the alternative host. The maturing developments in genomic DNA capture, cloning, and screening by DNA sequencing are allowing this approach to become more directed such that only novel natural product gene clusters can be identified from a population of collected metagenomic DNA before screens for activity.

The process of transferring foreign DNA into a new host for natural product access is more generally referred to as heterologous biosynthesis (Figure 8.13). Novel antibiotic gene clusters may be transferred to a heterologous host in an attempt to improve the prospect of enhanced product formation (especially in cases where the native hosts are incapable of facile culture). Furthermore, the molecular tools associated with the new host provide a basis for biosynthetic and process engineering and improved compound formation.

Native hosts also provide new opportunities given recent advances in DNA sequencing. Next-generation sequencing technology allows rapid genomic content elucidation for current and emerging natural product producers, and emerging bioinformatics approaches help to decipher the new sequence for embedded natural product gene clusters. Interestingly, well-established natural product producers are often revealed to be housing multiple natural product gene clusters (although only a few cluster pathways may be actively generating a product). There is great interest in better understanding and then manipulating the regulation mechanisms that determine biosynthesis, such that every possible compound is allowed to be produced and tested for therapeutic potential.

All of the above options hold great promise for ushering in a new era of productive natural product discovery. Successfully navigating the information developing from genomic and metagenomic sequences and converting this information to chemical output through either native or heterologous hosts is the basis for much of ongoing research. There is no doubt that those natural products produced in the past and highlighted throughout this chapter were only a small fraction of compounds contained within nature. Unlocking this untapped chemical potential is thus the next frontier in natural product research.

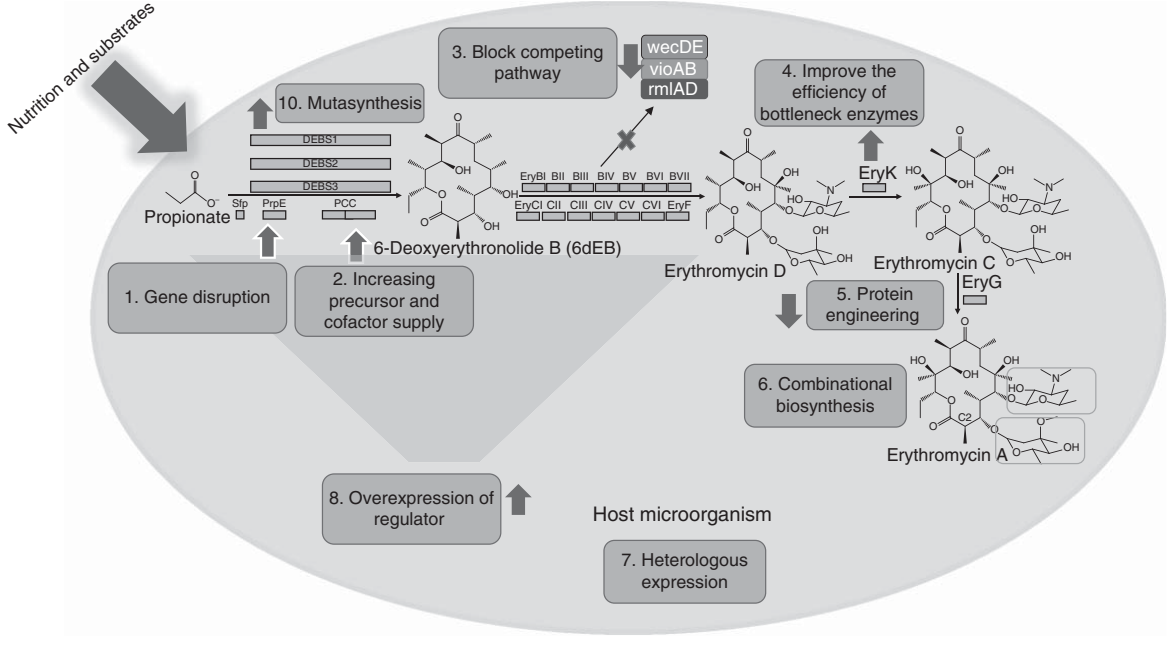


Figure 8.13 Various mechanisms (including native gene deregulation for enhanced native production, metabolic engineering, and heterologous biosynthesis) for new and enhanced natural product production featuring erythromycin biosynthesis as an example.

Further Reading

Aoki, H. and Okuhara, M. (1980). Natural β-lactam antibiotics. *Annu. Rev. Microbiol.* 34: 159–181.

Baltz, R.H., Miao, V., and Wrigley, S.K. (2005). Natural products to drugs: daptomycin and related lipopeptide antibiotics. *Nat. Prod. Rep.* 22 (6): 717–741.

Davies, J. and Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiol. Mol. Biol. Rev.* 74 (3): 417–433.

Demain, A.L. and Adrio, J.L. (2008). Contributions of microorganisms to industrial biology. *Mol. Biotechnol.* 38 (1): 41–55.

Demain, A.L. and Sanchez, S. (2009). Microbial drug discovery: 80 years of progress. *J. Antibiot.* 62 (1): 5–16.

Hamilton-Miller, J.M. (1973). Chemistry and biology of the polyene macrolide antibiotics. *Bacteriol. Rev.* 37 (3): 166–196.

Kishimoto, S., Sato, M., Tsunematsu, Y., and Watanabe, K. (2016). Evaluation of biosynthetic pathway and engineered biosynthesis of alkaloids. *Molecules* 21 (8): 1078.

Luo, Y., Cobb, R.E., and Zhao, H. (2014). Recent advances in natural product discovery. *Curr. Opin. Biotechnol.* 30: 230–237.

Mingeot-Leclercq, M.P., Glupczynski, Y., and Tulkens, P.M. (1999). Aminoglycosides: activity and resistance. *Antimicrob. Agents Chemother.* 43 (4): 727–737.

Schnappinger, D. and Hillen, W. (1996). Tetracyclines: antibiotic action, uptake, and resistance mechanisms. *Arch. Microbiol.* 165 (6): 359–369.

9

Pharmaceutical Proteins

Heinrich Decker[1], *Susanne Dilsen*[1], *and Jan Weber*[2]

[1] *Sanofi-Aventis Deutschland GmbH, Industriepark Hoechst, 65926 Frankfurt am Main, Germany*
[2] *GSK Vaccines GmbH, Emil-von-Behringstraße 76, 35041 Marburg, Germany*

CHAPTER MENU

9.1 History, Main Areas of Application, and Economic Importance

Through the work of Stanley N. Cohen with regard to the transformation of the intestinal bacterium *Escherichia coli* (*E. coli*), the foundation was laid for genetic engineering applications and thereof the production of human pharmaceutical proteins with recombinant microorganisms. This made it possible to manufacture and express proteins in almost unlimited quantities with great specificity. The first human protein using *E. coli* as a production host was the peptide hormone somatostatin, an antigrowth hormone that is used for the treatment of growth disturbance. As the first recombinant medicinal product, insulin came on the market in 1982 using *E. coli* as the host microorganism, which became in the 1980s the dominant platform for protein-based recombinant products, followed by *Saccharomyces cerevisiae* (*S. cerevisiae*). Microorganisms as hosts for the production of pharmaceutical proteins are characterized by high growth rates and relatively simple handling. More complex and glycosylated proteins can be produced in eukaryotic cells such as baker's yeast or *Pichia pastoris*. However, the

Industrial Microbiology, First Edition.
Edited by David B. Wilson, Hermann Sahm, Klaus-Peter Stahmann, and Mattheos Koffas.

glycosylation patterns in yeast can differ substantially from the pattern found in native human proteins. Proteins such as erythropoietin, a 34 kDa cytokine that stimulates the production of red blood cells, require a very specific glycosylation pattern influencing their physicochemical properties and pharmacological activity. For the production of complex and specific proteins, mammalian cell lines (CHO, mice, human, BHK, and Vero) are used, which are nowadays the prevalent system for the production of glycosylated recombinant pharmaceutical products that are close to the native human protein (Table 9.1).

The production of protein substances in recombinant microorganisms was an important milestone in the pharmaceutical industry. Up to this turning point, the corresponding active ingredients were extracted from animal or human organs: insulin from the pancreas of pigs and cattle, human growth hormone (hGH) from the pituitary, and the coagulation factor VIII from human blood plasma. The controlled production of active pharmaceutical ingredients (API) in microorganisms or cell culture ensures the safe supply to patients because in contrast to the limited availability of organs or blood preserves, the biotechnical production capacity is adapted to needs. In addition, risks arising through the use of human and animal tissue are minimized. These are, e.g. viruses or prions as a potential threat.

In the Federal Republic of Germany, a total of 207 medicinal products were approved in 2016 containing active substances that were produced by recombinant technologies. Approximately 40% of them are produced with microbial expression hosts, the other 60% derive from the expression using mammalian cell lines (see Table 9.1). A medicinal product usually consists of a pharmaceutically

Table 9.1 Expression hosts used for approved recombinant pharmaceutical drugs in Germany in 2016.

	Production host	Number of registered drugs
Microorganism	*Escherichia coli*	49
	Saccharomyces cerevisiae	31
	Hansenula polymorpha	2
	Pichia pastoris	1
	Vibrio cholera	1
Mammalian cell lines	Chinese Hamster ovary cells (CHO)	80
	Mice cells (e.g. NS/0)	22
	Human cells ss (e.g. HEK, PER.C6)	8
	Baby hamster kidney cells (BHK)	6
	African green monkey (Vero)	3
Insect cell line	Cabbage looper (Baculovirus system)	1
Transgenic animals	Rabbit	1
	Goat	1

CHO = Chinese Hamster Ovaria cell line; BHK = Baby Hamster Kidney cell line; Vero = kidney epithelial cell line from monkey.

Table 9.2 Overview of the annual turnover of the most important microbial pharmaceutical proteins in the year 2014.

Active pharmaceutical ingredient (API)	Therapeutic area	Sales 2014 (billion US$)
Insulins	Diabetes	24.0
Filgrastim and Pegfilgrastim	Chemotherapeutic induced Leucopenia	7.0
Interferon β-1a	Multiple sclerosis	5.5[a)]
Ranibizumab	Age-based macular degeneration	4.3
Somatropin	Growth restriction	3.4
Liraglutide	Diabetes	2.4
Peginterferon α and interferon α	Hepatitis B and C	1.7
Teriparatide	Osteoporosis	1.3

a) 2013.
Adapted from www.evaluategroup.com.

active ingredient (the so-called API), which is mixed with other materials, e.g. for preservation or better absorption and distribution of the active substance in the body. Therefore, an active substance can be made available in different forms as a medicine to patients. As a result, the 207 medicinal products on the German market are derived from 163 different active ingredients. For example, the company Lilly Deutschland GmbH provides the same active substance insulin Lispro (a fast-acting insulin, see Section 9.3) in two different formulations under the brand name Humalog™ and Humalog Mix™.

The worldwide sales of genetically engineered drugs (e.g. therapeutic proteins and antibodies) was 162 billion US$ in the year 2014, and about 30% of these sales were generated by products derived from genetically engineered microorganisms (see Table 9.2). The majority of the sales are generated with active ingredients that are obtained from mammalian cell cultures. From the top five best-selling biologics in the world, in 2014, the first four positions were occupied by antibodies and proteins produced with the use of mammalian cell culture technology (sales in 2014 : 40 billion US$). The first microbial recombinant protein from *E. coli* with a sales volume of 8.15 billion US$ (insulin glargine Lantus™) can be found in position 5. Microbial expression of insulin and insulin analogs makes up almost half of the market share of the marketed protein-based recombinant pharmaceutical products, with a turnover of 24 billion US$.

9.2 Industrial Expression Systems, Cultivation and Protein Isolation, and Legal Framework

9.2.1 Development of Production Strains

Many researchers used the model microorganisms *E. coli* and *S. cerevisiae* for their fundamental work on the investigation of physiology, genetics, and regulation. It is not surprising that these microorganisms have been used for the

manufacture of the first generation of biologics since the appropriate tools for the genetic modification of these strains were available in the 1970s and 1980s. The success story of antibodies began after the establishment of a methodology for the production of monoclonal antibodies in 1975. A further important step for the successful growth of therapeutic monoclonal antibodies was the development of efficient and more cost-efficient production methods and the protein engineering of the monoclonal antibodies to move from an initial murine-like protein sequence to a humanized or complete human monoclonal antibody.

The selection of a suitable expression system for a particular protein depends on various factors, such as the size and structure of the protein, and also its properties. Glycosylated complex proteins are usually produced in mammalian cells, as *E. coli* has no enzymes for the glycosylation of proteins. Sugar chains are appended starting in the endoplasmic reticulum (ER) of eukaryotic cells and continuing in the Golgi apparatus to folding polypeptide chains either on the residues L-asparagine (N-glycosylation) or L-threonine (O-glycosylation). Proteins produced in baker's yeast *S. cerevisiae* are characterized by a different glycosylation pattern compared to mammalian cells. The sugar chains produced by baker's yeast contain a high percentage of mannose and the proteins are partly hyper-mannosylated, while more complex sugar side chains with different sugar blocks are found in mammalian cells. For some proteins, it has been shown that the nonglycosylated protein produced in *E. coli* has the same biological activity as those produced in mammalian cells (e.g. human interleukin-2). These differences from the native human glycosylation pattern can potentially cause immunological reactions against the protein and influence the pharmacodynamics (for example, the binding to a receptor, the biological effectiveness) as well as the pharmacokinetics (the half-life of an active substance in the body). Both baker's yeast and *E. coli* can grow in a simple, cost-effective medium with additives such as yeast extract or peptone with high growth rates. Moreover, high cell densities of more than 50–200 g/l dry mass and thus high space-time yields can be achieved in industrial bioreactors by batch, fedbatch or continuous fermentations. The scale of bioreactors can reach up to 100 m^3 in commercial production.

When producing microbial proteins, there are essentially three options (Figure 9.1): (i) direct cytoplasmic expression, (ii) expression as a fusion protein, and (iii) expression and subsequent secretion. In the cytoplasmic expression, the protein is available intracellularly as insoluble inclusion bodies or as soluble protein. Inclusion bodies are produced in bacteria when translation rates are larger than the folding rate. They are solid particles of aggregated proteins. This phenomenon is used in the production of recombinant proteins through the application of strong promoters. In this case, proteins are protected in the inclusion bodies against proteolytic digestion but must be solubilized and folded correctly subsequently. In addition, proteins that are toxic for the producing cells can be manufactured more easily as inclusion bodies. These can be seen as visible particles via microscopy and can contain up to 30–50% of the total protein content of *E. coli* cells (Figure 9.2). Soluble, correctly folded proteins can be formed in the periplasm of *E. coli* or, in the case of baker's yeast, by secretion into the cultivation medium. In baker's yeast, the formation of disulfide

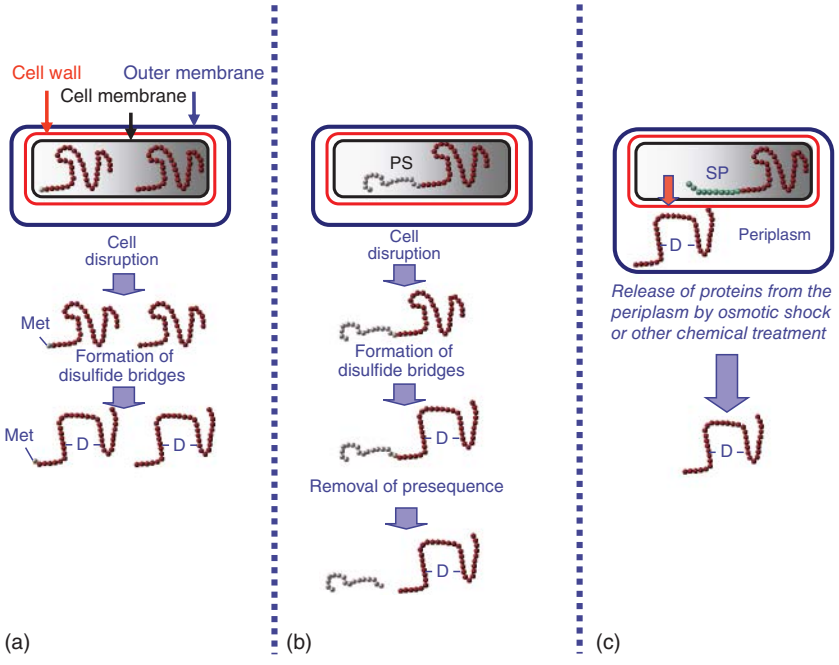


Figure 9.1 Different possibilities for protein production in *E. coli*: (a) direct expression in the cytoplasm, (b) fusion protein in the cytoplasm, and (c) secretion into the periplasm (PS = presequence; SP = signal peptide; Met = methionine, D = disulfide bridge).

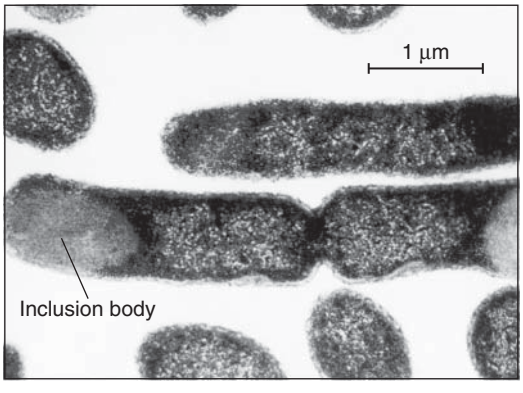


Figure 9.2 Electron micrograph of a recombinant *E. coli* K12 culture at harvest time. Gene expression was induced after an initial growth phase and the insulin fusion protein forms a clearly visible insoluble protein aggregate in the cytoplasm.

bridges and the folding of the protein in its native form take place only in the endoplasmic reticulum. In *E. coli*, the formation of disulfide bridges is heavily favored in the periplasm, as there is a suitable oxidative environment present, which allows the formation of the disulfide bridges. In the cytosol, the formation of disulfide bridges is difficult because reducing conditions prevail. For the selective release of a protein from the *E. coli* periplasm, an osmotic shock can be applied that leaves the bacterial cell otherwise undamaged. As only a small fraction of the *E. coli* proteins are located in the periplasmic space (4–10%),

subsequent purification of the target protein is simplified compared to a protein purified from the cytoplasm.

A further possibility for the production of proteins is the expression of a fusion protein (Figure 9.1). In this case, the target protein is merged with another protein. This second protein will be split off the target protein enzymatically or chemically in the course of the isolation. This method is used when the target protein is unstable but can be stabilized via a fusion protein. With the introduction of a second protein, we can also impact the solubility of the target protein or introduce a purification affinity tag to simplify purification (e.g. polyhistidine affinity tag). In addition, this method can be used to produce a homogeneous N-terminal protein. In the case of direct expression of a gene in *E. coli*, the target protein contains always an N-terminal *N*-formyl-methionine or methionine because of the AUG start codons. The removal of the *N*-formyl residue is catalyzed in *E. coli* by the enzyme peptide deformylase. The cleavage of the *N*-formyl residue is usually very efficient; however, in case of strong overexpression of a protein, a certain percentage can still contain the *N*-formyl residue. *E. coli* also has an enzyme for the cleavage of the N-terminal methionine (methionine-amino peptidase); the efficiency of the cleavage, however, is subjected to the following amino acid in position 2 of the protein. Some of the smaller amino acids such as glycine, proline, and alanine facilitate the separation of the methionine residue. Other amino acids such as arginine or phenylalanine inhibit the cleavage. As a result, a mixture of the native target protein and a methionine or *N*-formyl-methionine extended form is obtained (Figure 9.1).

Saccharomyces cerevisiae is able to correctly secrete folded proteins into the cultivation medium. As *S. cerevisiae* secrets only a limited number of proteins into the medium, subsequent purification of the target protein is simplified. Hereby, the natural secretion mechanism of the baker's yeast is used to secrete the recombinant proteins. Classically, the target proteins are genetically fused to the N-terminal prepro-signal sequence of the yeast α-mating factor to facilitate secretion into the medium. The fusion proteins are transported via the endoplasmic reticulum, Golgi apparatus, and secretory vesicles, are correctly folded, and have disulfide bridges formed. The prepart of the signal sequence provides the transport of the protein in the endoplasmic reticulum. This sequence is then removed by specific proteases (signal peptidases). The function of the prosequence is less clear but is involved in the further processing and secretion of the target protein. The prosequence is also split-off by specific proteases such as kexin. The amino acid sequence Lys–Arg or Arg–Arg is used as an example of an enzymatic cleavage site.

If the target sequence of the heterologous target protein is defined, the selection of the expression host and the optimization of the expression rate take place. There is a large choice of commercially available microorganisms. So far, almost exclusively *E. coli*, *S. cerevisiae*, *Hansenula polymorpha*, and *P. pastoris* are used for the production of recombinant pharmaceutical proteins as the most experience and wide ranging knowledge exists for these microorganisms. Different host strains are available for these microorganisms, which differ in their protein expression abilities as well as in other properties. For example, the deletion of

certain proteases may have a positive effect on the product formation and stability. Proteolytic degradation products need to be separated laboriously during the purification process and synthesis is not completely efficient with regard to formation of the target product, as a number of secondary products are formed. Often, the *E. coli* strains K or B or other derivatives are used. *E. coli* BL21 (an *E. coli* B derivative) is in comparison to *E. coli* K12-deficient regarding the two proteases *lon* and *ompT*, which facilitates the isolation of intact proteins. The *lon* protease is an intracellular protease that degrades proteins before cells are lysed. In contrast, the *ompT* protease abolishes extracellular proteins and can degrade proteins in the periplasm or after cell lysis. It will have to be seen whether, in the future, further microorganisms such as *Bacillus* that allows highly efficient protein secretion or *Aspergillus* and *Trichoderma* that promotes protein glycosylation will be used for the production of pharmaceutical proteins. These strains among others such as *Corynebacterium* and *Pseudomonas* are already used for other industrial applications in the food industry or industrial enzyme production at large scale. With the advancement of modern genomics to characterize and model genomes, we are now entering a new field of science, whereby we can improve and engineer microbial strains in order to produce soluble, correctly folded proteins that carry all necessary post-translational modifications.

The following topics need to be considered, if the expression rate is to be optimized:

(i) The promoter sequence
(ii) The initiation and termination of the translation
(iii) The copy number of the plasmid
(iv) The codon usage
(v) The selection of the protein sequence in fusion proteins
(vi) The selection of the signal peptide sequences for secreted proteins.

(i) With the choice of a suitable promoter, the expression of the genes and the expression level can be controlled and timed. A distinction is made between strong and weak promoters and inducible and constitutive promoters. Inducible promoters are active only if certain conditions are met such as the addition of an inducer. A commonly used promoter for gene expression in *E. coli* is the *lac* promoter, which is turned on by the addition of isopropyl-β-D-1-thiogalactopyranosid (IPTG) or lactose. These inducer molecules bind to the *lac* repressor, a protein releasing the *lac* promoter for transcription of the following genes by RNA polymerase. In large scale, the pBAD promoter is used that is induced by arabinose. L-Arabinose is cheaper than IPTG and less toxic. Through the variation of the L-arabinose concentration in the media, the expression level can be adjusted very precisely. Other alternatives are the *PhoA* or *trp* promoters, which are turned on by phosphate or tryptophan limitation. Through the addition of β-indole acrylic acid, the trp promoter can be fully induced as this acid inactivates the *trp* repressor protein completely. For the production of insulin in *S. cerevisiae*, the constitutive triose-phosphate isomerase promoter is applied. This ensures a constant expression level during the continuous process scheme.

(ii) For the initiation of translation, the Shine–Dalgarno sequence and the correct distance of this sequence to the start codon is important. Efficient terminators prevent the formation of excessively long unstable mRNA molecules and the unwanted transcription of adjacent genes.
(iii) The copy number of plasmids per cell can vary between a few molecules (e.g. pBR322 – 15–20 copies/cell) and hundreds of copies (pBluescript – 300–500 copies/cell). It determines the number of gene copies of the target protein per cell. The increase of the so-called gene doses can have a positive influence on the expression rate. For genes that are chromosomally integrated, the gene copy number can be increased by multiple integrations into the genome. Also, the integration site itself can have an impact on the expression level.
(iv) For some amino acids, different codons are available and their frequency of use is specific to the microorganism. As rarely used codons can slow down translation, it is recommended to adapt the gene sequence for the expression of a human protein in *E. coli* in a way that it corresponds largely to the codon usage of the host cell.
(v) The fusion partner of a protein can take over various tasks. It can influence the solubility of the target partner. It can stabilize the target protein against proteolytic digestion. It can contain motifs for affinity purification of the protein such as the so-called HisTag (poly histidine affinity tag). It can improve the expression rate or it can control the secretion or the localization of a protein in a specific cell compartment.
(vi) The signal sequence encodes in general for a short peptide that determines the destination of a protein such as the periplasm (*E. coli*), cell compartments (e.g. mitochondria), or the culture medium. For the transport of proteins through the cell membranes, specific transport systems exist. Specific signal peptidases cleave these C-terminal protein motifs during the transport through the membrane.

As a selection marker, antibiotic resistance on the plasmid such as β-lactamase (ampicillin resistance) or auxotrophic host strains (e.g. leucine auxotrophic strains) is used. For auxotrophic host strains, the chromosomal mutation is complemented by the plasmid and thus ensures the stability of the plasmid. It is also possible to integrate the sequence of the respective protein into the chromosome. Important for industrial strains is the stability of the host–vector system over several generations when scaling-up in an industrial environment and in the case of continuous processing, stability over a longer period of time.

After determining the expression vector and host cell, the manufacturing of the "master cell bank" needs to be carried out. From various individual clones that are available after transformation of the final plasmid, one clone (initial cell bank) is selected and used for the production of the master cell bank. The master cell bank lasts for the entire life cycle of the product and is part of the approval documentation that is submitted to the authorities. For this documentation, an extensive characterization of the cell bank is necessary. Among other things, the

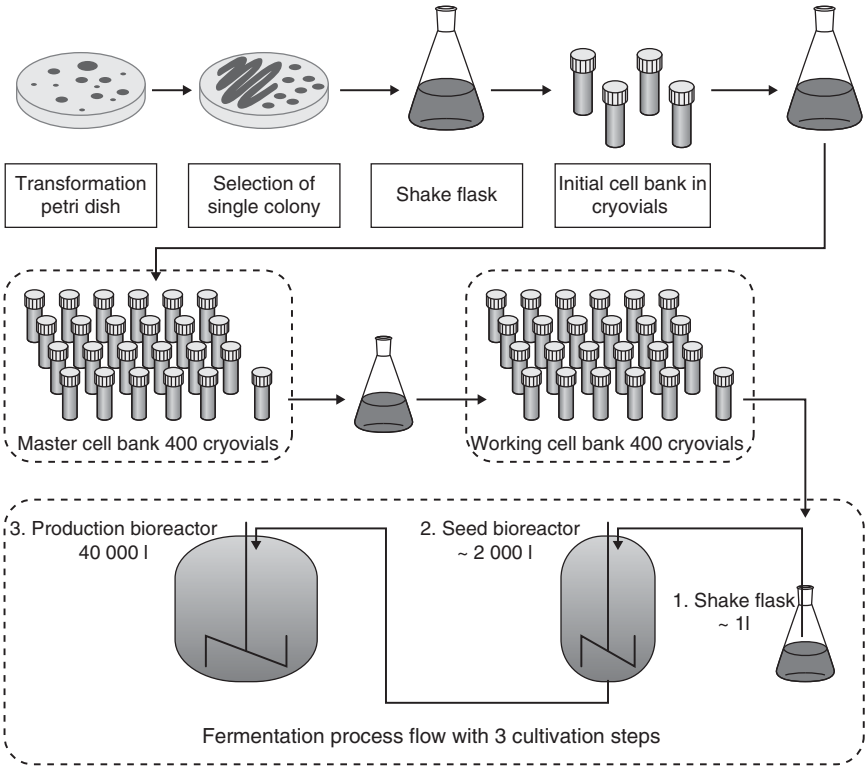


Figure 9.3 Preparation of a working cell bank via the initial cell bank and the master cell bank. The cell banks are stored at low temperatures in freezers or in or above liquid nitrogen. Production batches are usually started with an ampoule from the working cell bank. In the presented example, 160 000 production batches can be started with the present cell bank. If one batch is inoculated every day, the cell bank stocks will last for more than 400 years.

sequence of the plasmid, the copy number, the stability of the strain over several generations, the strain homogeneity, and the strain properties are studied. From the master cell bank that may consist of several hundred ampoules, working cell banks are derived (Figure 9.3). This measure ensures that the master cell bank is available for the entire life cycle of a product as any switch to a new master cell bank requires significant effort to demonstrate that the overall process performance and resulting product quality is comparable.

9.2.2 Isolation of Pharmaceutical Proteins

Biopharmaceutical manufacturing processes have developed rapidly in terms of product yield, productivity, and cost efficiency in the last decade. Through the increasing molecular and cellular understanding of the created expression systems, significantly improved fermentation yields have been achieved in combination with more exact process control. The expression level depends very much on the selected protein, expression host, fermentation process, and expression

pathway, but expression levels of 1–10 g/l have been achieved in microbial high cell density fermentation processes.

The capacity bottlenecks and the main cost drivers in microbial production processes are localized in the isolation and purification steps (the so-called *downstream processing*), and in particular in the multistage chromatographic steps.

After cell separation and, if necessary, cell disruption, different chromatographic procedures (e.g. ion exchange and size exclusion chromatography), membrane processes (ultra- and diafiltration), and physical processes such as precipitation and crystallization are applied. The reproducibility of the whole process is shown in the context of the process validation. This must demonstrate a high reproducibility of the entire process within predetermined limits. As the majority of pharmaceutical proteins are injected, the corresponding active ingredients must be manufactured to a high purity to avoid any side effects. A distinction is made between the process-related impurities and product-related impurities. The process-related contaminants come from the process itself, such as, for example, host cell proteins, endotoxins in the case of *E. coli*, or chemicals used in the production and purification of the protein. The depletion of these substances must be controlled and verified. For endotoxins, host cell proteins and DNA authorities have specified appropriate limits. Product-related impurities are derivatives of the actual target protein. They are formed, for example, through modification (e.g. proteolytic degradation products, deamidation of asparagine, or glutamine side chains) from biosynthetic precursor molecules (e.g. incomplete processing during the protein secretion) or during the manufacturing process itself (e.g. oxidation of methionine residues). At the end of the manufacturing process, a highly pure protein is produced, which meets all predefined quality specifications. The quality of the protein is the result of a defined and reproducible manufacturing process approved accordingly by the authorities. This ensures the purity, the safety, and efficacy of the product for the patient.

The manufacturing cost for a biopharmaceutical is divided into material and production costs. The proportion of total production cost (~70–80%) for microbial processes is usually significantly greater than the proportion for material cost (~20–30%). With respect to the production cost, the capital (equipment and building) and personnel costs (operators, maintenance, quality, and management) are the largest items.

9.2.3 Regulatory Requirements for the Production of Pharmaceutical Proteins

The manufacture of medicinal products is carried out in accordance with good manufacturing practice (*GMP*). Within this framework, the pharmaceutical and biotechnology companies ensure that their products meet specific requirements in terms of identity, effectiveness, quality, and purity.

The production includes related controls for the acceptance and release of raw materials, production, packaging labeling, quality control, product release, and storage and distribution of active substances. The authorities have made very general specifications for:

– Quality management
– Personnel
– Buildings and equipment
– Process equipment
– Documentation and records
– Materials management
– Production and in-process controls
– Packaging and labeling
– Storage and distribution
– Laboratory controls
– Validation and qualification
– Change controls
– Rejection and reuse of materials
– Complaints and recalls
– Contract manufacturers, agents, brokers, and sales persons.

These general requirements need to be interpreted by the manufacturers, codified, and implemented via detailed internal guidelines for the relevant products and systems. The implementation is assessed by the authorities on a regular basis by means of on-site inspections.

9.3 Insulins

9.3.1 Application and Structures

According to the World Health Organization (WHO) in 2014, there were 422 million diabetics. The trend is on the rise. The International Diabetes Federation (IDF) forecasts for the year 2030 worldwide an increase to almost 552 million diabetics.

There are two types of diabetes. In both cases, the main symptom is an increased blood sugar concentration. In type 1 diabetes, the insulin-producing β-cells of the pancreas degenerate, often from a very young age of the patients. As a result, the insulin necessary for the regulation of the blood sugar is no longer produced. In type 2 diabetes, insulin is produced but not sufficiently recognized by the cells. To maintain the blood sugar level low, the pancreas responds with an even greater production of insulin (the so-called insulin resistance). The β-cells in the pancreas are so heavily loaded that they are finally exhausted after years. An unhealthy lifestyle with too little exercise and unfavorable nutrition are among the main causes of type 2 diabetes. Although type 1 diabetics always require insulin, type 2 diabetic patients are usually treated with oral antidiabetic agents at the beginning of the disease and change to insulin in the later stages of the disease.

Insulin is composed of two peptide chains (the A-chain with 21 amino acids and the B-chain with 30 amino acids), which are connected by two disulfide bridges (Figure 9.4). A third disulfide bridge connects amino acids 6 and 11 of the shorter A-chain with each other (Figure 9.4). In the β cells of the pancreas, the so-called

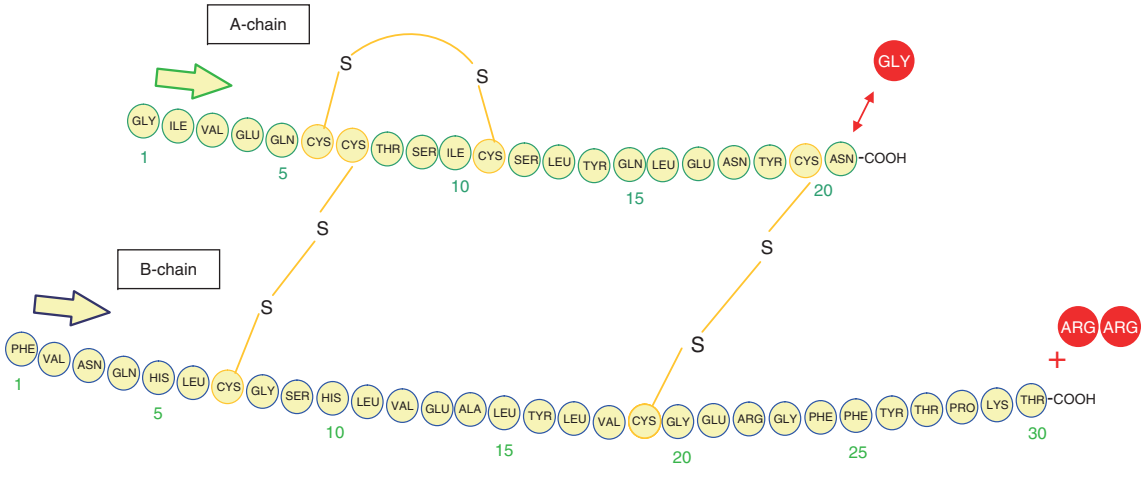


Figure 9.4 Structure of native human insulin. A-chain (1–21); B-chain (1–30). The molecule has three disulfide bridges, two disulfide bridges connecting the A- and B-chains (all amino acids are indicated in the three-letter code). The long acting basal insulin analog glargine (Lantus™) differs from human insulin only by a prolonged B-chain (two additional amino acids arginine at positions B31 and B32) as well as an amino acid exchange on the A-chain (glycine instead of asparagine at position A21), see red markings. The fast acting insulin analogs have the following differences compared to human insulin: (1) Insulin lispro (Humalog™) at position B28: lysine instead of proline at position B29: proline instead of lysine, (2) Insulin aspart (NovoRapid/NovoLog™) at position B28: aspartic acid instead of proline, and (3) Insulin glulisine (Apidra) at position B3: lysine instead of asparagine at position B29: glutamic acid instead of lysine.

preproinsulin molecule is initially formed. At the N-terminal end of the preproinsulin, a signal sequence is attached, which is followed by the B-chain, then the C-peptide, and finally the A-chain. Through formation of the three disulfide bridges, the previously stretched molecule is folded. In the endoplasmic reticulum and in the Golgi apparatus, the signal sequence and the C-peptide of the preproinsulin are split enzymatically. The insulin is now in its native structure.

The industrial production of insulin has a long history. After the successful extraction of insulin by Banting and Best in 1921, the commercial production and the distribution of this insulin was started very rapidly by companies such as Hoechst AG and Eli Lilly by 1923 and by Novo Nordisk in 1925. Insulin from the pancreas of pigs was extracted and purified. However, the primary sequence of the porcine insulin differs from the human sequence by one amino acid and by three amino acids for bovine insulin. This can have side effects such as allergies for some patients. In 1982, the first highly pure recombinant insulin from *E. coli* came on the market, with an identical sequence to the human insulin (Humulin™). The biotechnical production of this peptide hormone ensured the supply safety for millions of patients around the world. According to the international diabetes foundation (IDF) in 2015, 415 million patients are suffering worldwide from type 1 or type 2 diabetes. Today, the supply security is guaranteed by sufficient production capacities in the whole world. Nevertheless, an increase of up to 642 million patients in 2040 is expected by IDF.

In addition to human insulin and insulin preparations of animal origin, there is now a large number of genetically modified insulins, known as insulin analogs. With regard to the analogs, individual amino acids of the human insulin protein are replaced and thus molecules are created with additional desired properties. The onset and duration of efficacy can thus be virtually "tailor-made." Therefore, a distinction is made between depot insulins (e.g. insulin glargine, Lantus, or insulin degludec, Tresiba™), which act over a long period of time, and those whose onset and effect are rapid and of short duration (e.g. insulin glulisine, known under the brand name Apidra™, insulin lispro Humalog, or insulin aspart, Novorapid™). The differences between the insulin analogs compared to human insulin are shown by means of the example of insulin glargine (Figure 9.4).

9.3.2 Manufacturing Processes by *Escherichia coli* and *Saccharomyces cerevisiae*

Insulin can be produced with different manufacturing processes. Depending on the microorganism, the preparation and purification of the hormone is different. Two principle production processes are currently used: production of a fusion protein in *E. coli* and production of a precursor protein, the single-chain insulin precursor with *S. cerevisiae* (Figure 9.5). Another possible procedure that is not described in any further detail here is that of the separate production of the A- and B-chain. This procedure is based on the separate production of A- and B-chain in two different *E. coli* strains and coupling of the two chains after fermentation and product isolation with a chemical step.

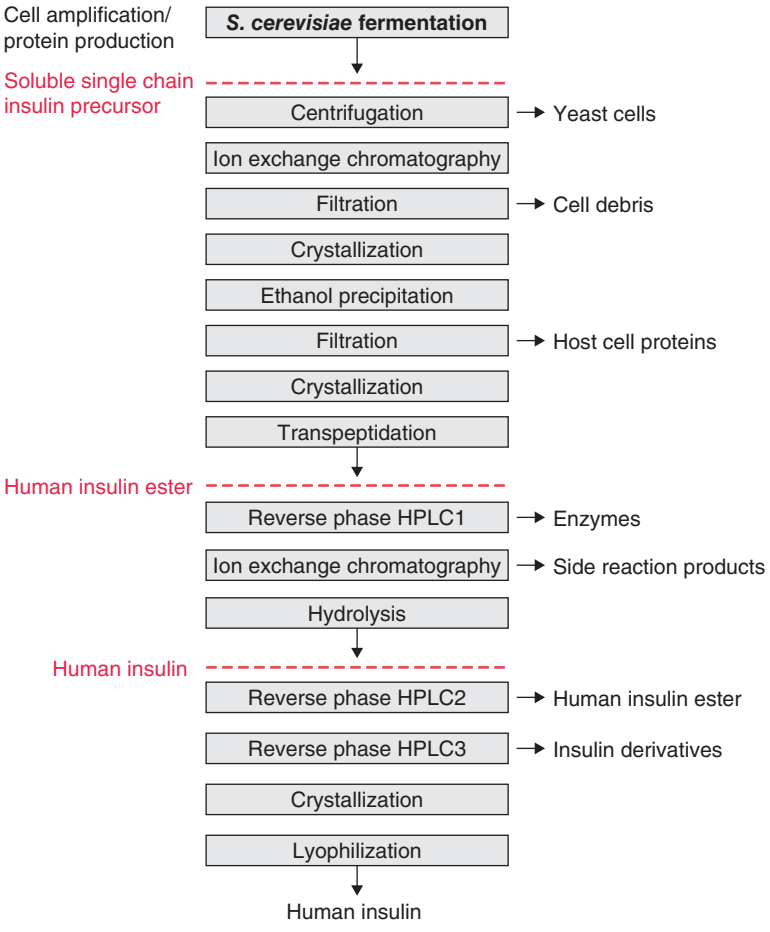


Figure 9.5 Process scheme for the production of human insulin with (a) *S. cerevisiae* and (b) *E. coli*.

9.3.2.1 Production of a Fusion Protein in *E. coli*

The fusion protein is located intracellularly as an insoluble inclusion body (Figure 9.2). The cultivation takes place in large, e.g. 5–60 m^3, reactors with genetically modified *E. coli* strain in a high-cell-density process. In the *E. coli* production strain, a plasmid is introduced that contains the genetic sequence of an insulin fusion protein. With the help of an inducible promoter, gene expression is controlled. From a certain cell density, the protein production is started by the addition of an inducer (Figure 9.6a). In addition to the monitoring and control of the physicochemical parameters such as pH value, pO_2, temperature, a good mass, and heat transfer in the bioreactor are important. High cell densities require high aeration levels in the bioreactor and a glucose supply that is adapted to support cell growth and product formation in an optimal way to avoid by-product formation such as acetate. An efficient mixing of the fermentation suspension prevents the occurrence of local concentration gradients of nutrients

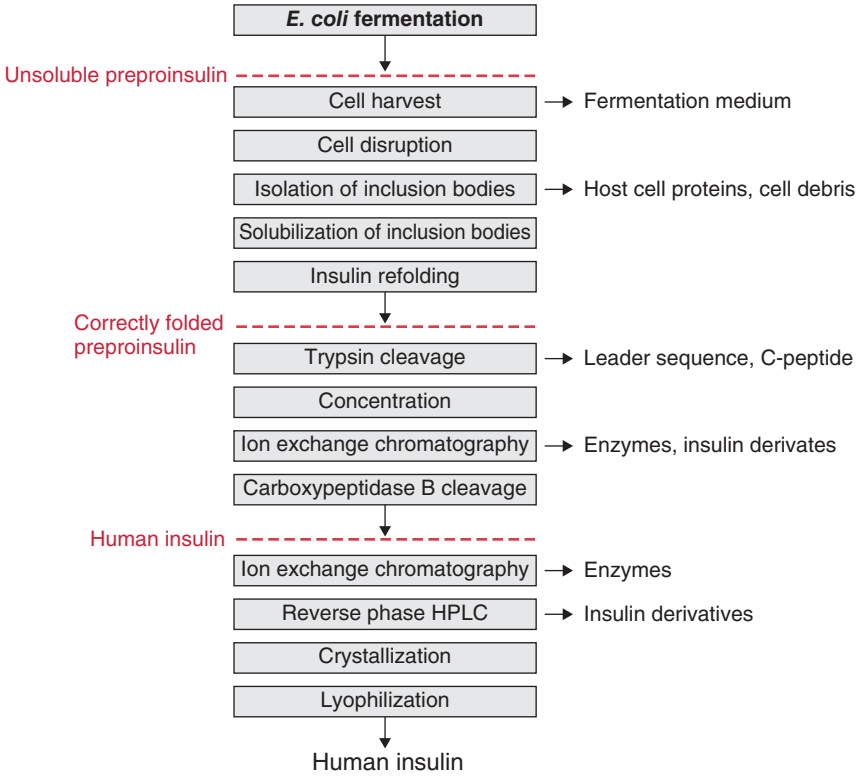


Figure 9.5 (*Continued*)

and facilitates the removal of heat that is generated by the microorganisms in considerable amounts.

After the end of cultivation, the microorganisms are harvested by centrifugation and the concentrated cell suspension is washed to remove cultivation media components (Figure 9.7). The cells are disrupted mechanically in a high-pressure homogenizer and the inclusion bodies are isolated by centrifugation. The inclusion bodies that exist as insoluble aggregates are solubilized in a denaturing agent and reduced completely. For the correct formation of disulfide bonds, the completely denatured and reduced protein is converted to the correctly folded form under oxidative conditions. After the refolding of the fusion protein, the presequence and C-peptide are cleaved through proteolytic processing with the serine protease trypsin. Trypsin splits the protein chain on the C-terminal side of lysine and arginine. After the cleavage, the insulin is in its native form. However, located at the terminus of the B-chain, there is still an alkaline amino acid that must be split off by the metalloprotease carboxypeptidase B in the further course of the process. Carboxypeptidase B splits basic amino acids such as lysine and arginine on the C-terminus of proteins. The purification of the native insulin is performed by several chromatographic purification steps. The final purification step leads to highly pure insulin, which is isolated under acidic conditions, crystallized, and finally lyophilized (Figures 9.5 and 9.7).

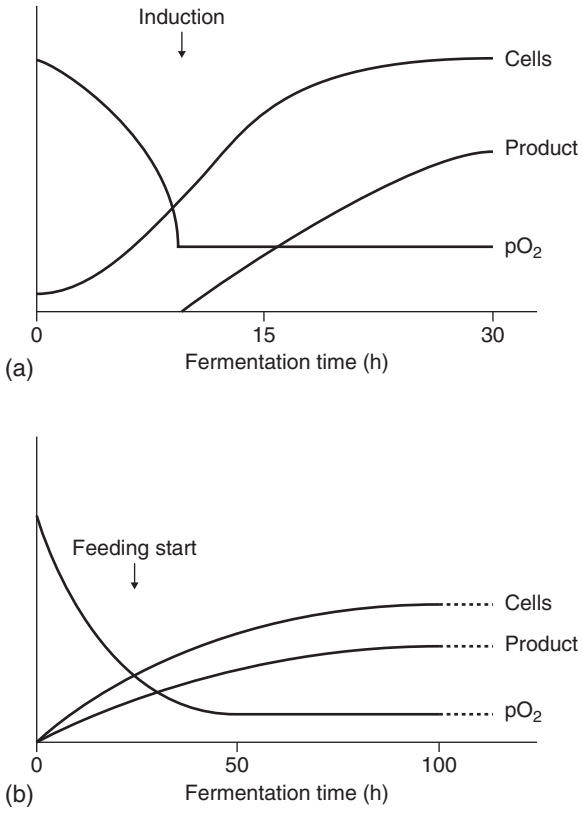
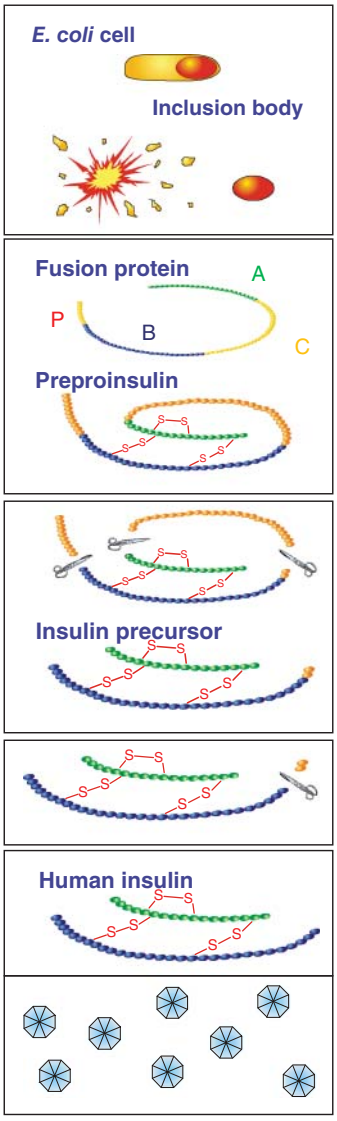


Figure 9.6 Microbial insulin production. (a) Fermentation parameters for insulin production with *E. coli*. The fusion protein is present intracellularly as an insoluble inclusion body. After a growth phase in fed-batch operation, insulin production is chemically induced. After about 24–40 hours, the cell suspension is harvested. (b) Fermentation parameters during the start-up phase during insulin production with *S. cerevisiae*. When stationary conditions are reached after about 72 hours, the cultured suspension is continuously withdrawn from the fermenter. The continuous operation takes about three weeks.

9.3.2.2 Production of a Precursor Protein, the So-Called Mini Proinsulin with the Host Strain *S. cerevisiae*

The use of *S. cerevisiae* as a host microorganism for insulin production has two fundamental differences compared to the above-described procedure: (i) The yeast cells secrete the insulin single-chain precursor into the cultivation medium and therefore cell disruption is not necessary. (ii) The disulfide bonds are correctly formed by the yeast cells, which omit the folding step. The insulin precursor molecule contains a signal sequence that directs the molecule into the secretory pathway. The signal sequence and leader peptide are removed by cellular proteases of the yeast secretory pathway and a single-chain insulin precursor is secreted to the cultivation medium. The single-chain precursor consists of the two insulin chains B (1–29) and A (1–21) connected by a very short connecting (=C) peptide, which can vary in its length. In a first step, the peptide bridge between B-chain and A-chain is removed enzymatically. Then, the B-chain is extended by threonine in a further enzymatic reaction to yield human insulin (position 30, Figure 9.4).

The cultivation of the baker's yeast takes place in a continuous fermentation process in a simple yeast extract containing medium. The proinsulin encoding plasmid vector is located in high copy number. The expression of the target gene is done with the help of a constitutive promoter, which ensures that the promoter



1. Isolation of cells by centrifugation or microfiltration
2. Cell disruption by high pressure
3. Isolation of inclusion bodies with insulin fusion protein and solubilisation and reduction of the fusion protein
4. Refolding and formation of disulfide bridges of preproinsulin
5. Enzymatic cleavage of folded preproinsulin to remove presequence and C-peptide with trypsin
6. First concentration and purification of insulin precursor
7. Ion exchange chromatography
8. Enzymatic cleavage with carboxypeptidase
9. Ion exchange chromatography
10. Reverse phase chromatography
11. Crystallization and lyophilization

Figure 9.7 Processing of insulin inclusion bodies and subsequent process flow for the purification of human insulin (A = A-chain of the insulin, B = B-chain of the insulin, C = peptide bridge or connecting peptide, P = presequence, scissors symbolizing the cleavage sites for proteases).

is always active and gene expression is at a constant level. Thus, the insulin precursor protein from the baker's yeast is continuously produced and processed. The production fermenter is operated first in fed-batch mode and after about three days transferred to a continuous operating mode (Figure 9.6b). The dilution rates are relatively low in comparison to the maximum specific growth rate of the production strain. Optimal growth conditions are at pH 5 and a temperature

of 32 °C. As cell growth and product formation are closely dependent on each other, it is important to ensure that the yeast is always in the aerobic metabolic state. Sugar surplus and/or a lack of oxygen are avoided by precise monitoring of the respiratory quotient. In the case of sugar surplus, the baker's yeast also produces ethanol under aerobic conditions ("Crabtree effect"), and this status is not desirable during continuous production.

The processing begins with the separation of the yeast cells from the cultivation medium by centrifugation with a separator or decanter (Figure 9.5). For prepurification and concentration of the insulin precursor protein, the supernatant is transferred to a cation exchange chromatography column. The insulin precursor protein is eluted from the column with the help of a pH gradient and filtered in order to remove residual yeast cells. The target protein from the eluate is isolated by crystallization. The crystals are dissolved to a pH slightly above neutral and ethanol is added. Under these conditions, insulin is very soluble, and impurities, especially yeast proteins, are precipitated and removed from the solution by filtration. An additional crystallization step leads to a product with more than 90% purity. The insulin intermediate, however, still contains impurities from the cultivation medium (yeast proteins and DNA).

In the next step, the single-chain insulin precursor is cleaved into the A- and B-chain enzymatically, for example by adding lysyl-endopeptidase (*Achromobacter lyticus*) or trypsin (e.g. porcine or bovine origin). The cleavage occurs after lysine-29 in the B-chain and after a lysine residue in the C-peptide. The insulin precursor molecule is still missing the threonine in position 30 of the B-chain, which can be inserted by means of an enzymatic transpeptidation. Hereby, threonine-*t*-butyl ester is enzymatically coupled at the C-terminus of the B-chain. The resulting insulin ester is then purified by means of reverse-phase chromatography and anion exchange chromatography, which helps to remove the enzyme used in the transpeptidase reaction and to remove the by-products formed during this reaction. The native insulin is formed by cleavage of the *t*-butyl ester group from the threonine-*t*-butyl ester in position B30 by a hydrolysis reaction. The control of the parameters temperature and pH is of high importance in order to not destroy the disulfide bonds.

The final cleaning of the insulin takes place via reverse-phase chromatography and crystallization. Then, the human insulin is transferred in a stable form by lyophilization. The last steps of the process are basically comparable to the purification procedures when *E. coli* is used as a host microorganism. At the end of the process insulin, the insulin purity is typically more than 99.5%, as measured by RP-HPLC.

9.4 Somatropin

9.4.1 Application

Somatropin is an human growth hormone (hGH) formed in the α cells of the frontal lobe of the pituitary, in particular during sleep. Somatropin is administered as a medicine for children with short stature if caused by a reduced secretion

of the endogenous growth hormone. Somatropin is also used in adults if a growth hormone deficiency is based on a pituitary insufficiency. The market volume of the largest manufacturers was more than 3 billion US$ in 2014 (Table 9.2).

9.4.2 Manufacturing Process

Somatropin is a protein with 191 amino acids and is produced predominantly with recombinant *E. coli*. The first processes for the production of Somatropin were based on the formation of inclusion bodies resulting in a complex downstream processing with cell disruption, isolation, and dissolving of the product. Today's processes are using protein secretion technologies. The recombinant protein is secreted into the periplasm of the bacterium by appending a signal sequence and then released by osmotic shock. The individual manufacturing steps for the production of Somatropin are described in Figure 9.8.

Somatropin is produced by numerous manufacturers as a so-called *biosimilar*. In 2006, somatropin was approved in the EU as the first *biosimilar* under the brand name Omnitrop™.

Biosimilars are medicines that are produced by living cells that are highly similar to another already approved medicine (the "reference medicine"). They can be

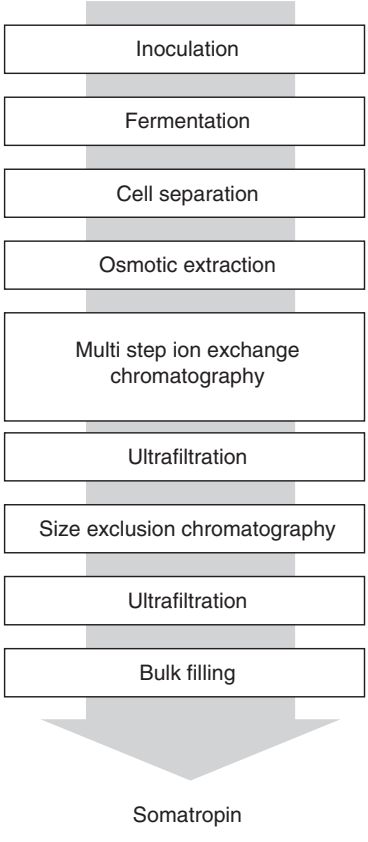


Figure 9.8 Procedure for the production of somatropin with *E. coli*. The product is secreted into the periplasm.

brought onto the market after patent expiry of the original product. Registration of a biosimilar is more complex compared to small molecule generics as similarity to the originator has to be demonstrated in order to ensure the safety and efficacy of the product. This is the reason why new specific approval procedures for biosimilars were created by the authorities.

9.5 Interferons – Application and Manufacturing

Interferons are proteins primarily formed by white blood cells. They are characterized by stimulating the immune system and having antiviral and antitumor effects. They act as cytokines of the innate, also called nonspecific immune system. Out of 17 different interferons known, only few are of commercial interest. The interferon sales amounted to 5.5 billion US$ in 2013 (Table 9.2). Interferon α-2a harbors a lysine residue in position 23, which is replaced by arginine in interferon α-2b. Interferon α-2a was approved in 1987 and was one of the first recombinant proteins to be on the market. The mature interferon α-2a protein is composed of 165 amino acids and has two disulfide bonds between amino acids 1 and 98 and 29 and 138. In the direct expression of interferon α-2a in *E. coli*, one methionine is attached to the N-terminus of the protein (Figure 9.1) that is only partly removed during the production in *E. coli*. Even though the biggest part of the molecule is identical to the human protein regarding the amino acid sequence, a small part contains this additional methionine and has a length of 166 amino acids. The recombinant interferon α-2a is different from the human version because of the lack of O-glycosylation because in *E. coli*, this type of protein modification does not exist.

Interferon α-2a is produced in *E. coli* intracellularly. After cell disruption, the formation of disulfide bonds and then purification by several successive chromatography steps takes place. To extend the duration of efficacy of these two interferons, improved, PEGylated active substances (PEG, polyethylene glycol) were developed and approved by the authorities in 2000 (PEG interferon α-2b) and in 2002 (PEG interferon α-2a). These active substances are formed by attaching a side chain from a branched polyethylene glycol polymer. The underlying reaction mechanism for the PEGylation of proteins is shown schematically in Figure 9.9 as an example. The linkage of the 40 kDa large branched polyethylene glycol polymer takes place at the free ε-amino group of lysine residues via an amide bond. The degree of substitution is 1 mol of polymer/mol of protein. Through this modification, the size of the interferon molecule increases from approximately 20 to 60 kDa and a significantly extended half-life is achieved. As a consequence, the drug can be administered less frequently.

Interferon β-1b is produced in *E. coli* K12. The recombinant protein differs in three points from the human version. The protein is not glycosylated, the N-terminal methionine is missing, and the amino acid cysteine was replaced by serine in position 17. A disulfide bond connects the cysteine in position 31 with cysteine in position 141. The recombinant protein is formed as an inclusion body.

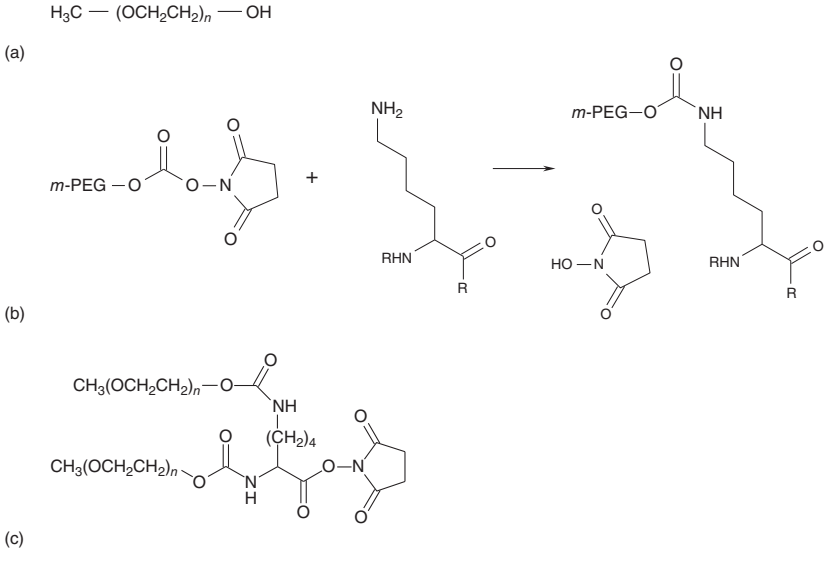


Figure 9.9 Reaction mechanism for the PEGylation of a pharma protein. Basic structures (a): linear mono-methoxy PEG-OH chain (*m*-PEG), (b): activated *m*-PEG chain and representation of the reaction mechanism. Linking of an *m*-PEG-succinimidyl carbonate with the ε-amino group of a lysine residue of a protein. The *N*-hydroxysuccinimide ester is cleaved during the reaction. (c) Structure of the branched activated PEG reagent for Pegasys™. The activated PEG reagent contains two *m*-PEG chains (20 kDa each). The two *m*-PEG chains are coupled to the α- and ε-amino groups of lysine residues.

Table 9.3 Interferons produced in *E. coli*.

Interferon	Modification	Use	Amino acids per protein	Market approval
IFN α-2a	—	Cancer	165	1987
IFN α-2a	PEGylation	Hepatitis B/C	165	2002
IFN α-2b		Cancer, hepatitis B/C	165	2000
IFN α-2b	PEGylation	Hepatitis C	165	2000
IFN β-1b	—	Multiple sclerosis	165	1995
IFN γ-1b	—	Immune system stimulant	140	1992

After isolation, the inclusion body must be transferred into the native folded form and then processed to high purity by chromatographic steps. Recombinant interferon γ-1b produced in *E. coli* contains 140 amino acids. The native protein contains no disulfide bridges but exists as a homodimer: two identical protein molecules form a dimer linked noncovalently with each other. An overview of the various interferons produced in *E. coli* and their use is shown in Table 9.3.

9.6 Human Granulocyte Colony-Stimulating Factor

9.6.1 Application

The granulocyte colony-stimulating factor (G-CSF) is a peptide hormone consisting of 174 amino acids. It is distributed as a cytokine in the case of inflammation of the body and stimulates the formation of neutrophils within the bone marrow.

The humane G-CSF is a glycoprotein that is glycosylated at position 133 (threonine). It has a molecular mass of 19.6 kDa. The glycosylation makes approximately 4% of the total mass. In addition to glycosylation, two disulfide bridges represent another key element of the post-translational modification.

As an active ingredient, recombinant G-CSF can be produced either with mammalian cells (CHO cells; Lenograstim) or with *E. coli* (Filgrastim). Lenograstim is identical to the human G-CSF with regard to the amino acid sequence and the glycosylation at position 133. Filgrastim has an additional methionine attached at the N-terminus of the amino acid sequence and has no glycosylation.

G-CSF also exists in a PEGylated form (Pegfilgrastim). In this modification, a 20 kDa mono-methoxy-polyethylene-glycol is bound covalently to methionine at the N-terminus of filgrastim (Table 9.4).

If, as a result of a chemotherapy (cancer treatment), white blood cells are reduced (neutropenia), G-CSF is administered to stimulate the growth of neutrophil granulocytes, which are a type of white blood cells. This is done to reduce the risk of infections, which can be a side effect of reduced levels of white blood cells. G-CSF can be used either as a preventive measure if a strong decrease in the neutrophils is expected, or therapeutically, if the number of neutrophils is already low.

Table 9.4 Different active substances of the granulocyte colony-stimulating factor (G-CSF) and the expression systems applied.

Expression system	**CHO**	***E. coli***	***E. coli***
Active substance	Lenograstim	Filgrastim	Pegfilgrastim
Amino acids	174	175	175
Modification	Glycosylation at position 133	No	PEGylation of N-terminus
Molecular mass (kDa)	19.6	18.8	39

Table 9.5 The active substance filgrastim is commercialized under various brand names in Germany.

Brand name	**Active substance manufacturer**
Filgrastim Hexal™	Hexal
Zarzio™	Sandoz
Accofil™	Accord
Grastofil™	Apotex
Ratiograstim™	Ratiopharm
Tevagrastim™	Teva
Nivestim™	Hospira

All drug manufacturers use *E. coli* as expression system.

In addition to the original product Neupogen™ from Amgen, the active substance filgrastim is sold under various brand names from different manufacturers on the market (see Table 9.5).

9.6.2 Manufacturing Process

Filgrastim is produced as inclusion body in a fermentation process starting from a cell bank through several precultures comparable to the production of recombinant human insulin with *E. coli*. After cell harvest and cell disruption, the inclusion bodies are isolated. After the folding reaction, the native protein is purified in several chromatography steps to high purity.

In the case of Pegfilgrastim, a 20 kDa mono-methoxy-polyethylene-glycol is bound covalently to the N-terminus.

9.7 Vaccines

9.7.1 Application

In addition to pharmacologically active substances, several vaccines on the market are also manufactured by recombinant microorganisms. Table 9.6 gives an

Table 9.6 Overview of vaccines containing recombinant antigens currently on the market and manufacturer.

Disease	Pathogen	Brand name	Manufacturer	Expression system
Hepatitis A/B	Viral hepatitis A/B	Ambirix™	GlaxoSmithKline Biologicals	*S. cerevisiae*
		Twinrix™	GlaxoSmithKline Biologicals	*S. cerevisiae*
	Hepatitis B virus	Engerix™-B (surface protein)	GlaxoSmithKline Biologicals	*S. cerevisiae*
		Fendrix™	GlaxoSmithKline Biologicals	*S. cerevisiae*
		HBVAXPRO™	Sanofi Pasteur MSD	*S. cerevisiae*
Uterine cervical cancer	Human papillomavirus (HPV)	Gardasil	Sanofi Pasteur MSD	*S. cerevisiae*
		Silgard™	MSD Sharp & Dohme	
Cholera	*Vibrio cholerae*	Dukoral™	Crucell Sweden AB	*Vibrio cholerae*
Meningitis	*Neisseria meningitidis*	Bexsero™	GlaxoSmithKline Biologicals	*E. coli*

overview of recombinant microbial vaccines currently on the market. The baker's yeast *S. cerevisiae* is frequently used as an expression system. For the production of vaccines, the immunogenic parts of a pathogen are targeted to be produced by genetically modified microorganisms. These immunogenic parts are isolated and if necessary mixed with appropriate amplifiers, so-called adjuvants, and are applied for active vaccination mostly intramuscularly to protect against pathogens. The protein (antigen) is recognized by the body as a foreign substance. The body reacts with an immune response and the formation of lymphocytes, which start synthesizing antibodies against the corresponding antigen. The protection against the antigens remains for a long period of time through the so-called memory cells. By this mechanism, an infection can be prevented in the case of a reoccurrence of the antigen.

9.7.2 Manufacturing Procedure Using the Example of Gardasil™

Cervical cancer is the second most common cancer in women. This form of cancer is caused by infection with human papilloma viruses (HPV).

The HPVs belong to the nonwrapped, double-stranded DNA viruses. They are pathogens that infect the cells of skin and mucous membrane. Some of the known HPV types are responsible for the development of normal skin-warts (papilloma). About 10–15 HPV types can cause changes of the cells of the cervix, which can develop through precursors toward a cancer disease. The discovery of this relationship has allowed the development of prophylactic vaccines against HPV infection.

The development of the vaccine under the brand name Gardasil™ started in the beginning of the 1990s. After the initial registration in the year 2006, vaccination with Gardasil became widespread. Gardasil includes virus-like particles (VLP) for the main capsid L1 protein of HPVs. Gardasil is a fourfold vaccine against the HPV types 6, 11, 16, and 18.

The manufacturing process consists of two parts: (i) cultivation and harvest of a recombinant yeast *S. cerevisiae* and (ii) purification of the VLPs, including the binding of the purified VLPs to an aluminum-containing adjuvant. The fermentation process consists of pre- and main fermentation. In the prefermentation, the same media are used for all four HPV types. During fermentation, growth and glucose concentration are monitored. After the main fermentation in an appropriate cultivation medium, the cells are harvested by microfiltration. The cell concentrate is then divided into portions and frozen.

The purification process begins with the thawing of the frozen cell concentrate and the release of the VLPs by cell disruption and subsequent microfiltration. The cell lysates are incubated and the VLPs are purified using cross-flow filtration, chromatography, and ultrafiltration. For all four types, the final purification step consists of a buffer exchange and a sterile filtration after which the so-called final, in aqueous phase, dissolved product (final aqueous product, FAP) is manufactured. The FAP is adsorbed on amorphous aluminum hydroxyphosphate sulfate for each of the four types for the production of the four monovalent bound bulk products (MBAPs).

Aluminum hydroxyphosphate sulfate is used as an adjuvant. An adjuvant is a substance that reinforces the effect of another substance. With regard to Gardasil, the immunogenic effect of the four VLPs is strengthened unspecifically by the addition of aluminum hydroxyphosphate sulfate.

9.7.3 Manufacturing Process Based on the Example of a Hepatitis B Vaccine

Hepatitis B is an infection of the liver with the hepatitis B virus (HBV). The infection is either acute (in 90% of the cases) and the self-healing process takes place within 4–6 months, or it can become chronic if hepatitis persists more than half a year. Worldwide 300–420 million people are estimated to be chronically infected by the WHO with hepatitis B and up to a million people die each year from this disease. In approximately one-third of the world population (>2 billion), antibodies are detectable as a sign of a passed HBV infection. As a consequence of the chronic course of the disease (chronic persistent hepatitis), there is the risk to get liver cirrhosis and hepatocellular carcinoma. The treatment of chronic hepatitis B is difficult. This is why preventive vaccination is the most important measure to avoid infection and to reduce global virus carriers.

The HBV is one of the partial double-stranded enveloped DNA viruses that almost exclusively infect liver cells. The infectious virions are surrounded by an outer lipid bilayer where three viral surface membrane proteins (LHBs, MHBs, and SHBs) are stored. The lipid bilayer probably originates from the endoplasmic reticulum. The surface proteins of the virus are also called HBsAg (*hepatitis B surface antigen*) because they play a role in the immune defense against the virus

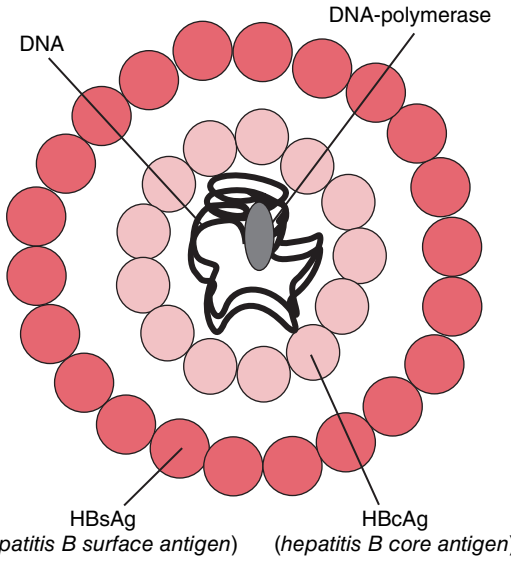


Figure 9.10 Schematic representation of the hepatitis B virus with the HBcAg- and HBsAg-antigens.

(Figure 9.10). If antibodies against the surface proteins are detectable in the blood, this is a sign of the self-healing process. Within the virus envelope, there is a nucleocapsid localized, formed by the HB-core protein (HBc). The nucleocapsid surrounds the viral genome. The complete composition of the HBV occurs in the endoplasmic reticulum and Golgi apparatus and the excretion from the liver cell through exocytosis.

The recombinant hepatitis B vaccine HBVAXPRO consists of a highly purified HBsAg, which is adsorbed to aluminum adjuvants. The inserted plasmid encodes for a protein of a 24 kDa large, so-called SHBs (*small hepatitis B surface antigen*). The antigen is produced by a recombinant *S. cerevisiae* strain in a fermentation process. After cell disruption and subsequent purification, the antigen is formulated and transferred in the final container.

9.8 Antibody Fragments

The production of antibody fragments in *E. coli*, the so-called fabs (fragment antigen binding), gains in importance in recent years. A fab contains the antigen binding domain of an antibody without the so-called FC part of a full IgG antibody (Figure 9.11). Although only higher cells have been used for the industrial production of complete antibodies so far, two fragment antibodies produced with *E. coli* are approved already. These are the fab-fragments Ranibizumab (Lucentis™, Novartis, Table 9.2) for the treatment of macular degeneration and Certolizumab (Cimzia™, UCB) for the treatment of rheumatoid arthritis. Ranibizumab binds the vascular endothelial growth factor (VEGF) and thus prevents the formation of blood vessels. Ranibizumab is a treatment for age-related macular degeneration, an eye disease, and has been available on the market since 2006 in the United States. The heterodimer has a size of 48 kDa and is nonglycosylated. It

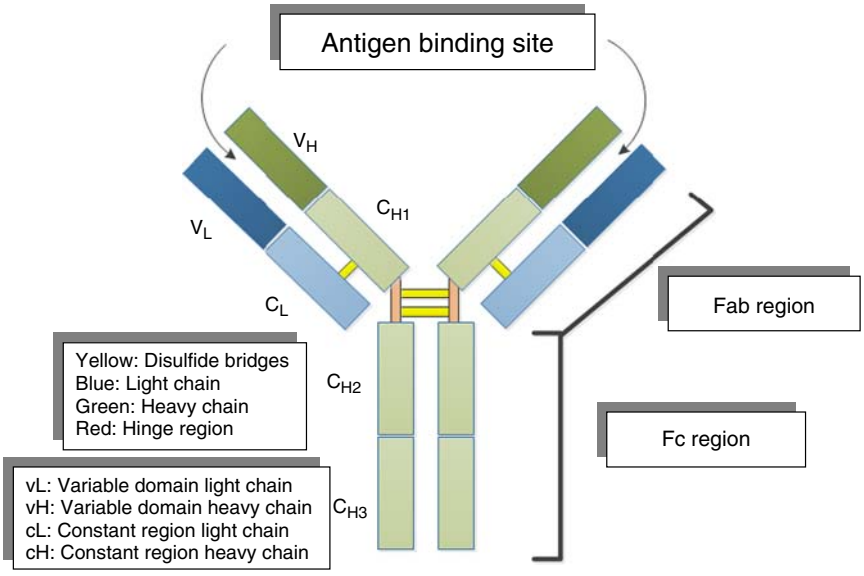


Figure 9.11 Schematic representation of an IgG antibody, with the individual regions that are already industrially used for the production of recombinant proteins from microorganisms or which may be used in the future.

is relatively small in comparison to a full IgG antibody that can be up to 150 kDa. The expression is conducted in *E. coli* where the heavy and light chains of the antibody are secreted into the periplasm. The light chain of the native protein is 214 amino acids in length and is connected via a disulfide bond with the heavy chain (231 amino acids). In addition, the molecule possesses four intramolecular disulfide bonds. In the oxidative milieu of the periplasm, the correct folding of the two protein chains occurs and forms the heterodimer. The mature protein is then released from the periplasm by an osmotic shock and purified over several successive purification steps.

9.9 Enzymes

Enzymes represent a further important product class. For example, the enzyme uratoxidase (rasburicase, Fasturtec™) is used for the treatment of side effects in cancer therapy. When undergoing chemotherapy, there may be an increased formation of uric acid, which precipitates in the kidney because of its low solubility, resulting in a failure of the renal function. Uratoxidase catalyzes the conversion of uric acid to allantoin. Allantoin is soluble in water and is easily excreted by the kidney. As this enzyme is not synthesized by humans, the gene was isolated from an *Aspergillus* strain and cloned into *S. cerevisiae*. The protein is a tetramer with identical subunits of a size of 34 kDa. After fermentation, the cell mass is concentrated, the yeast cells are disrupted, and the uratoxidase purified over several chromatographic steps.

Another enzyme used in medical applications and produced in microorganisms is reteplase. Reteplase is derived from the human plasminogen activator t-PA and consists of 355 amino acids. The natural t-PA molecule is a serine protease with a chain length of 527 amino acids. Reteplase is used to treat patients with acute myocardial infarction and accelerates the resolution of blood clots. Reteplase is formed in *E. coli* K12 as an inclusion body. The protein is purified after folding through different chromatographic steps.

9.10 Peptides

When the production of smaller peptides is required, use of recombinant production in microorganisms is in competition with chemical synthesis. The production of very small peptides with a chain length of only 30–40 amino acids – often with no pronounced secondary structure and no disulfide bridges – is difficult in microorganisms as these can be degraded intracellularly by proteases. In chemical solid-phase synthesis, a peptide is built linearly, step by step, using activated amino acids on a carrier resin, detached from the carrier afterward and purified by chromatographic procedures. The production costs for chemical synthesis increase with increasing chain length. As a rule of thumb, from a chain length of 30–40 amino acids and above, recombinant production of peptides in microorganisms should be considered. An example is the active ingredient teriparatide (Forteo™, Table 9.2), a 34 amino acid fragment of the human parathyroid hormone that stimulates bone formation and received market authorization in 2003 for the treatment of osteoporosis. The active substance is a protein with a molecular mass of 4118 Da. The structure of the peptide reveals a helical region. This active substance is biologically produced in *E. coli*. Another recent example for a recombinant peptide is Liraglutide (Victoza™), which is a 34 amino acid long analog of the human glucagon-like peptide-1 (GPL1) launched in 2010. The peptide chain of this product is produced in *S. cerevisiae* and chemically modified after some purification steps with a fatty acid attached to the ε-amino group of the Lys residue in position 26. The fatty acid group is designed to promote binding to human serum albumin and prolong the half-life of the molecule.

9.11 View – Future Economic Importance

In 2014, 19 biopharmaceuticals derived from recombinant DNA technology have been approved in the United States. Of the 19 approved products, 4 (21%) are based on microbial expression systems. As "work horses," *E. coli* and *S. cerevisiae* are still prevalent.

A further competitive element in the pharmaceutical industry is becoming more and more relevant, the so-called *biosimilar* ("similar biological medicinal products"). A biosimilar is a biological product that is highly similar to an existing approved reference product and should not show any clinically meaningful differences compared to the reference product. They are approved based on

the same standards of pharmaceutical quality, safety, and efficacy that apply for biological medicines. The global market volume for biosimilars was 3 billion US$ in 2014 and it is expected that the volume of this market will increase more than 10-fold by 2020.

In EU in 2016, there are seven biopharmaceuticals approved as biosimilars: Epoetin (α and ζ), etanercept, follitropin, and infliximab produced by cell culture techniques and glargine, somatropin, and filgrastim produced by microbial processes. In the European and US markets, additional biosimilars are expected in the coming years, in particular in the area of the insulins.

For the commercial manufacture of monoclonal antibodies, only mammalian cells (mostly CHO cells) are currently used. With the emergence of smaller, less complex antibody formats (fab-fragment antibodies, nanobodies),microbial systems are of increasing interest for commercial production. Currently, more than 65 antibody fragments are being evaluated in preclinical and clinical studies.

Another interesting development are the so-called DARPins (designed ankyrin repeat protein) and anticalins. Both molecular classes are artificial proteins that recognize and bind to antigens and therefore mimic the antigen binding properties of antibodies. DARPins are derived from proteins that have one or more ankyrin-repeat motifs. These motifs have been demonstrated in a large number of natural proteins. DARPins consist of several of these predominantly 33 amino acid-long motifs, of which 7 amino acids are variable. Depending on the number of motifs, these molecules have a molecular mass from 14 to 21 kDa. DARPin libraries are created using molecular biological methods by random mutagenesis. DARPins that can bind a target protein can be selected, for example, by the phage display. Anticalins are also artificial proteins that are derived from the naturally occurring lipocalins, which is a widespread protein class. Anticalins have a molecular mass of 20 kDa and are therefore also significantly smaller than antibodies. The generation of molecular libraries takes place by random mutagenesis as well. Anticalins recognize and also, in contrast to antibodies, bind low molecular mass compounds. The small size of the DARPins and anticalins facilitates tissue penetration in comparison to conventional antibodies and one could imagine the targeted transport of drugs as a field for study.

Both protein classes can be easily produced in *E. coli* in high concentrations and demonstrate a high level of stability. The extent to which this molecule class can displace fab-fragments and antibodies from their current leading role in medical treatments should be of interest over the upcoming years.

Microbial expression systems will constantly evolve and enhance in their ability to produce recombinant protein drugs. *E. coli* and yeast as expression systems are genetically tractable; a very robust and fast fermentation technology has been developed for these two microorganisms that are scalable up to high cubic meter scales. The limiting properties on the other hand are the missing secretion in the supernatant for *E. coli* and the lack of human-like glycosylation of proteins in yeast. However, with modern molecular biology methods and metabolic engineering, it is now possible to modify *E. coli* strains or other hosts genetically so that a glycosylation of proteins or the secretion of proteins in the supernatant is possible. With these advances, it will be possible in the future to engineer yeast or filamentous fungi to produce human therapeutic proteins with a specific or

native human glycosylation pattern. Regarding the secretion of proteins with *E. coli*, new technologies such as ESETEC 2.0™ (*E. coli* secretion technology) from Wacker Biotech GmbH (a wholly-owned subsidiary of Wacker Chemie AG) show concentrations in the supernatant of up to 5 g/l heterologous protein for use in clinical studies.

Further Reading

Dingermann, T. (2008). Recombinant therapeutic proteins: production platforms and challenges. *Biotechnol. J.* 3: 90–97.

Internet search performed September 2016. www.prnewswire.com and www .evaluate.com.

Mollerup, I. (1999). Insulin, purification. In: *Encyclopedia of Bioprocess Technology* (eds. M.C. Flickinger and S.W. Drew), 1491–1498. New York: Wiley.

Prestka, S. (2007). The interferons: 50 years after their discovery, there is much more to learn. *J. Biol. Chem.* 282: 20047–20051.

Walsh, G. (2014). Biopharmaceutical benchmarks. *Nat. Biotechnol.* 32: 992–1000.

10

Enzymes

David B. Wilson[1], Maxim Kostylev[2], Karl-Heinz Maurer[3], Marina Schramm[4], Wolfgang Kronemeyer[5], and Klaus-Peter Stahmann[4]

[1]†, Cornell University, Department of Cellular and Molecular Genetics, Ithaca, NY 14850, USA
[2]University of Washington, Department of Microbiology, Seattle, WA 98195, USA
[3]AB Enzymes GmbH, Feldbergstraße 78, 64293 Darmstadt, Germany
[4]Brandenburgische Technische Universität Cottbus-Senftenberg, Institut für Biotechnologie, FG Technische Mikrobiologie, Universitätsplatz 1, 01968 Senftenberg, Germany
[5]QIAGEN GmbH, Qiagenstraße 1, 40724 Hilden, Germany

CHAPTER MENU

10.1 Fields of Application and Economic Impacts

10.1.1 Enzymes are Biocatalysts

Biocatalysts are either living cells, e.g. bacteria, which consist of complex networks of metabolic pathways, or single enzymes. Intermediate forms are immobilized, permeabilized cells where several enzymes are overproduced and entrapped on the one hand or cascades of enzymes on the other hand. Industrial enzymes are exclusively proteins, macromolecules that consist of several hundred amino acids. Ribozymes, ribonucleic acid polymers with catalytic activity, are not used in industry. More than 5000 types of chemical reactions are known to be catalyzed by enzymes. Educts, called substrates, are converted to different molecules called products. In general, enzymes are highly selective for their substrate and product chemo-, regio-, and stereospecificity.

† Deceased

Industrial Microbiology, First Edition.
Edited by David B. Wilson, Hermann Sahm, Klaus-Peter Stahmann, and Mattheos Koffas.

Enzymes lower the activation energy by stabilizing reaction intermediates, which results in an increased reaction rate. Importantly, thermodynamics determine the direction of the chemical reaction, while the enzyme only accelerates the rate of conversion of substrates to products. In comparison with a spontaneous chemical reaction, enzymes can accelerate conversion rates 10^5–10^8-fold. An example of a fast enzyme is catalase from *Escherichia coli*, which catalyzes decomposition of H_2O_2 with a turnover number greater than $10^5\ s^{-1}$. Industrial enzymes work in the range of $10^2\ s^{-1}$. Selectivity of enzymes is particularly impressive for some DNA polymerases. For example, the enzyme of the hyperthermophilic archaeum *Pyrococcus furiosus* has an error rate of 10^{-6}.

10.1.2 Advantages and Limitations of Using Enzymatic Versus Chemical Methods

Advantages of enzymes can be mild reaction conditions (temperature, neutral pH, and atmospheric conditions). Selectivity allows conversion of substrates without protection of functional groups, yielding stereo- and regiochemically defined reaction products. This means a reduced number of steps in an enzymatic production process. Having fewer steps reduces the number of reactors needed, shorter process times, and higher yields.

There are a number of challenges associated with using enzymes for industrial applications, however. Most enzymes need aqueous reaction conditions and only a few (e.g. lipases) tolerate organic solvents. Even in optimized systems, enzymes tend to rapidly lose activity because of poor stability and unfolding of the three-dimensional structure. To extend their half-life, enzymes can be immobilized on supports, such as microporous beads. However, although immobilization can enable enzyme recycling up to a hundred times, it creates an additional cost factor. The interaction of immobilized enzymes with insoluble or high molecular weight substrates can also be limited. The need for cofactors or mediators to be recycled in stoichiometric amounts can contribute other costs and limitations for some enzymes.

The chemical process for the conversion of nitrogen (N_2) and hydrogen (H_2) to ammonia (NH_3) led to Nobel Prizes for Fritz Haber (1918), Carl Bosch (1931), and Gerhard Ertl (2007). Essential is an iron-containing catalyst applied at a pressure of >100 bar and a temperature of >400 °C. Worldwide, more than 150 million tons of ammonia were produced in 2016, with 85% used as fertilizers, e.g. for corn agriculture. In nature, all conversion of N_2 to ammonia is carried out by archaea and bacteria. Interestingly, nitrogenase, the enzyme catalyzing the assimilation of N_2, e.g. in *Azotobacter* sp. also needs iron but works at 25 °C and 1 bar.

Nitrogen fixation is not yet possible with enzymes alone. Nitrogenase is a complex system of several protein subunits that coordinate metal–sulfur clusters. Its redox reaction might become possible when it is immobilized on electrodes. However, many other enzymes, mostly hydrolases, are used in household and industry.

10.1.3 Brief History of Enzyme Used for the Industrial Production of Valuable Products

Understanding of enzymes and their potential applications originates in the fundamental studies by Payen, Buchner, and Fischer in the nineteenth century. In 1833, the French chemist Anselme Payen was the first who discovered an enzyme he called diastase, a plant amylase from malt extract. Later, in 1876, Friedrich Kühne used the protease trypsin from pancreas for cheese preparation.

In 1894, Emil Fischer proposed the **key-lock principle** to describe the enzyme–substrate relationship. Then, in 1897, Eduard Buchner showed that enzymes can also work independently of cells. He demonstrated that the alcoholic fermentation can happen in the presence of yeast cell extract and no living cells. These studies led to the suggestion for naming these catalysts "enzyme" from Greek "zyme," which means "from baker's yeast." In 1901, Jokichi Takamine filed the first enzyme patent for takadiastase, an amylase, as a drug to support digestion, and thus drove the advancing industrial enzyme research. Takamine was the first to use microorganisms for enzyme production. Patented in 1907, Otto Roehm used proteolytic enzymes for leather production. Later, in 1913, pancreatic enzyme trypsin was used as an additive for washing clothes to improve results. Up to that point, laundry was a laborious process requiring high temperatures, extensive manual labor, and a lot of time. Today, addition of enzymes to laundry detergents reduces the number of chemicals and use of energy for heating. Although the granulated enzymes make up less than 1% (w/w) of laundry detergents, they greatly improve the detergent performance.

Since 1959, the **induced fit model** (Figure 10.1) described by Koshland is used to describe the molecular mechanism of interaction between enzyme and substrate. The essential concept of the model is that both enzyme and substrate

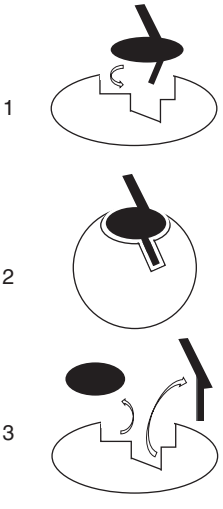


Figure 10.1 Induced fit mechanism for enzyme kinetics. An enzyme (white) is mostly a protein that binds (1) its substrate (black). Both are set under tension during reaction (2). After reaction – here cleavage of a substrate – the products are released (3) and the enzyme reverts to its starting conformation.

are not rigid, and both adjust during their interaction. Although thousands of three-dimensional structures have been solved using X-ray scattering and NMR spectroscopy, the rules that govern folding of an amino acid chain into an active enzyme are not completely understood.

Although the scientific classification of organisms reflects evolution, enzymes are classified strictly by catalytic properties. There are seven enzyme classes, which are divided further into subclasses (Figure 10.2). Enzymes are assigned systematic EC-numbers (*E*nzyme *C*ommission within International Union of Biochemistry and Molecular Biology; IUBMB) to reflect their specific properties. For example, the enzyme with the four-digit code EC 1.1.1.1 is alcohol dehydrogenase. It belongs to class 1 called oxidoreductases. Most enzymes applied industrially today belong to class 3, called hydrolases. Hydrolases need stoichiometric amounts of water to catalyze cleavage of the glycosidic bond in polysaccharides, the peptide bond in proteins, the backbone of nucleic acids, or the ester bond of lipids. Because the direction of the reaction is determined by thermodynamics, hydrolases can also be used for condensation reactions. This can be accomplished by removing water from the reaction mix. In this way, lipases can be used to form esters (e.g. to restructure triglycerides like cocoa butter equivalents) while peptidases can be used to elongate peptide chains (e.g. for insulin analogs).

Today, more than 500 enzymes are produced and sold. The largest variety is used for scientific applications (e.g. for molecular techniques). Additionally, more than 500 industrial products are made using enzymes. The company Novozymes (Denmark) is the largest commercial enzyme producer, followed by DuPont (USA). An increasing diversity of industrial enzymes makes it difficult to get a comprehensive overview. Table 10.1 provides examples of enzymes used in common household products. In Table 10.2, examples of important industrial processes that rely on enzymes are listed. The total market value of enzymes in 2017 is estimated at >5 billion US$ (Table 10.3).

10.1.4 Diverse Ways That Enzymes Are Used in Industry

Microbial enzymes are applied in industry for large-scale processes or for expensive specialty chemicals. The latter can be precursors or end products.

An example of a large-scale product is fructose syrup. Xylose isomerase normally catalyzes the conversion of D-xylose to D-xylulose with highest affinity. However, in industry, this enzyme is used to convert D-glucose to D-fructose. Fructose is 30% sweeter than glucose and it is added to food at more than 10 million tons/yr. Xylose isomerase is produced industrially in *Bacillus* and *Streptomyces* species. Enzymes for food applications must meet the highest safety requirements. Therefore, they are produced under the guidance of good manufacturing practice (GMP). A key issue is the safety of the production strain. Food ingredients produced with enzymes of recombinant microorganisms can be considered "Generally Recognized As Safe" (GRAS) based on FDA (Food and Drug Administration, USA) regulations.

In 2017, GRAS notice of the sugar substitute D-allulose was granted by FDA. The document consists of seven parts. Part II describes three different production processes developed by SamYang Corporation (Seoul, South Korea). In one

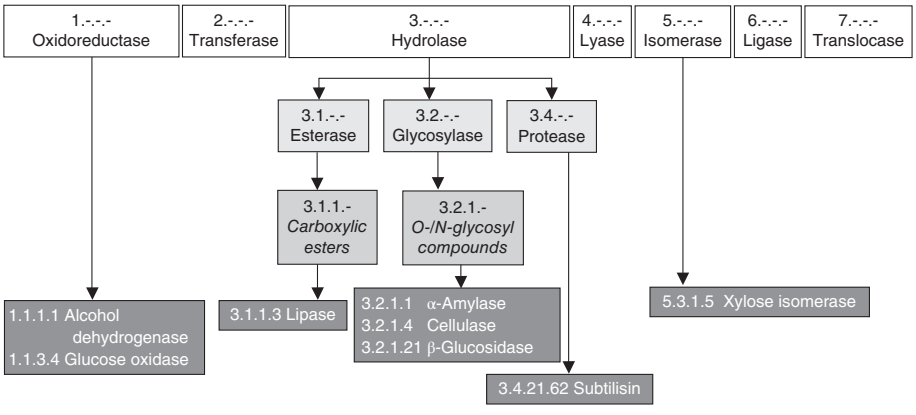


Figure 10.2 Classification of selected enzymes. Each enzyme has a code number that characterizes the reaction type as to class (1st digit) and subclasses (2nd, 3rd, and 4th digit). About 90% of microbial enzymes relevant for application belong to hydrolases.

Table 10.1 Microbial enzymes used in household applications (listed is a selection of examples).

Enzyme containing product	Enzyme class	Activity	Process in households
Detergents	Protease	Protein stain removal	Laundry
			Dish washing
			Contact lenses cleaning
	Amylase	Starch stain removal	Laundry
			Dish washing
	Lipase	Lipid stain removal	Laundry
			Waste water treatment
	Cellulase	Color clarification Anti-redeposition Pilling prevention	Laundry Biopolishing of cotton clothes in textile industry
Personal care products	Amyloglucosidase	Antimicrobial (with glucose oxidase)	Tooth paste
	Glucose oxidase	Selective oxidation	Blood sugar determination

Table 10.2 Microbial enzymes applied for industrial production processes.

Product	Enzyme applied	Company	Annual production (t/yr)
Chemicals			
Acrylamide	Nitrile hydratase	Mitsubishi Rayon Co., Ltd.	>10 000
Fine chemicals			
Aspartame	Thermolysin	Holland Sweetener Company	>1000
Chiral amines	Lipase	BASF	>1000
Enantiopure alcohols			>100
(*R*)-Mandelic acid	Nitrilase		>10
L-Amino acids	L-Acylase	EVONIK	>100
(2*R*,3*S*)-3-(*p*-Methoxyphenyl)glycidyl methylester	Lipase	DSM	>100
S-Ibuprofen	Lipase	Sepracor	<1

In 2014, more than 100 industrial processes used at least one enzyme. This table is a selection of common examples.

Table 10.3 Estimated market of microbial enzymes in 2017.

Enzyme	Example for gene origin/production microorganism Bacteria or Fungi	Annual production (kg/yr)	Market value (million US$/yr)
Cellulase, complex	*Trichoderma reesei* (F)	$> 10^6$	>1000
Protease	*Bacillus licheniformis*	$> 10^6$	>1000
Phytase	Thermophilic bacterium/*Aspergillus niger* (F)	$> 10^5$	>500
Amylase	Thermophilic bacterium/*Bacillus subtilis*	$> 10^5$	>200
Lipase	Engineered fungal gene/*Aspergillus oryzae* (F)	$> 10^4$	>100
Xylose isomerase	*Thermomyces lanuginosus/Aspergillus oryzae* (F)	$> 10^3$	>100
DNA polymerase	*Thermus aquaticus/Escherichia coli*	$< 10^0$	>100
Others			>2000
Total			>5000

Mass of active protein is given for seven enzymes selected out of more than 500 with economic impact. Complex: several proteins with complementary catalytic properties.

of these processes, fructose syrup is passed into nonviable immobilized recombinant *Corynebacterium glutamicum* cells harboring D-allulose 3-epimerase from *Clostridium scindens*. The fructose is converted at 50 °C to D-allulose, a nondigestable carbohydrate having 70% sweetness of sucrose.

Expensive precursors for chemical synthesis of pharmaceuticals and specialty chemicals are also considered fine chemicals. Chiral amines play a role in stereoselective organic synthesis. They are used as resolving agents, building blocks, or chiral auxiliaries. Although classically available through racemic resolution with optically active acids, chiral amines can also be obtained using enzymatic approaches. About half of existing pharma compounds are chiral molecules. Specialized suppliers offer such chiral building blocks based on customer requests. Enantiopure intermediates represent a market of more than 10 billion US$.

Pharmaceuticals with new properties are expected to produce an income of more than a billion US$ per year for the parent company. In the production process, the inherent inefficiency of kinetic resolution (maximum 50% yield) can be overcome by asymmetric reactions catalyzed by improved microbial enzymes providing up to 100% yield. Sulopenem is a β-lactam antibiotic developed by PFIZER (USA). It has potent activity against multidrug resistant bacteria, and in 2019, it is planned to be brought to market by Iterum Therapeutics (Ireland). Asymmetric reduction of tetrahydrothiophene-3-one with a wild-type reductase gives the desired (*R*)-alcohol, a key component in sulopenem. A combination of random mutagenesis was used to improve the enantioselectivity of a ketoreductase toward tetrahydrothiophene-3-one. The best variant increased enantioselectivity from 63% ee to 99% ee (*e*nantiomeric *e*xcess).

10.2 Enzyme Discovery and Improvement

10.2.1 Screening for New Enzymes and Optimization of Enzymes by Protein Engineering

The two primary sources utilized for the screening of new industrially relevant enzymes are (i) isolated microbes and (ii) metagenomic DNA, both of which are usually obtained from environmental samples (Figure 10.3).

From a wealth of more than 10^6 microbial species of archaea, bacteria, and fungi, which are proposed to inhabit the biosphere, more than 10^4 can be cultivated in a laboratory. Although this is only 1%, it is useful to screen among them. Amylase-secreting *Bacillus subtilis* can be isolated within a few days from most soil sources using a minimal medium containing starch as a sole source of carbon and energy. Microorganisms that produce enzymes with desired properties (e.g. high-temperature tolerance and stability) can be selected and isolated from unique ecological niches (e.g. a thermal vent) using appropriate substrates or indicators. The DNA polymerase isolated from the thermophilic bacterium *Thermus aquaticus* (growth optimum of about 70 °C) had sales of 500 million US$ in 2009. Although *Taq* DNA polymerase gene can be expressed

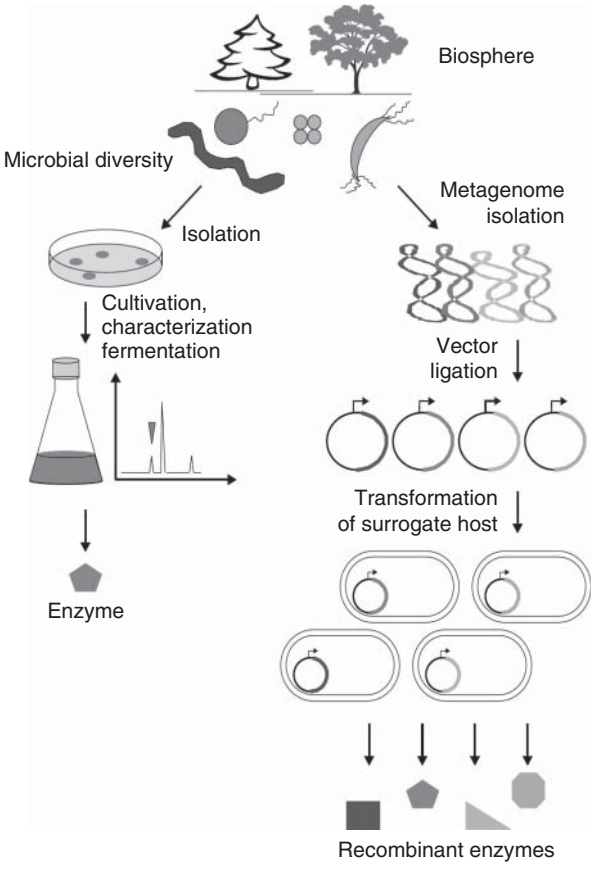


Figure 10.3 Classical versus molecular screening and development of microbial enzymes. Source: Lorenz et al. (2002). Modified with permission of Elsevier.

in highly efficient *E. coli* strains today, a part of the production is still performed with *T. aquaticus*. A reason for this is that it is difficult to get rid of the *E. coli* DNA. The polymerase becomes unstable when the host DNA is removed or replaced with synthetic DNA.

Successful cultivation of an isolated microbe does not necessarily result in the expression of all genes, some of which could encode enzymes with desired properties. However, because of recent advances in sequencing technologies, there are more than 2000 microbial genomes available in government-supported databases, such as the NCBI database. Two approaches help to discover new enzymes from the wealth of available sequences. "Genome hunting" (or "Genome mining") is based on searching for open reading frames in the genome of a given microorganism. Putative genes are then cloned, overexpressed, and assayed for activity. "Data mining" is based on alignment among all sequences deposited in databases. The presence of conserved regions among sequences can be indicative of functional domains. Such homologous sequences can be considered as candidates for further characterization.

Metagenomics is the alternative strategy to conventional microbe screening. In this approach, isolation and cultivation of the microbes is not needed. Instead, DNA sequences of interest can be cloned and expressed in *E. coli*, the workhorse of molecular biology, or in other suitable host microorganisms. Preparation of genomic libraries from environmental DNA and the systematic screening of such libraries for open reading frames can reveal putative genes that encode novel enzymes (Figure 10.3). DNA from volcanic vents, arctic tundra, cow rumen, and termite guts have yielded microbial genes for lipases, oxidoreductases, amidases, amylases, nitrilases, β-glucosidases, decarboxylases, and epoxide hydrolases. Although metagenomics has been employed for more than two decades, not a single enzyme has overcome the hurdles to industrial application to date. An interesting question is that of regulatory approval, if the gene source must be stated as unknown.

Natural enzymes do not necessarily fulfill commercial process requirements and they usually need fine tuning to achieve industrial scale production. Common challenges that have to be addressed are substrate/product inhibition, stability, substrate specificity, or enantioselectivity. Development of improved biocatalysts is challenging and complex (Figure 10.4). Two approaches for the optimization of enzymes are (i) rational redesign of well-studied enzymes and (ii) randomly performed combinatorial methods that search for the desired functionality in libraries.

Directed evolution (see Section 10.2.2) increased the activity of glyphosate-*N*-acetyltransferase by 10 000-fold and at the same time its thermostability increased fivefold. Examples of proteins from directed evolution on the market are green fluorescence protein (GFP) of Clontech and Novo Nordisk's LipoPrime® lipase.

10.2.2 Classical Development of Production Strains

If a microorganism is isolated and found to produce an interesting enzymatic activity, classical tools can be applied to improve its performance. A first step

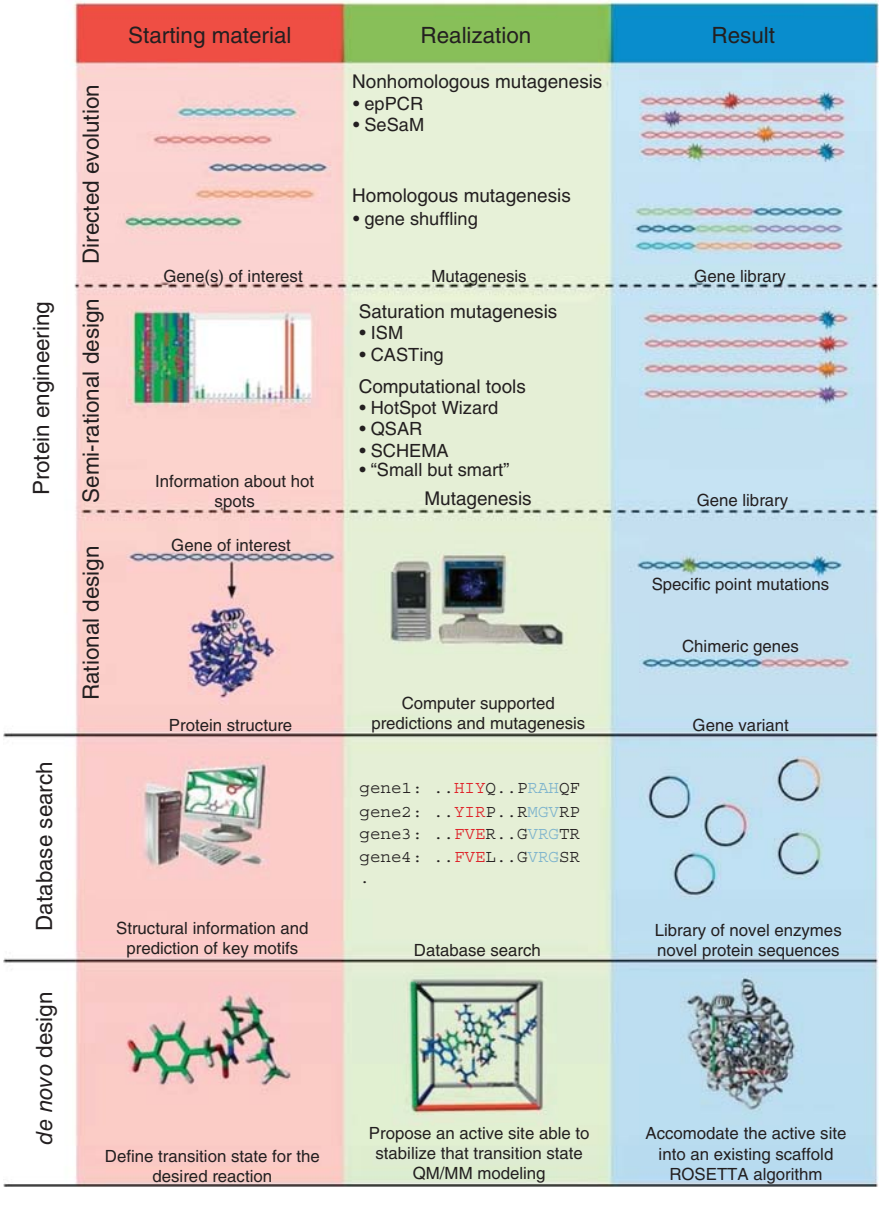


Figure 10.4 Enzyme optimization strategies via protein engineering, database search, or *de novo* design. Source: Davids et al. (2013). Modified with permission of Elsevier.

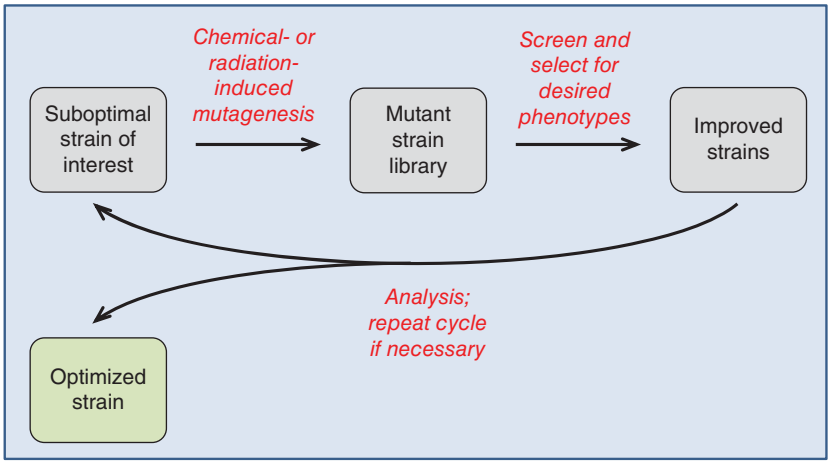


Figure 10.5 Classical strain development via directed evolution.

can be analysis of positive or negative effectors. A negative effector of cellulase production by fungi can be glucose, which commonly causes downregulation of genes encoding polysaccharide-degrading enzymes. This phenomenon is called catabolite repression. An example is the filamentous fungus *Trichoderma reesei*, which is used commercially for the production of cellulolytic enzymes. Its cellulase production was greatly increased by screening for mutants that are resistant to 2-deoxyglucose, a structural analog of glucose that works as a regulatory effector but cannot be metabolized. A positive effect on cellulase production is caused by the presence of disaccharides or under starving conditions, which generates low growth rates (e.g. $0.03\,h^{-1}$). More than 10 transcriptional factors identified in *T. reesei* were patented recently after comparative transcriptional studies of mutants.

Isolated enzyme-producing strains can be improved via the process called directed evolution, which involves multiple cycles of random mutagenesis and selection (Figure 10.5). Physical (e.g. ultraviolet irradiation at 254 nm) and chemical (e.g. *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine) mutagens are commonly used to induce elevated mutation rates in strains of interest. Mutated strains are then subject to screening and selection for the improved production of enzymes. Additional cycles, sometimes using alternative mutagens, can be added as needed (Figure 10.5). Many currently used commercial production strains are based on mutants obtained using directed evolution.

10.2.3 Genetic Engineering of Producer Strains

Although in some applications, (e.g. pectinase aided extraction in wine making), products of genetically modified organism (GMOs) (see Chapter 2) are not allowed, approximately 90% of industrial enzymes are recombinant versions.

Escherichia coli is commonly used as an enzyme production host for several reasons: (i) availability of strong and tight induction systems, (ii) fast growth, (iii) cultivation in cheap medium to high biomass concentrations, and (iv)

ability to reduce unwanted proteolytic degradation. *E. coli* can accumulate heterologous proteins up to 50% of its dry cell mass. However, there are also drawbacks: (i) inability for glycosylation, (ii) disulfide bridges can be formed in the periplasm only, (iii) toxic cell wall pyrogens have to be eliminated, and (iv) some overproduced proteins do not fold properly and aggregate in so-called inclusion bodies, which are insoluble. As maximum titers are in the range of 5 g/l, *E. coli* is most commonly used for the production of expensive enzymes such as restriction endonucleases. Using the addition of appropriate signal sequences to the recombinant enzyme gene, it is possible to direct its production to the periplasm, which has the advantage of a simplified purification process because only the outer membrane needs to be lysed. However, for many industrial enzymes, it is important to be secreted from the host microorganism. In such cases, the lack of a good secretion system in *E. coli* makes it an undesirable production host.

Bacillus subtilis and *Bacillus licheniformis* are Gram-positive bacteria. Consequently, transport through the cytoplasm membrane results in secretion into the medium. Up to 25 g/l amylase or protease is reported to accumulate extracellularly within 48 hours. To minimize proteolysis of the recombinant enzymes, several protease genes have been deleted in production strains.

The budding yeast *Saccharomyces cerevisiae* is a common eukaryotic host used for the production of recombinant enzymes. It has a very well-established genetic system and several so-called shuttle vectors have been developed. These vectors can be constructed in *E. coli* and then introduced in *S. cerevisiae* using various transformation methods. Advantages of *S. cerevisiae* include its tolerance to low pH, high sugar concentration, and osmotic stress. It is also reported to secrete up to 10 g/l protein into the medium. Recombinant *S. cerevisiae* strains are used to produce some of the amylolytic enzymes needed for first-generation bioethanol. On the other hand, hyperglycosylation of the recombinant protein, mainly by α-1,3-linked mannose residues, can negatively affect enzyme activity. There is also a lack of tight promoters for the downregulation of recombinant enzyme expression during growth.

Another budding yeast commonly used as an expression system is *Pichia pastoris*, which can reach more than 20 g/l for intracellular proteins and about 15 g/l for secreted proteins. Advantages of this methylotrophic yeast over *S. cerevisiae* are as follows: (i) it can grow on methanol as a sole carbon and energy source, which can prevent contamination by other organisms, (ii) expression can be regulated using an efficient and tightly regulated methanol inducible promoter, (iii) integration of multiple copies of foreign DNA into chromosomal DNA, and (iv) its better secretion ability. On the other hand, a major disadvantage is that methanol is not feasible for food or feed-relevant applications and its handling and storage pose a hazard because of its high flammability. Similar in properties is the closely related yeast *Hansenula polymorpha*. Using its promoter of the methanol oxidase gene (MOX), recombinant protein expression reached more than 30% of the total cell protein.

Although filamentous fungi are more complex because of their multicellular structures and physiological differentiation, they play an important role in enzyme production. Up to 100 g/l active protein can be accumulated extracellularly, but such high titers require up to 10 days. Most commonly used

filamentous fungi in industry are *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*. *A. oryzae* has been used for the production of *Mucor* rennin and a recombinant phytase. Additionally, recombinant lipases from three fungi, *Rhizomucor miehei*, *Thermomyces lanuginosus*, and *Fusarium oxysporum*, have been produced in this host. *Aspergillus awamori* has been used to produce glucoamylase from *A. niger*. *Acremonium chrysogenum* has been used to produce a *Fusarium* alkaline protease. A major advantage of filamentous fungi is their excellent secretion ability. The intracellular mechanism of transport happens at the endoplasmic reticulum (ER). Subsequently, vesicles are formed and transported to the hyphal tip. There, fusion with the cytoplasm membrane leads to release of the enzymes into the environment (Figure 10.6).

10.3 Production Process for Bacterial or Fungal Enzymes

Cultivations on production scale are performed in stirred vessels of 10–1000 m^3. Both batch and fed-batch processes are performed. As typical bacteria such as *Bacillus* strains represent the minimum in complexity of life and transport distances are short, they usually have fast growth. With growth rates up to 0.8 h^{-1}, a cultivation is finished after two days.

Compared to bacteria, fungi have a 10-fold larger cell diameter, which results in a 1000 times larger cell volume. In addition, fungi, as eukaryotes, have different types of organelles, which are absent in bacteria. Vesicle transport along the cytoskeleton is ATP-consuming. Therefore, growth rates are in the range of 0.2 h^{-1} and the cultivation process needs about a week. To obtain a sufficient dosage of the target gene, in bacteria as well as in fungi, multiple genomic integrations are possible. Once genes are integrated in the genome, they tend to be stable and no selection for the recombinant genes is required during cultivation. The use of antibiotic resistance markers, e.g. as a tool to reach high copy numbers of plasmids, will not be allowed to comply with the future regulation of food enzymes in Europe.

Media are inexpensive bulk products from agriculture, e.g. hydrolyzed corn starch. To minimize quality variations, large batches are bought. Various inorganic salts are also added depending on the microorganism's requirement. Operational parameters such as temperature, pH, feed rate, oxygen consumption, and carbon dioxide formation are measured and controlled. Besides the costs for media, energy costs for air supply by stirring are relevant. As bacteria grow faster than fungi, a higher stirring velocity is needed to get the oxygen dissolved into the water phase. Intensive cooling is needed to avoid overheating. To reduce costs associated with cooling, cultivation at higher temperatures is preferred (37 °C instead of 30 °C).

10.4 Polysaccharide-Hydrolyzing Enzymes

Cellulose and starch are quantitatively the most important polysaccharides on earth. Plants produce 10^{10} tons of cellulose for rigid cell walls per year. Starch is

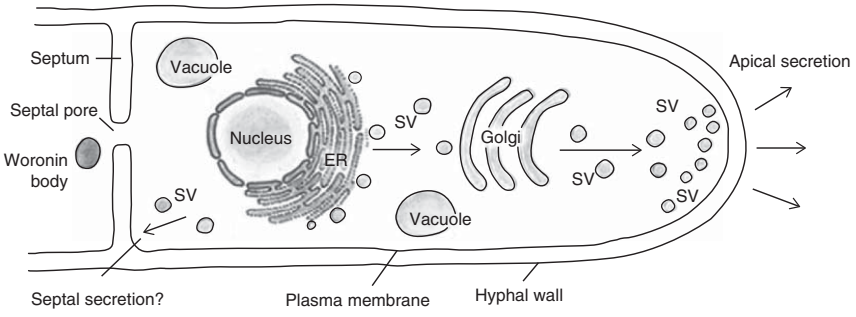


Figure 10.6 Secretion of enzymes in filamentous fungi like *Trichoderma reesei*. The terminal cell in hyphae is producing enzymes. They are secreted into the lumen of the endoplasmic reticulum (ER) where post-translational modifications, e.g. glycosylation, are carried out. Via Golgi secretory vesicles (SV) are produced, transported to the tip and fused to the plasma membrane so that their content is released.

produced for fast storage of carbon and energy, as well as for its fast liberation of simple sugars (e.g. in grains for development of the plant embryo). The primary function of cellulose is mechanical and structural stability. The degradation of both polysaccharides is a naturally occurring process. To utilize plants for energy and nutrients, microorganisms have evolved diverse arrays of efficient enzymes, which we can use as powerful catalysts in industry.

10.4.1 Starch-Cleaving Enzymes Produced by *Bacillus* and *Aspergillus* Species

Starch is a macromolecule of glucose. It consists mainly of α-1,4-glycosidically linked molecules that form double helices. Tightly coiled helices form granula in the plant cells' cytosol. Starch from corn can have a mass of 10^8 Da. Many of these helices can be linked via α-1,6-glycosidic branches (Figure 10.7).

Four types of enzymes are needed for the hydrolysis to glucose. **α-Amylases** cleave in an endo manner. They are specific for α-1,4-glycosidic bonds and randomly cleave the macromolecule, forming smaller oligomers. **β-Amylases** act in an exo manner. They start at the reducing end of the helices and release the disaccharide maltose. **Pullulanases** cleave the α-1,6-glycosidic bonds at the branching points. **Glucoamylases**, also called α-glucosidases, start at the nonreducing end and release glucose from maltose or from oligosaccharides.

Starch-cleaving enzymes are used as baking aids. The released maltose from starch is catabolized by the yeast. The released carbon dioxide increases bread volume, improves dough preparation, and helps to develop a nice tan and crust

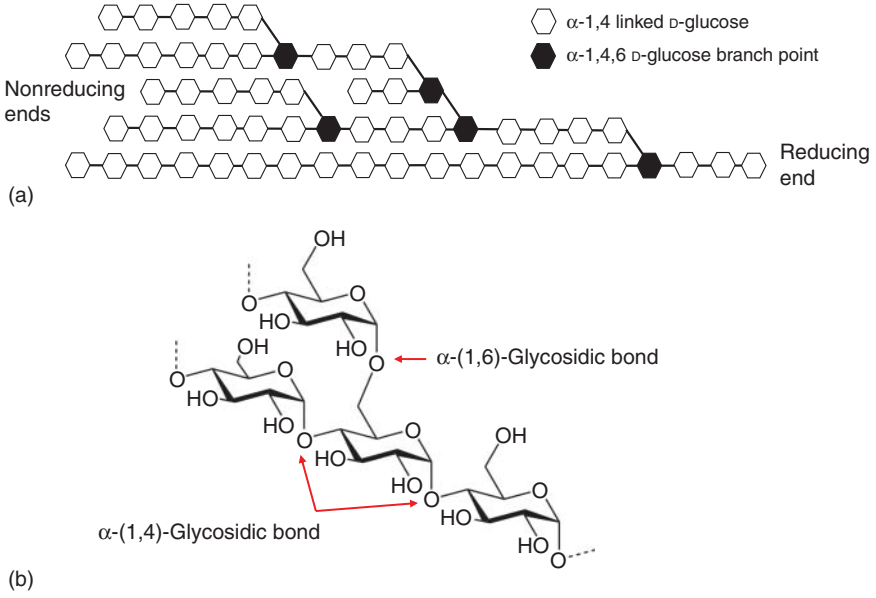


Figure 10.7 Molecular structure of starch. (a) Superstructure made of amylose chains and (b) types of bonds.

of the bread. Additionally, maltogenic α-amylase is used to avoid starch retrogradation, the formation of a polysaccharide superstructure stabilized by hydrogen bonds. This helps to prevent staling of the bread and has been described as a so-called fresh-keeping effect. However, α-amylase is not considered a preservative.

The production of HFCS (high-fructose corn syrup) from corn plays a role in sweetening of soft drinks and many foods. To cleave the starch into dextrin and oligosaccharides, α-amylases from *B. licheniformis* are used. The dextrins are then hydrolyzed further using glucoamylase and pullulanase. Partial isomerization of glucose to fructose is catalyzed by immobilized glucose isomerase (see Section 10.1.4).

Ethanol is the most utilized liquid biofuel. In countries with surplus agricultural capacity, ethanol is usually produced from starch because of its low price and easily available raw material (e.g. corn). Because *S. cerevisiae*, the industrial ethanol producer, cannot degrade starch, saccharification to maltose and glucose is performed enzymatically.

α-Amylases and glucoamylases are produced commercially in recombinant Bacillus strains (Table 10.4). The α-amylases for industrial use (e.g. the textile and detergent industry) are characterized by a high specific activity and temperature stability. Some such enzymes have been derived from inherently heat-stable amylases of *B. licheniformis* and *Bacillus stearothermophilus*. Performance and stability were enhanced by random mutagenesis using DNA shuffling and subsequent screening (Figure 10.8). The molecular mechanism of increased stability is not fully understood yet.

α-Amylases from filamentous fungi such as *A. niger* or *A. oryzae* are less heat stable. However, their use can be advantageous because of their high activity and stability at pH 3, which prevents bacterial contamination during enzymatic processing.

Downstream processing of the amylases starts with a separation of the biomass by separators, rotary drum filters, or filtration. Depending on the application,

Table 10.4 Industrial applications of bacterial amylases.

Industrial application	Microbial source	Role of amylase
Starch liquefaction and saccharification	*B. stearothermophilus, B. licheniformis*	Hydrolysis of starch to shorter dextrins, maltose and glucose
Baking	*B. stearothermophilus*	Conversion of starch in dough to fermentable sugars
Detergents	*B. licheniformis*	Digestion of starch-containing foods in stains to water soluble dextrins
Textiles	*Bacillus* sp.	Removal of starch sizing agent from woven fabric
Biofuel production	*B. subtilis*	Conversion of starch in feedstock to smaller fermentable sugars
Paper	*B. subtilis*	Partial hydrolysis of starch to reduce viscosity

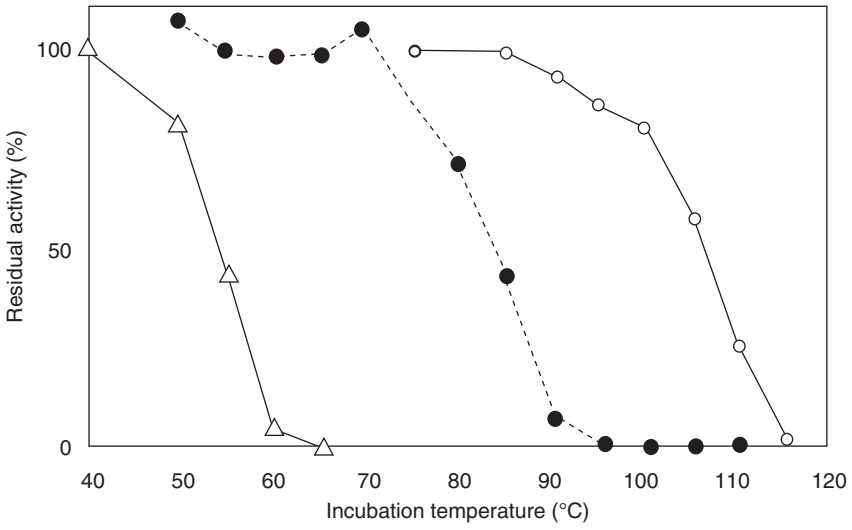


Figure 10.8 Temperature resistance of α-amylase from *Bacillus licheniformis* after single amino acid replacement mutagenesis. Depending on the type and location of the mutations, the effect can be either destabilizing as in D204K (Δ) or stabilizing as in N265Y (○) in comparison with the wild type (●).

the cell-free supernatants are concentrated by ultrafiltration and dried to stable liquid products or processed into solid particles. Purification by chromatography is not needed.

10.4.2 Cellulose-Cleaving Enzymes: A Domain of *Trichoderma reesei*

Cellulose is, in contrast with starch, a very stable macromolecule. This is reflected in the tensile strength of cotton, for example. Because cellulose is resistant to enzymatic saccharification, it is considered to be recalcitrant. Cellulose exists in the form of fibrils, bundles of β-(1, 4)-D-glucan polymers. These fibrils have species-dependent supramolecular characteristics, such as lateral dimensions and degree of polymerization (DP). Unlike starch, polymer chains of cellulose have no branching, and this enables them to pack tightly in crystalline structures that are stabilized by extensive interchain hydrogen bonds. The extent of crystallinity can differ within the fibril, such that regions near the surface and the end of the fibril are typically the least constrained. Depending on the starting material and the isolation procedure used, fibrils can assemble into larger aggregates that constitute a cellulose network, such as in the wood pulp fiber walls after isolation. This network has a complex orientation in space. Access to the individual polymer chains is considered to be the rate-limiting step in enzymatic hydrolysis of cellulose (Figure 10.9).

Based on over 50 years of research, it is clear that a complex system of enzymes is needed for the efficient saccharification of cellulose (Table 10.5). All cellulases hydrolyze the β-(1, 4)-D-glycosidic bonds of the cellulose chain, but they differ

in properties, such as the mode of action and processivity (consecutive hydrolysis without the release of the cellulose chain). **Exocellulases** initiate hydrolysis from the chain ends and then processively release units of cellobiose (the repeating unit) from the cellulose chain. **Exocellulases** are classified based on whether they initiate hydrolysis from the reducing (e.g. *T. reesei* CBHI) or nonreducing (e.g. *T. reesei* CBHII) end of the cellulose chain. **Endocellulases** randomly initiate hydrolysis anywhere along the polymer chain, shortening its length. Cellulose chains with DP ≤6 are soluble. Most endocellulases are considered to act on cellulose with low processivity. **β-Glucosidases** hydrolyze cellobiose and other soluble cellooligosaccharides to glucose, completing the enzymatic saccharification of cellulose (Figure 10.9c). The different types of cellulases degrade cellulose in a synergistic manner, whereby the total activity of a mixture of enzymes is greater

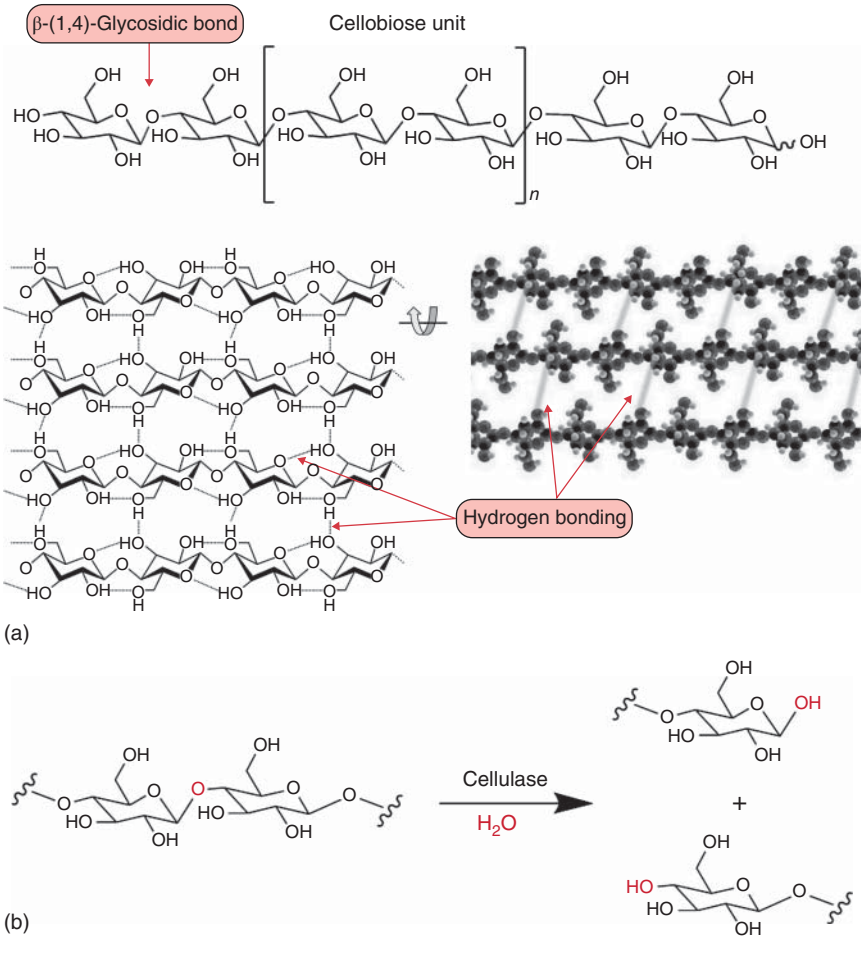
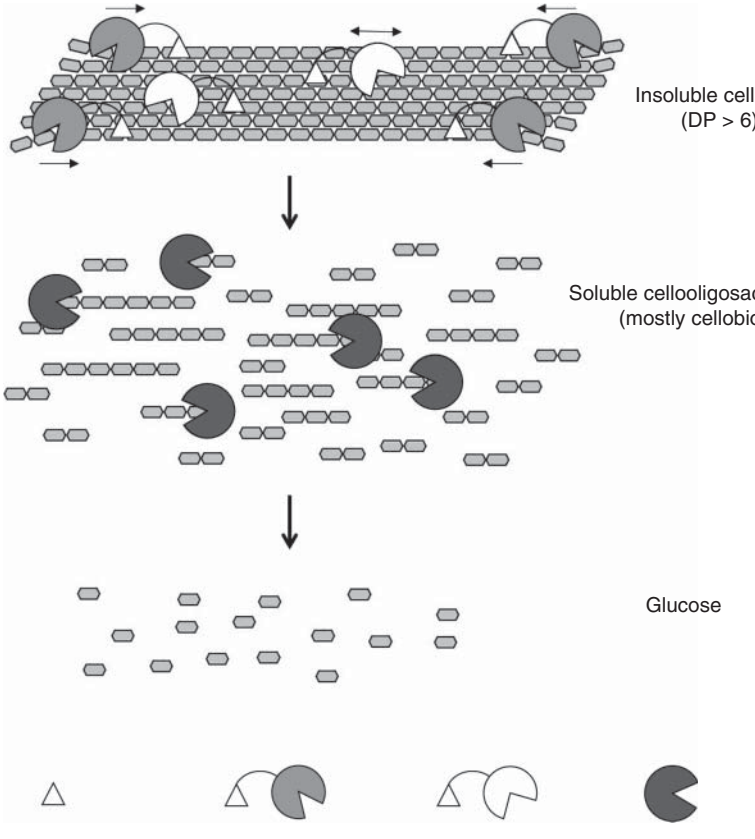


Figure 10.9 Cellulose (a) structure and recalcitrance, (b) hydrolysis by (c) four types of cellulases. (continued on the next page).



(c)

Figure 10.9 (*Continued*)

than the sum of the individual enzyme activities. In reflection of this, many cellulolytic microorganisms produce an array of cellulases with different properties.

One of the most studied cellulolytic microorganisms is the saprophyte *T. reesei*, which is the main source of industrial cellulases for commercial cocktails (e.g. Accelerase 1000). Sequencing of its genome revealed about 9000 genes. Two hundred genes are predicted to encode glycosyl hydrolases, 10 of which are known cellulases. Current strains of *T. reesei* can produce up to 100 g of extracellular protein/l.

10.4.3 Production Strains

The worldwide need for alternative fuel sources became a pressing issue after the oil crisis in the 1970s. As a result, there was a surge of research into the potential of ethanol production from cellulose-rich biomass, with a focus on fungal

Table 10.5 Major cellulases produced by *Trichoderma reesei* (database: www.cazy.org)

Classical name	EC number	CAZy name	Activity type
Bgl1 Bgl2	3.2.1.21	GH3 Cel3A GH1 Cel1A	β-Glucosidase; hydrolyzes cellobiose and soluble cellooligosaccharides to glucose
EGI EGII EGIII EGV	3.2.1.4	GH7 Cel7B GH5 Cel5A GH12 Cel12A GH45 Cel45A	Endocellulase; hydrolyzes β-(1,4)-glycosidic bonds anywhere along the cellulose chain, creating new chain ends and soluble oligosaccharides
CBHI	3.2.1.91	GH Cel7A	Exocellulase; hydrolyzes β-(1,4)-glycosidic bonds from the reducing chain ends, releasing cellobiose
CBHII	3.2.1.91	CH Cel6A	Exocellulase; hydrolyzes β-(1,4)-glycosidic bonds from the nonreducing chain ends, releasing cellobiose
EG7	1.14.99.54	AA9	Lytic polysaccharide monooxygenase (LPMO); oxidatively cleaves β-(1,4)-glycosidic bonds, creating new chain ends; disrupts crystallinity at the cellulose surface

enzyme use for the saccharification of cellulose. However, even today, companies cannot produce bioethanol from cellulose in a cost-effective manner (in competition with oil). Enzymatic saccharification continues to be one of the primary drivers for the high costs. Although the price of commercial cellulase cocktails has dropped significantly over time, cellulase production continues to be expensive. In addition, plant cellulose is part of a complex structure that also includes large amounts of lignin and hemicellulose, both of which can impede cellulase activity. Currently, lignin and hemicellulose are removed using different physicochemical pretreatments, which themselves are expensive. Besides reducing the cost of these processes directly, another potential way to address this issue is to use ligninases and hemicellulases to enzymatically break down at least some of the lignin and hemicellulose. Hemicellulases are already added in some of the modern commercial cellulase cocktails. The efforts to reduce the costs associated with cellulase production are ongoing. This is done via the fine-tuning of the enzyme cocktails themselves (e.g. adding new enzymes) and the improvement of production strains, such as *T. reesei*.

Trichoderma reesei was originally described as an isolate of *Trichoderma viride* but later recognized to differ significantly from it. It was then renamed in honor of the Natick laboratory (Massachusetts, USA) researcher Elwyn T. Reese, who carried out important early studies of enzymatic cellulose hydrolysis. In *T. reesei*, as in other cellulolytic microorganisms, production of cellulases is tightly controlled and is repressed in the presence of simple carbon sources such as glucose. This is known as catabolite repression. To improve *T. reesei* cellulase production for the more economic production of bioethanol, efforts to isolate catabolite-derepressed mutants were initiated.

All *T. reesei* strains used at present go back to a single isolate obtained from decaying cotton canvas of an army tent on the Solomon Islands in World War

II. A significantly improved strain, RUT-C30, was obtained in three rounds of mutagenesis and screening. The first cycle of UV mutagenesis and screening was followed by another cycle using the chemical mutagen *N*-methyl-*N*-nitroso-*N*′-nitroguanidine. This led to the isolation of partially derepressed strains. Following another round of UV mutagenesis and screening for resistance to the antimetabolite 2-deoxyglucose (a structural analog of glucose), the RUT-C30 isolate was obtained. It produced high levels of extracellular cellulases and was resistant to catabolite repression by glucose and glycerol.

Understanding the genetic and physiological changes that are responsible for the ability of RUT-C30 to secrete elevated amounts of protein in a catabolically derepressed manner can aid the development of rational approaches for strain improvement in the future. Electron microscopy analysis of this isolate revealed a more than fivefold increase in its endoplasmic reticulum content compared to the wild-type strain. RUT-C30 also has significant genomic rearrangements, which are reflected in the changed size of its chromosomes.

More recent studies comparing DNA between the wild-type strain and RUT-C30 have revealed many more changes than expected. RUT-C30 has been found to have 25 large deletions, which total about 100 kb of genomic DNA. It also has over 200 single nucleotide variants. Altogether, 43 genes were affected by the deletions and mutations in RUT-C30. Most of the genes are not directly involved in cellulase production, however. Instead, they play a role in other cell processes, such as metabolism, vesicle transport, and signaling, most of which can indirectly affect protein production and secretion.

Because of the ability of RUT-C30 to produce large amounts of extracellular protein, there has been interest in using it as a host for the production of heterologous proteins. Successful examples are the expression of genes encoding a phytase from *E. coli* or fungal pectinases. Recombinant techniques that have been traditionally used in *T. reesei* do not work well in RUT-C30 because of its genetic and physiological changes. Today, various molecular techniques are developed to, e.g. allow targeted genomic integration and identify improved enzyme producers. Furthermore, sexual reproduction of *T. reesei* has been recently achieved, leading to the possibility of developing new strains via crossing of different mutants and natural isolates.

10.5 Enzymes Used as Cleaning Agents

Enzymes have contributed to the improvement of modern household and industrial laundry and dish washing detergents. Addition of enzymes to detergents results in (i) better cleaning performance, (ii) rejuvenation of cotton fabric by cellulases, (iii) reduced energy consumption due to lower washing temperatures, and (d) phosphate-free and less alkaline waste water. In general, fewer chemicals are needed.

To remove stains, including those from blood, grass, egg, and fat, a number of different hydrolases are added to laundry detergents. The major classes are proteases, lipases, amylases, mannanases, cellulases, and pectinases. In dish washing, the major enzyme classes are bacterial proteases and amylases.

Proteases were the first enzymes used in laundry. The following subchapters will focus on them.

10.5.1 Subtilisin-Like Protease

Proteases catalyze the hydrolysis of peptide bonds. Based on mechanistic features, they are classified into six groups: either aspartate, cysteine, glutamate, metallo, serine, or threonine, which play an essential role in the active site. Proteases are also classified either as exo- or endo-cleaving, based on whether they catalyze the removal of the terminal amino acid or cleave within a polypeptide, respectively. Alternatively, proteases are classified based on the optimal pH for their activity as acid, neutral, or alkaline.

Proteases in the food industry enhance flavor in dairy, meat, and fish products. In the leather industry, they are applied during soaking and hair removal. In medical treatments, they are used for osteoarthritis, removal of necrotic tissue, and wound healing. For personal care, they are active in contact lenses cleaning solution.

The performance of a detergent protease depends on (i) stain degradation activity; (ii) compatibility with surfactants, oxidizing and complexing agents, perfumes, other enzymes; and (iii) shelf life, i.e. stability.

Subtilisin-like proteases are alkaline serine proteases with a molecular mass of 18–90 kDa. Their outstanding properties are high stability and a broad substrate specificity. A disadvantage is that they need higher temperature, which conflicts with the contemporary goal of using lower washing temperatures.

A directed evolution campaign was performed with *Bacillus sphaericus* subtilisin using random mutagenesis followed by recombination of variants. A 7-fold increase in the turnover number (k_{cat}) at 108 °C and 10-fold increase in catalytic efficiency was achieved.

A chimeric enzyme generated by replacing a stretch of 12 amino acids in the psychrophilic Antarctic Bacillus TA39 protease with the corresponding amino acid sequence of the mesophilic subtilisin Savinase1 showed a higher specific activity for synthetic substrates and a broadened substrate profile at 21 °C.

A directed evolution campaign using iterative rounds of Sequence Saturation Mutagenesis (SeSaM) was performed. In the first round of SeSaM, variants were identified with increased activity at 15 °C but accompanied by decreased stability at 58 °C. The amino acid substitutions identified were independently saturated to elucidate the effect of each position on the increased activity at 15 °C. Three amino acid exchanges correlated with increased activity at 15 °C. In parallel, screening for increased stability at 58 °C resulted in a variant harboring Ser39Glu, Asn74Asp, and Asp87Glu. Both sets of mutations were combined in the hybrid variant MF1. MF1 showed 1.5-fold improved k_{cat} and a 100-fold increased half-life at 60 °C (224 minutes) in comparison to BgAP wild-type protein (Figure 10.10).

Although the above examples are promising, they have significant limitations for industrial applications. The k_{cat} values of the enzymes were measured with low-molecular-weight peptide substrates, which are very different from denatured high-molecular-weight proteins attached to a textile surface and potentially

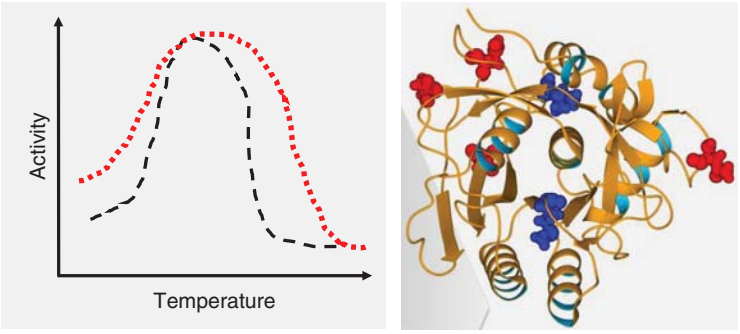


Figure 10.10 *Bacillus gibsonii* protease variant MF1 after directed evolution to high activity at 15 ° C and stability at 58 °C. (a) Variant temperature profile (dashed red) compared to the wild-type enzyme (dashed black). (b) The result of four amino acid exchanges (red) found via screening for stability combined with another two (blue) for low-temperature activity are shown in a hypothetical model. Source: Vojcic et al. (2015). Modified with permission of Elsevier.

cross-linked to sugars by aging or heating. Thus, impressive improvements of an enzyme's activity with synthetic substrates do not necessarily predict its performance in a detergent or other applications.

10.5.2 *Bacillus* sp. Production Strains and Production Process

Subtilisin is produced using strains from the genus *Bacillus*. The species can be *B. subtilis*, *B. licheniformis*, *Bacillus lentus*, or other *Bacilli*. *Bacilli* have a high protein secretion ability and because they are Gram-positive, they can export proteins directly into the extracellular medium, unlike *E. coli*.

Nowadays, genetically modified microorganism (GMM) strains are used. Handling takes place under containment in sealed installations. The manufacturing process can be described as a three-step process: cultivation, recovery, and enzyme formulation.

An aerobic submerged culture in a stirred-tank reactor is the typical industrial process for subtilisin production. Online control of pH, temperature, DO (dissolved oxygen), and foam formation is performed. Supply of oxygen is done through a sparger. Stirrers mix gas, biomass, and suspended particulate substrates.

There are four cultivation types: batch culture, fed-batch culture, perfusion batch culture, and continuous culture. In a batch culture, the microorganisms are inoculated in a fixed volume of medium. In a fed-batch culture, concentrated components of the nutrient are gradually added to a preculture, which results in an increasing volume. In a perfusion batch culture, the addition of the culture and withdrawal of an equal volume of used cell-free medium is performed. In the continuous culture, fresh medium is added into the batch system at the exponential phase of the microbial growth with a corresponding withdrawal of the medium containing the product. For subtilisin production, fed-batch culture

is predominant. The feed contains nutrients for maintenance of the *Bacilli* and inducers for enzyme production. A typical inducer is casein, a protein from milk.

All subtilisins are extracellular after cultivation. The first step in their purification is separation of the bacterial cells. That can be done by filtration, e.g. rotary drum filtration. A rotary vacuum filter consists of a several meters long rotating drum covered by a filter medium. The drum is immersed partly into the broth. As the drum rotates, the broth is pulled on the filter and rotated out of the liquid/solids suspension as a cake. When the cake is rotating out, it becomes dry as the vacuum continuously removes the enzyme-containing liquid from it. Finally, the cake is discharged as solid and the drum rotates to another separation cycle. The enzyme-containing liquid is normally concentrated by ultrafiltration. At sufficiently high concentration, protein precipitates out of the solution.

The protein is then dried to a nondusting material that can be stored and handled safely. Once the protein is dry, it is important to minimize losses in enzymatic activity during transport, storage, and usage. This is achieved via the use of stabilized liquid concentrates or coated granulates of the protein, which can mix well with the detergent powder or tableting material. Chemical stabilizers are available to protect the enzymes thermally and chemically, e.g. by reversibly inhibiting protease activity.

Formulations enhance stability by counteracting the primary forces of denaturation, catalytic-site deactivation, and proteolysis. Denaturation occurs by physical unfolding of an enzyme's tertiary protein structure. To minimize unfolding, the formulator can alter the protein's environment to induce a compact protein structure. This is done most effectively by adding water-associating compounds such as sugars, polyhydric alcohols, and lyotropic salts, which detach water molecules from the protein surface via "preferential exclusion." The best ways to combat active site inactivation are to ensure sufficient levels of any required cofactors, to add reversible inhibitors, and to exclude reactive or oxidizing species from the formulation.

Enzymes are respiratory sensitizers, which can cause allergic reactions. Therefore, dry granular enzyme additives in powdered laundry detergents should be formulated with regard for worker safety. Through formulation improvements, enzyme granules have become increasingly resistant to physical breakage and formation of airborne dusts upon handling. Two processes that produce the most attrition-resistant granules to date are high-shear granulation and fluidized-bed spray coating. These processes use binders, coatings, and particle morphologies. The goal is nonfriable particles, which protect enzymes during storage and allow for their ready release in solution during application.

10.6 Feed Supplements – Phytases

The goal of feed enzyme application is to deliver the desired dose of active enzyme to the site of action, namely the gastrointestinal tract of the animal. Phytases and xylanases enable better direct degradation of feed compounds. Additionally, both have an indirect effect. In total, feed conversion rate can increase by 25% with the addition of supplementary enzymes.

Xylanases added to wheat, rye, and triticale-based animal feeds enable the degradation of insoluble polysaccharides and therefore reduce viscosity. Additionally, protein digestibility in wheat is increased. This is attributed to the release of protein from the xylan-rich thick cell wall protein-rich aleurone layer. Because starch- and cellulase-degrading enzymes were explained in detail above, this subchapter focuses on phytases.

10.6.1 Fields of Applications of Phytase

Phytic acid represents 60–80% of total phosphorus in plant feeds. Phytases are hydrolytic enzymes that initiate its stepwise dephosphorylation to myo-inositol (Figure 10.11). The poor digestion of phytate by monogastric animals causes two problems: (i) loss of bivalent cations complexed by phytates and (ii) environmental pollution.

To reduce a need for dietary supplementation of inorganic phosphorus, phytase is added to animal feed. Recycling of plant phytates can help address the eventual depletion of rock phosphorus deposits, which will increase the price of inorganic phosphorus in the future. Nutritional values of phytases in replacing inorganic phosphorus supplementation and improving bioavailabilities of calcium, iron, and zinc are documented.

10.6.2 Phytase in the Animals Intestine

Because of the lack of significant phytate degrading activity in the gastrointestinal tract, single-stomach (monogastric) animals such as swine, poultry, and fish require extrinsic phytase to make phytate phosphate available for growth and development. In ruminant animals such as cattle and sheep gut, microbes produce phytases, which can partially digest phytate.

The first commercialized phytase was launched into the market in 1991. Now, the enzyme is present in about 75% of all diets for monogastric animals.

Because animal cells contain intracellular partially phosphorylated myo-inositols that are dephosphorylated at position 2, a 2-phytase is probably available. Commercial phytases can be classified according to their origin, the carbon in the myo-inositol ring of phytate at which the ester bond is cleaved (3- or 6-phytases), or based on the presence of any protection against the high temperatures present in feed production (coated or uncoated). Phytases from fungi as well as from bacteria are applied (Table 10.6). Phytases act only on soluble phytate. Under physiological conditions, a pH-dependent equilibrium of soluble and insoluble phytate exists. Phytase activity at pH 3.0 is needed. Stability of the phytase under the pH conditions of the stomach and its susceptibility to pepsin degradation are important properties (Table 10.7).

Thermal stability is a further issue as feed pelleting is performed at temperatures between 60 and 95 °C. Recovery after pelleting can be above 90%. Phytase might exert larger and broader socioeconomic impacts when tailored for species and diets. Loss of phytase activity during feed pelleting arguably remains a limiting factor (Table 10.8).

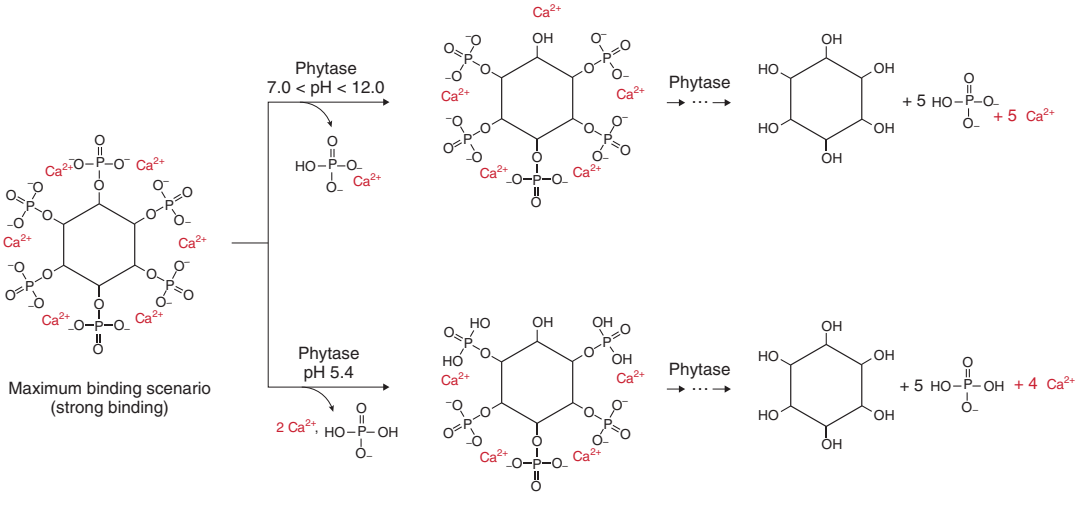


Figure 10.11 Chemical reaction catalyzed by phytase. The substrate, phytate, is a "mine" of phosphoric acid needed for the animal breeding and available in plant feed. The enzyme cleaves the ester bonds to release phosphoric acid bound on inositol and, indirectly, cations such as calcium ions, also needed by the animals.

Table 10.6 Fungi and bacteria as gene donors and production strains for phytases on the market in 2012.

Microorganism			
Gene donor	**Expression host**	**Product**	**Company**
Aspergillus niger	*Aspergillus niger*	Natuphos	BASF
	Trichoderma reesei	Finase P/L	AB Vista
Penicillium funiculosum	*Penicillium funiculosum*	Rovabio	Adisseo
Escherichia coli (B)	*Trichoderma reesei*	Finase EC	AB Vista
	Pichia pastoris	OptiPhos	Enzyvia
		Quantum	AB Vista
	Schizosaccharomyces pombe	Phyzyme XP	DuPont
Peniophora lycii	*Aspergillus oryzae*	Ronozyme P	Novozymes/DSM
Citrobacter braakii (B)		Ronozyme HP	

Table 10.7 pH stability and protease tolerance of phytases produced in different hosts.

Treatment	**% of initial activity**		
Acidic pH	***Peniophora lycii***	***Aspergillus niger***	***Escherichia coli***
pH 2, 37 °C, 1 h	40–50	70–85	90–95
+ BSA	45–50	75–85	95–100
+ 2 kU pepsin			
pH 2, 37 °C, 1 h	15–25	40–50	90–100

BSA is bovine serum albumin, a cheap protein.

10.6.3 Production of a Bacterial Phytase in *Aspergillus niger*

Phytase production became possible with the application of recombinant techniques. Scientific milestones were the isolation of phytase-producing fungal (Figure 10.12) and bacterial strains, the characterization of the purified enzymes, cloning of the corresponding genes, and their overexpression. New-generation phytases have improved stability that is needed for activity in the animal intestine.

Use of microbial phytases for animal feed application needs the acceptance of genetically modified products by consumers. Although phytase supplementation results in substantial reductions in total phosphorus excretion, a slight increase of soluble phosphorus in the excreta is possible. That has to be addressed in the wastewater treatment.

Both traditional and biotechnological approaches have been applied successfully to search for novel phytases and to overproduce them efficiently. Protein engineering using structure-based rational design and directed evolution has

Table 10.8 Storage stability of phytase produced in *Escherichia coli* and *Aspergillus oryzae*.

Formulation	% initial activity	
Feed pelleting	***Escherichia coli***	***Aspergillus oryzae***
30 days	>90	>90
60 days	>85	>85
120 days	>70	>70
Pure, coated		
30 days	>90	>70
Pure, uncoated		
30 days	>90	>35

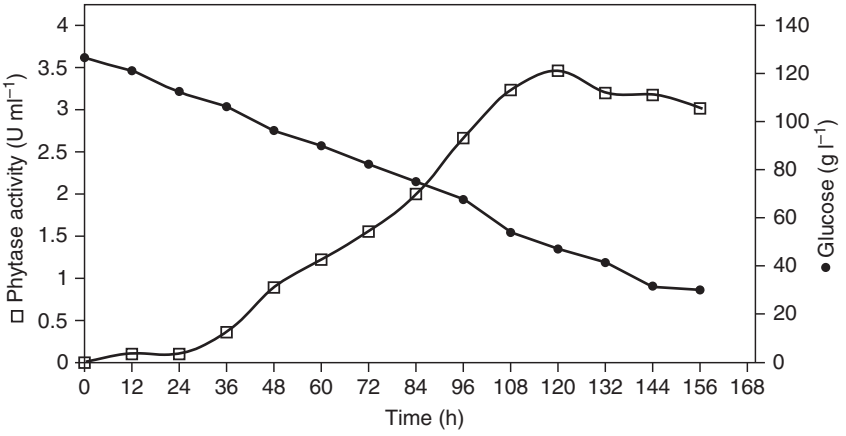


Figure 10.12 Time course of phytase production by *Aspergillus ficuum*.

resulted in functional-target enhancements of fungal and bacterial phytases with commercial value. Either the expression of fungal genes in fungi or of bacterial genes in bacteria works. Today, the gene sequences can be obtained (and modified) using bioinformatics and then synthesized chemically. Codon usage and secretion signals can be optimized for the heterologous expression host.

Interestingly, some bacterial phytases are produced heterologously by fungi. An example is a gene originating from *E. coli* but overexpressed in *A. niger* or in *T. reesei*. This is driven primarily by two economic considerations: (i) high extracellular enzyme titers and (ii) robustness of the enzyme production process because of acidic cultivation conditions. Acid culture conditions simplify sterile procedures, saving investment, and operating costs. Additionally, bacterial protein overexpression is often limited by the formation of inclusion bodies.

In case of fungal phytases, the bacterial production is limited by the lack of adequate posttranslational modification. Glycosylation improves half-life of many

enzymes (e.g. by resistance against proteolysis). Indeed, artificial deglycosylation was shown to reduce both molecular mass and thermostability.

10.7 Enzymes for Chemical and Pharmaceutical Industry

10.7.1 Examples for Enzymatic Chemical Production

Acrylamide is as monomer needed for the production of polymeric materials. Addition of water to acrylonitrile is one possible production process. Use of a reduced copper or vitriol catalysts results in poor yield and can lead to unwanted polymerization and conversion to acrylic acid. These problems are overcome by the use of nitrile hydratase from *Rhodococcus* sp. More than 10 000 t/yr are produced by nitrile hydratase entrapped in cross-linked bacterial cells. In this approach, purification of the enzyme is not needed. The granulated product is used in a process that takes less than 20 hours. The substrate concentration is kept low by a feeding strategy to avoid uncontrolled heat production caused by the strong exothermic reaction. Stability of the enzyme is also negatively affected by the substrate as well as by the product. Today, new generations of enzymes modified by deletion and exchange of more than 10 amino acids show improved stability and performance. At a temperature of 10 °C it is possible to achieve acrylamide concentrations above 500 g/l.

Aspartame (L-Asp-L-Phe-methyl ester) is an artificial sweetener 100 times sweeter than sucrose. In the gastrointestinal tract, it is degraded into its components, the two amino acids and methanol.

The regio- and stereoselective catalysis of a protease is needed to get only the desired dipeptide formation when an L-aspartic acid derivative and the methylester of a D/L-phenylalanine racemate are mixed. It is advantageous to push the reaction equilibrium to the product peptide precipitation. A typical enzyme used is thermolysin from *Bacillus thermoproteolyticus*. This metalloprotease needs one Zn^{2+} for catalysis and four Ca^{2+} for stability. Optimal conditions for activity are 70 °C at pH 8.

10.7.2 Production of (*S*)-Profens by Fungal Lipase

An example of a product produced with the use of highly selective lipases is ibuprofen. This substance is an analgesic and anti-inflammatory drug that can be produced chemically as a racemic mixture. The *S*-enantiomer of ibuprofen is over 100 times more effective than the *R*-enantiomer in the inhibition of prostaglandin biosynthesis. Additionally, the *R*-enantiomer generates undesired effects in the gastrointestinal tract, in lipid metabolism, and membrane function.

Enantioselective resolution of racemates of profens can be carried out by lipases, mainly those from *Candida antarctica* or *Candida rugosa*. Two enzymatic approaches to perform the racemate resolution in order to obtain the optically pure (*S*)-acids are

(a) enantioselective hydrolysis of 2-aryl-propionate ester racemates in aqueous media catalyzed by microbial lipases with preference for the (*S*)-isomer;
(b) enantioselective esterification of 2-aryl-propionic acid racemates in organic media catalyzed by microbial lipases with preference for the (*R*)-isomer.

Both approaches have been successfully employed in the efficient production of (*S*)-ibuprofen, (*S*)-naproxen, (*S*)-ketoprofen, and (*S*)-flurbiprofen, reaching high enantiomeric excess (i.e. ee > 99%).

10.8 Enzymes as Highly Selective Tools for Research and Diagnostics

The market for enzymes used in laboratories can be split in two parts. The major part consists of analysis and engineering of nucleic acids. This sector is growing rapidly, in part because of the emerging practice of using the so-called personalized medicine, in which an individual's genetic profile is used to guide diagnosis, prevention, and treatment of diseases. For example, enzymes are used in the differential analysis, amplification, and sequencing of DNA isolated from a patient's tumor. A less significant sector includes analytical test systems for metabolites, e.g. citric acid in food and other substances (Table 10.9).

10.8.1 Microbial Enzymes for Analysis and Engineering of Nucleic Acids

This field is broad and consists of more than 300 different enzymes of highest quality. Heterologous expression of genes for such enzymes (e.g. in *E. coli*) is a minor issue. More than 90% of the effort goes to downstream processing and quality management. In most cases, getting rid of unwanted side products, such as host DNA from a recombinant DNA polymerase, is not possible in a single purification step. Most frequently, more than two different techniques are applied, e.g. chromatography and precipitation.

Proteinase K, an endopeptidase degrading keratin, is an extracellular enzyme produced by the fungus *Tritirachium album*. This fungus is able to grow on keratin, an extremely tough protein (skin, hair, and nails) as the sole source of carbon and nitrogen. This enzyme is used by laboratories to get rid of interfering proteins during DNA preparation. Specifically, complete removal of nucleases is important. Purified preparations are sold for prices above 1000 US$/g. As *T. album* produces other unwanted proteases, recombinant production is performed in *E. coli*. In that host, the overexpressed proteinase K aggregates in inclusion bodies, which can be isolated after cell lysis by centrifugation. They are then solubilized and the proteinase is allowed to refold in a complex buffer system. This process is challenging because the folded protein contains two disulfide bridges and two bound calcium ions. Interestingly, the last step is autocatalytic processing from an inactive precursor into the active protease by release of the propeptide. Altogether, this strategy avoids toxic interference with the host microorganism.

Table 10.9 Selected enzymes produced by microorganisms for research and diagnostics.

Activity/enzymes	Estimated number[a)]	Exemplary methods	Field of application	Production source[b)]
Nucleic acid amplification				
DNA-dependent DNA polymerases e.g. *Taq DNA polymerase*	<20	PCR, isothermal amplification, sequencing	Medical diagnostics, biotechnology research, forensic science	*Ec*
RNA-dependent DNA polymerases e.g. AMV reverse transcriptase	<10	Reverse transcription	Medical diagnostics, biotechnology research	*Ec*
RNA polymerases e.g. T7 RNA polymerase	<10	*In vitro* transcription	Biotechnology research, medical diagnostics	*Ec*
Nucleic acid hydrolysis				
Restriction endonucleases e.g. *Eco*RI, *Hin*dIII	>200	DNA digestion for cloning	Biotechnology research, medical diagnostics, forensic science	*Ec*
DNases e.g. DNase I	<20	Removal of DNA during protein purification	Biotechnology research	bp; *Ec*; *Pp*
RNases e.g. RNase H	<10	Removal of RNA during DNA purification	Biotechnology research	*Ec*
Protein hydrolysis				
Proteases e.g. trypsin	<30	Peptide mapping by LC–MS/MS	Biotechnology research	*Ea*; *Bl*; *Pp*; bp
Metabolite modification				
Various enzyme classes e.g. glucose oxidase	>100	Metabolite assay	Medical diagnostics, food and beverage analysis, biotechnology research	Various
Reporter activity				
Various enzyme classes e.g. horseradish peroxidase	<20	ELISA	Medical diagnostics, biotechnology research	hr; bp; *Ec*

[a)] Estimated number of commercially relevant enzymes in the respective group.
[b)] Selected production strains or other sources are stated. *Ec, Escherichia coli*; bp, bovine pancreas; *Pp, Pichia pastoris*; *Ea, Engyodontium album* (formerly *Tritirachium album*); *Bl, Bacillus licheniformis*; pp, porcine pancreas; hr, horseradish.

Restriction endonucleases catalyze the cleavage of specific DNA sequences. They are used for mapping and cloning of DNA. Their names begin with a three-letter acronym in which the first letter corresponds to the first letter of the genus from which the enzyme was isolated and the next two letters correspond to the first two letters of the species name. Extra letters or numbers could be added to indicate individual strains or serotypes. Thus, the enzyme *Hin*dII was one of four enzymes isolated from *Haemophilus influenzae*.

In addition, each major type of enzyme can contain subtypes. This especially applies to the type II enzymes, of which more than 3500 have been characterized. Type II endonucleases are divided into seven subtypes. Type II enzymes usually form homodimers (or sometimes multimers) and they recognize palindromic sequences four to eight nucleotides in length. An example is *Eco*RI, an enzyme that recognizes a sequence of six nucleotides. Both strands are cleaved after the first base: 5′G↓AATTC and 3′CTTAA↓G. Experimental evidence suggests that the dimerized enzyme binds unspecifically to the DNA double strand and, after linear diffusion along the strand, couples hydrolysis of the DNA backbone with a stronger interaction at the recognition site.

Today, more than 200 different enzymes are on the market. A particularly important consideration for production and downstream processing is the minimization of unwanted DNases and the so-called "star activity" (alternative DNA cleavage). This has been achieved with optimized buffers.

***Taq* DNA polymerase**, produced by *T. aquaticus* or recombinantly by *E. coli*, has sales of about 500 million US$/yr. A significant number of diagnostic tests, e.g. for infectious diseases and oncology, is based on polymerase chain reaction (PCR) technology using DNA polymerases. The advantage of *Taq* DNA polymerase is its activity at 72 °C, which results in fast DNA amplification and its stability at 95 °C, a temperature needed for each cycle in PCR to melt DNA double strands before annealing of the primers to start the next amplification cycle.

A disadvantage of this enzyme is the lack of proofreading activity, which is a feature of some other DNA polymerases. One such example is found in the hyperthermophilic archaeon *Pyrococcus furiosus*. *Pfu* DNA polymerase has superior thermostability and immediately removes an incorrect nucleotide incorporated at the 3′ end of the growing DNA strand. Consequently, PCR products amplified with this enzyme have much fewer errors compared to the *Taq* DNA polymerase. Typical results are an error rate of 1 in 1.3 million base pairs. However, *Pfu* DNA polymerase is slower than *Taq* DNA polymerase.

In vitro DNA polymerase amplification yields decrease with increasing amplicon size because the enzyme can come off the template DNA strand before it is fully copied. To address this issue, some DNA polymerases have been fused, via a short linker, with a thermostable DNA-binding protein, such as that from *Sulfolobus solfataricus* or a DNA-binding domain of the *P. furiosus* ligase (Figure 10.13).

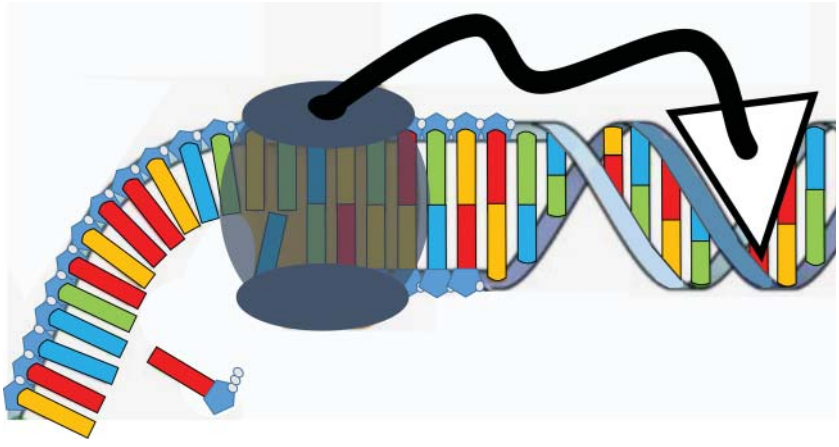


Figure 10.13 DNA polymerase with fused DNA binding protein. Schematic picture of a *Pyrococcus*-like proofreading polymerase (gray barrel) fused via a linker (black snake) with a double-stranded DNA-binding domain (white triangle). Such an artificial polymerase shows improved properties in polymerase chain reaction (PCR).

10.8.2 Specific Enzymes for Quantitative Metabolite Assays

In more than 30 test kits produced by different companies for the analysis of food or other complex substances, specific enzymes are used to quantify acids (e.g. citric acid), sugars (e.g. lactose), or other compounds (e.g. ammonia).

In many cases, reactions of one or more enzymes are coupled to a dehydrogenase reaction that specifically produces or consumes NAD(P)H so that its quantity can be easily detected in a photometer at 340 nm.

To determine citric acid, three enzymes are applied. In the first reaction, citric acid is converted by citrate lyase to oxaloacetate and acetate. In the presence of the enzymes L-malate dehydrogenase and L-lactate dehydrogenase, oxaloacetate and its decarboxylation product pyruvate are reduced to L-malate and L-lactate, respectively. The amount of NADH oxidized is stoichiometrically proportional to the amount of citrate.

Monitoring glucose in blood can prolong life expectancy by enabling diabetics to manage episodes of hypo- or hyperglycemia. Blood glucose concentrations are in the range of 5 mM for healthy patients, increasing up to 40 mM in diabetics. At the Children's Hospital in Cincinnati in 1962, the first generation of glucose biosensors was developed. These sensors were based on an electrochemical approach, using the enzyme glucose oxidase. **Glucose oxidase** catalyzes the oxidation of glucose to gluconolactone in the presence of oxygen, while producing hydrogen peroxide and water as by-products. Glucose oxidase requires a cofactor flavin adenine dinucleotide (FAD) for this oxidation process. FAD becomes reduced to $FADH_2$ during the redox reaction. Subsequent reaction with oxygen to produce H_2O_2 regenerates the FAD cofactor. This reaction occurs at the anode, where the number of transferred electrons can be correlated with the amount of H_2O_2 produced and hence the concentration of glucose. In second-generation sensors, oxygen was replaced with a synthetic electron

redox mediator. In third-generation biosensors, the manufacturer claims that the electron is transferred directly from the enzyme to the electrode. Glucose oxidase from *A. niger* is the only enzyme used. Most important are its stability and the simultaneous selectivity for D-glucose. Although its crystal structure has been solved, the mechanism of electron transfer is not fully understood.

10.9 Perspectives

10.9.1 L-DOPA by Tyrosine Phenol Lyase

L-DOPA (3,4-dihydroxyphenyl-L-alanine) is in the first line of treatment for Parkinson's disease, which is caused by deficiency of the neurotransmitter dopamine. With the aging of population, the demand for L-DOPA is increasing. Its current world market volume is about 100 billion US$/yr. Extraction from plants and chemocatalysis are the main sources for L-DOPA. Various whole-cell biocatalysis with *Erwinia herbicola* or enzymatic routes using tyrosinase, tyrosine phenol lyase (TPL), or *p*-hydroxyphenylacetate 3-hydroxylase as biocatalysts have been developed. TPL-mediated biocatalysis might show the greatest potential because it catalyzes the synthesis of L-DOPA from common chemicals in one step. There is a need for a robust catalyst that can form a C—C bond between pyruvate and pyrocatechol and converts the keto acid to the amino acid at concentrations above 100 g/l. A major challenge is to immobilize TPL on a support for increased half-life, while minimizing a diffusion barrier so that a high conversion rate can be maintained. Because solubility of L-DOPA in water is low, it can precipitate out during the process.

10.9.2 Activation of Alkanes

The C–H activation of alkanes is a challenge in metal–organic chemistry and it has also been investigated for enzymatic catalysis. Enzymes that can carry out such a biocatalytic oxidation are P450-monooxygenases. In an ideal case, they would use air as an oxygen source. The hydroxylation of cyclohexane is an interesting objective. The next step of interest is cyclohexanone formation. Cyclohexanone itself is further transformed by the biocatalytic Baeyer-Villiger oxidation into a lactone, a precursor for polymers such as Nylon-6. Several basidiomycetes, e.g. *Agrocybe aegerita*, produce extracellular heme-dependent enzymes. However, the main challenge for a recombinant expression platform is the supply of a coenzyme at sufficient titers.

10.9.3 Enzyme Cascades

Atorvastatin, a cholesterol-lowering drug that had global sales of more than 10 billion US$ in 2010, can be produced by a sequence of chemical and enzymatic steps. A cascade of three enzymes, a ketone reductase, a glucose dehydrogenase, and a halohydrin dehalogenase, is needed to reach regioselectivity. Halohydrin dehalogenases are of interest because of their epoxide ring-opening activity enabling the formation of C—C, C—N, or C—O and C—S bonds. Out of 50

possible candidates, one promising enzyme is a halohydrin dehalogenase that derives from *Agrobacterium radiobacter*. Protein engineering might lead to variants with decreased K_M of about 2 mM and increased k_{cat}/K_M of about 400/(s mM) using a complex substrate where a specific chlorine has to be substituted with a cyano group.

References

Davids, T., Schmidt, M., Böttcher, D., and Bornscheuer, U.T. (2013). Strategies for the discovery and engineering of enzymes for biocatalysis. *Current Opinion in Chemical Biology.* 17(2): 215–220.

Lorenz, P., Liebeton, K., Niehaus, F., and Eck, J. (2002). Screening for novel enzymes for biocatalytic processes: accessing the metagenome as a resource of novel functional sequence space. Current opinion in biotechnology, 13(6), 572–577.

Vojcic, L., Pitzler, C., Koerfer, G. et al. (2015). Advances in protease engineering for laundry detergents. *New Biotechnol.* 32 (6): 629–634.

Further Reading

Adrio, J.L. and Demain, A.L. (2014). Microbial enzymes: tools for biotechnological processes. *Biomolecules* 4 (1): 117–139.

Bornscheuer, U.T., Huisman, G.W., Kazlauskas, R.J. et al. (2012). Engineering the third wave of biocatalysis. *Nature* 485 (7397): 185.

Lei, X.G., Weaver, J.D., Mullaney, E. et al. (2013). Phytase, a new life for an "old" enzyme. *Annu. Rev. Anim. Biosci.* 1 (1): 283–309.

Payne, C.M., Knott, B.C., Mayes, H.B. et al. (2015). Fungal cellulases. *Chem. Rev.* 115 (3): 1308–1448.

Sarrouh, B., Santos, T.M., Miyoshi, A. et al. (2012). Up-to-date insight on industrial enzymes applications and global market. *J. Bioprocess. Biotech.* 4: 002.

Van der Maarel, M.J.E.C., van der Veen, B., Uitdehaag, J.C.M. et al. (2002). *J. Biotechnol.* 94: 137–155.

Wenda, S., Illner, S., Mell, A., and Kragl, U. (2011). Industrial biotechnology—the future of green chemistry? *Green Chem.* 13 (11): 3007–3047.

Wilson, D.B. (2008). Three microbial strategies for plant cell wall degradation. *Ann. N.Y. Acad. Sci.* 1125 (1): 289–297.

Zhang, P.Y. and Lynd, L.R. (2004). Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. *Biotechnol. Bioeng.* 88 (7): 797–824.

11

Microbial Polysaccharides

Volker Sieber[1], *Jochen Schmid*[2], *and Gerd Hublik*[3]

[1] *Technical University of Munich – Campus Straubing for Biotechnology and Sustainability, Chair of Chemistry of Biogenic Resources, Schulgasse 16, 94315 Straubing, Germany*
[2] *Norwegian University of Science and Technology, Department of Biotechnology and Food Science, Sem Sælands vei 6-8, 7491 Trondheim, Norway*
[3] *Jungbunzlauer Austria AG, Plant Pernhofen, 2064 Wulzeshofen, Austria*

CHAPTER MENU

11.1 Introduction

Microbial polysaccharides belong to the class of biopolymers, which consist as carbohydrate macromolecules synthesized by microbes. Polysaccharides make up one of the three major classes of biopolymers, next to polypeptides (proteins) and polynucleotides (deoxyribonucleic acids), and among these are industrially the most important based on the tonnage of their production. Microbial polysaccharides can have different biological functions. They can be used for intracellular storage of energy and carbon source (e.g. glycogen), they can provide structure and stability to the cells (e.g. K30 O-Antigen, a capsular polysaccharide on the cell surface), and they can be secreted by microbial organisms into the environment. Accordingly, the latter are termed "extracellular bacterial polysaccharides" (EPS). The function of these EPS typically lies in the formation of a matrix surrounding the microorganisms for providing adherence to surfaces, protection against xenobiotic molecules, and water retention, and EPS are the main component microbial biofilms (Figure 11.1). Plant or animal pathogens very often produce high amounts of EPS, as their function supports attack of the host.

Of the three different types of microbial polysaccharides, EPS are industrially by far most important. They have unique biophysical properties and, being secreted, can be produced and purified most easily.

Industrial Microbiology, First Edition.
Edited by David B. Wilson, Hermann Sahm, Klaus-Peter Stahmann, and Mattheos Koffas.

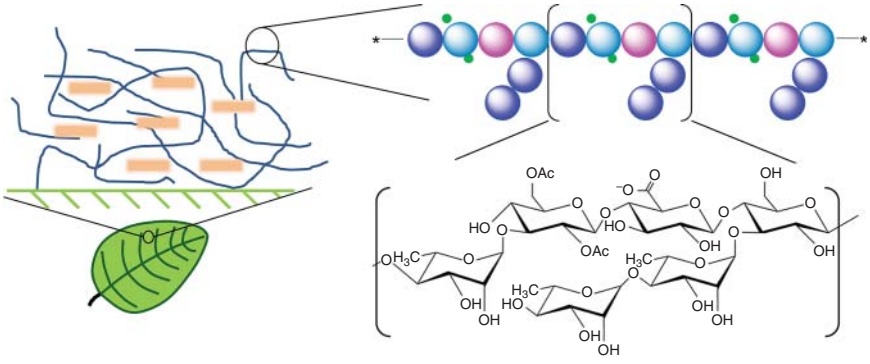


Figure 11.1 Schematic drawing of a biofilm on a tree leaf. The first magnification shows the bacteria embedded in extracellular exopolysaccharides, deoxyribonucleic acids, and proteins. The second magnification displays the structure of diutan, a heteropolysaccharide with a complex subunit including substituents, as produced by *Sphingomonas* sp. In the last zoom, the chemical structure of the diutan polysaccharide is displayed (not drawn to scale).

Polysaccharides are built of carbohydrate units that are linked to each other by glycosidic bonds. Unlike proteins and polynucleotides, whose biosynthesis is template based and as such leads to identical copies of polymer molecules, the biosynthesis of polysaccharides in general and of EPS in particular is achieved by a complex interplay of enzymes. Their structure is determined by factors such as specific substrate recognition of these enzymes, precursor availability, general media compositions or process parameters, and finally by chance leading to molecules with slightly varying subunits and polymer lengths. Microbial polysaccharides can generally be divided into homopolysaccharides and heteropolysaccharides. The former are built up entirely by glucose monomers, such as dextran and glucan. The heteropolysaccharides on the other hand consist of more complex subunits made of commonly four to eight monosaccharides (see for example, Figure 11.1). These monosaccharides can be different types of carbohydrates. Neutral aldoses such as glucose, mannose, and galactose; sugar acids such as glucuronic acid, and deoxy sugars such as rhamnose or fucose are typical constituents of microbial polysaccharides. In addition, monomers can carry substituents. Especially, carboxylic acids such as acetic acid or pyruvic acid are linked via ester or ketal bonds to the free hydroxyl groups. Homo- and heteropolysaccharides can be either linear or branched. Hyperbranching is less common for the extracellular polysaccharides because of their highly specific biosynthesis. The single polymer strands can form superstructures, such as double or triple helices, which mainly determine their rheological behavior.

Industrially important exopolysaccharides come from bacteria and fungi. Nature offers a plethora of different variants. Of the bacteria alone, more than 6000 different exopolysaccharide producing species have been described. The number of species and polysaccharide variants used in industry is much smaller. Table 11.1 gives an overview of the major products and their applications.

Table 11.1 Overview of major commercial microbial polysaccharides and their fields of application.

Polysaccharide	Microorganism	Main components	Industries	Application	Market Price estimation
	Bacteria				
Xanthan	*Xanthomonas campestris*	Glc, Man, GlcA	Food, oil, health care, personal care	Thickener, stabilizer in dressings, sauces or tooth paste, oil drilling	165 000 mt 3.5–10 US$/kg
Gellan	*Sphingomonas elodea* ATCC 31461	Glc, Rha, GlcA	Food	Gelling agent, thickener	4000 mt 25–50 US$/kg
Dextran	*Leuconostoc mesenteroides*	Glc	Pharma	Blood plasma extender	<500 mt
Welan	*Sphingomonas* sp. ATCC 31555	Glc, Man, Rha, GlcA	Construction, oil	Oil drilling, concrete viscosifier	300 mt 12–25 US$/kg
Diutan	*Sphingomonas* sp. ATCC 53159	Glc, Rha, GlcA	Construction, oil	Thickener, oil drilling, flow control of concrete	200 mt 12–25 US$/kg
Curdlan	*Agrobacterium* sp. ATCC 31749	Glc	Food	Gelling agent	N.A.
Succinoglycan	*Sinorhizobium meliloti*	Glc, Gal	Oil	Thickener of water-based drilling muds	N.A.
Hyaluronic acid	*Streptomyces pyogenes*	GlcA, GlcNAc	Pharma, personal care	Cosmetics, plastic, and eye surgery	1 bn US$
	Fungi	Glc	Oil, personal care	Enhanced oil recovery, cosmetics	1500 mt 12–20 US$/kg
Scleroglucan	*Sclerotium rolfsii* *Sclerotium glucanicum*				
Schizophyllan	*Schizophyllum commune*	Glc	Oil, personal care	Enhanced oil recovery, cosmetics	N.A.
Pullulan	*Aureobasidium pullulans*	Glc	Pharma, personal care	Pharma, personal care	N.A.

Gal = Galactose; Glc = Glucose; GlcA = Glucuronic acid; GlcNAc = Glucosamine; Man = Mannose; Rha = Rhamnose, mt = metric ton; N.A. = not available.

11.2 Heteropolysaccharides

11.2.1 Xanthan: A Product of the Bacterium *Xanthomonas campestris*

11.2.1.1 Introduction

Xanthan gum is by far the most important and most studied microbial polysaccharide. Many of the details of polysaccharide synthesis, production, etc., that are described for xanthan in this chapter can be extended to other microbial polysaccharides in general.

Xanthan was discovered in the late 1950s in the research laboratories of the US Department of Agriculture by Allene Jeanes and coworkers during a systematic program of bacterial screening for microbial biopolymers. The bacterium *Xanthomonas campestris* (strain Northern Regional Research Lab (NRRL) B-1459), a plant pathogen, was finally selected. It produces a high molecular mass exopolysaccharide, which protects the bacterium from detrimental environmental influences. This polymer, then named xanthan, proved to have technically and economically intriguing properties. The industrial importance of xanthan gum is based on its exceptional qualities as a rheology control agent in aqueous systems and as a stabilizer for emulsions and suspensions. Its numerous areas of application cover a broad spectrum and range from the food industry to oil recovery. Since then, many improvements in polysaccharide production and recovery have been made. Today, xanthan gum is the commercially most important microbial polysaccharide. Worldwide consumption of xanthan in 2017 was estimated at 165 000 mt, produced by companies in Europe (Jungbunzlauer), China (Deosen, Fufeng, Jianlong, and Meihua), and the United States (CPKelco, Archer Daniels Midland (ADM), DuPont, and Cargill). Food and personal care utilization accounts for about 35–40% of xanthan usage, 50–55% are used for oilfield applications (strongly depending on the oil price), and the remainder is used for other technical purposes.

11.2.1.2 Regulatory Status

Detailed investigations with respect to toxicology and safety have shown that xanthan is a safe food additive. It was cleared by the US Food and Drug Administration in 1969, permitting the use of xanthan in food products without any specific quantity limitation. In 1980, the European Commission approved xanthan under the number E415. In 1986, the acceptable daily intake rate of xanthan was changed by the joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) expert committee to "not specified" (no quantitative limit) confirming its status as a safe food additive.

11.2.1.3 Structure

Xanthan is an anionic branched heteropolysaccharide composed of sugar molecules D-glucose, D-mannose, and D-glucuronic acid in a molar ratio of 2 : 2 : 1 (Figure 11.2). The backbone of the molecule is a β-(1,4)-D-glucan and such is identical to cellulose. A trimer of mannose–glucuronic acid–mannose is branching off the C3-hydroxyl group of every second glucose. The inner mannose

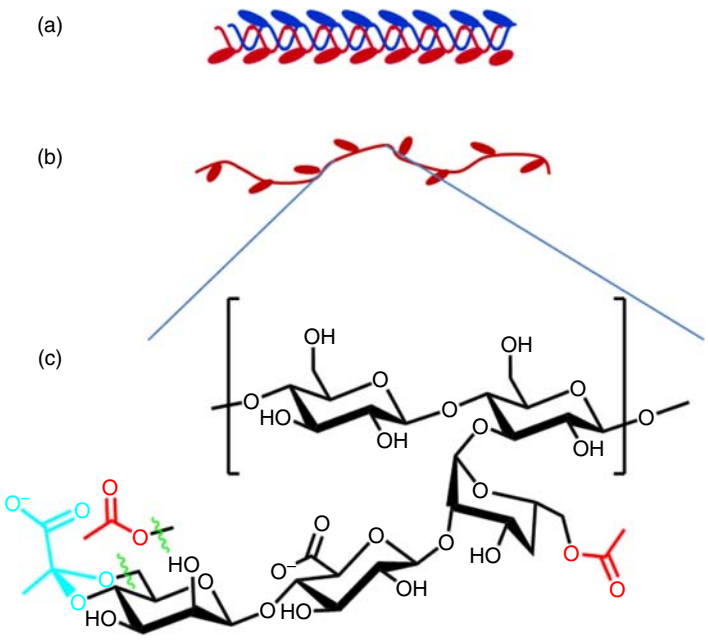


Figure 11.2 Model of secondary structure of the xanthan molecule as double helix (a) and single strand (b). The zoom displays the primary (chemical) structure of a repeating unit of xanthan, including the β-(1,4)-linked glucose backbone, the side chain with the internal mannose carrying an acetyl group (red), the following glucuronic acid, and the terminal mannose decorated either with acetyl (red) or pyruvyl group (blue) (c). Not drawn to scale.

residue is partially esterified with acetate at the C6-atom. The outer mannose can also carry such an acetate group or, alternatively, a pyruvate, which is bound via a ketal bond to the C4- and C6-hydroxyl group. As such, the pyruvate residue provides an additional negative charge in contrast to the acetate, which is bound via an ester bond. Not every subunit carries a substituent. Instead, commercial untreated xanthan typically contains only between 0.6 and 0.75 mol of acetate and 0.35–0.4 mol of pyruvate per subunit with strong variations depending on the growth conditions.

Many scientific authors support the model that native xanthan exists as a double helix, stabilized by hydrogen bonds plus ionic bridges via bivalent cations. Changes in temperature and/or ionic strength induce a reversible conformational transition of the secondary structure from the ordered (helix) to a disordered form (random coil), resulting in the separation of the xanthan strands. The downstream processing of xanthan gum therefore strongly influences the conformational status of the molecule and this in turn influences the apparent molecular mass. Moreover, the published results of molecular mass measurements of xanthan strongly vary with the applied method. Unfortunately, there are no validated molecular mass standards available on the market; therefore, published results should be considered with caution. Low-angle or multiangle laser light scattering is the most convenient method for measuring the molecular mass of

polysaccharides. The published molecular mass of xanthan ranges between 2 and 9 million Da.

11.2.1.4 Biosynthesis

Xanthan biosynthesis requires a high number of enzymes that are either directly or indirectly involved in the assembly of the polymer strands. To build up biomass and produce energy glucose is mainly metabolized via the Entner–Doudoroff pathway and the citric acid cycle. The synthesis of the polymer by linking the sugar molecules together is based on the utilization of high-energy precursors in the form of sugar nucleotides (Uridine diphosphate (UDP-glucose), UDP-glucuronic acid, and GDP-mannose). Their formation is straightforward from glucose, requiring only a few steps (Figure 11.3). Glucose is first phosphorylated at position C6 to form glucose-6-phosphate (G6P), which is converted to glucose-1-phosphate (G1P). This is coupled to Uridine triphosphate (UTP) by releasing pyrophosphate, which is cleaved immediately, shifting the equilibrium toward formation of UDP-glucose. Formation of GDP-mannose also starts from G6P, which is isomerized to mannose-6-phosphate (M6P) in two steps via fructose-6-phosphate. Formation of GDP-mannose from M6P is analogous to the UDP-glucose formation from G6P, with an isomerization to form mannose-6-phosphate (M1P) first, only that guanosine triphosphate (GTP) instead of UTP is involved. UDP-glucuronic acid is obtained from UDP-glucose by action of a single enzyme via the direct oxidation at C6. For the synthesis of each precursor, two ATP equivalents are necessary, so that 10 molecules of ATP are required for the pentasaccharide subunit. In the cell metabolism, one molecule of glucose can be converted to one molecule of pyruvate and one molecule of acetate (or its precursor acetyl-CoA). Under aerobic conditions, enough NADH to obtain the 10 ATP molecules that are required is available. When assuming each subunit carrying one pyruvyl and one acetyl group, the pentasaccharide subunit can such be obtained from exactly six molecules of glucose without much loss or gain of energy or other metabolites. Hence, the bacteria can produce xanthan without burdening their cellular metabolism just as long as sufficient supply of glucose and oxygen is provided.

The assembly of the xanthan subunits and their polymerization is shown in Figure 11.3. The biosynthesis of many other heteropolysaccharides follows the same general principle (the so-called Wzx/Wzy-pathway). First, the pentasaccharide subunit is assembled at the cytosolic side of the inner membrane. A hydrophobic isoprenoid (dolichol-pyrophosphate) acts as an anchor to the membrane. Stepwise, each sugar unit is added to the anchor starting with the glucose backbone followed by the three branching sugars and finally the acetyl or pyruvyl residues. Each single reaction requires its own enzyme (glycosyltransferases, acetyltransferases, and ketalase). Note that there are two acetyl transferases because acetate can be linked to the inner as well as outer mannosyl residue. Once the subunit is complete, a so-called flippase (Wzx-protein) moves it into the periplasm. There, the subunits are polymerized (via the Wzy-polymerase) and the growing polymer chain is secreted. The exact mechanism of the polymerization and the role of the putative three enzymes involved in this process are still not fully understood. Also, the trigger for

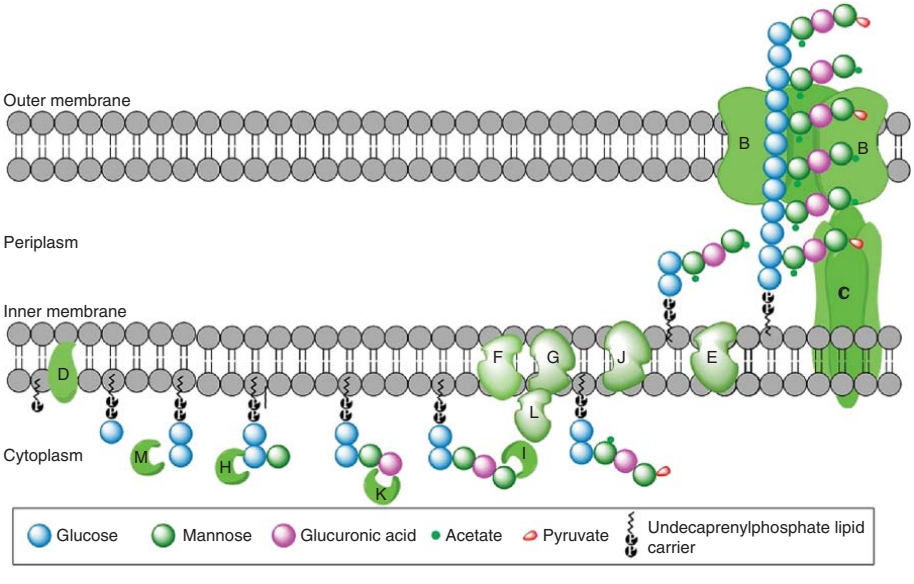


Figure 11.3 Subcellular localization of xanthan synthesis enzymes (green) in *Xanthomonas campestris*. The initiating glycosyltransferase GumD transfers a glycosyl-P residue from UDP-glucose to a lipid carrier. Glycosyltransferases GumM, GumH, GumK, and GumI add a second glycosyl residue, two mannosyl residues, and a glucuronyl residue to form the carbohydrate structure of a repeat unit. The terminal mannosyl residue can be decorated with a pyruvyl group by GumL and both mannosyl moieties can be acetylated by GumF and GumG, respectively. Repeat units are translocated by flippase GumJ to the outer face of the inner membrane and are polymerized by polymerase GumE. The polymer is exported by GumC. When GumC contacts GumB, complexes of both proteins can form pores, which permit export of the xanthan strands.

secretion and the termination of chain elongation are unknown. Altogether, 12 enzymes are necessary for the assembly and polymerization. The genes coding for these enzymes are clustered together in the genome in the so-called gum-operon and are termed *gumB*, *gumC* up to *gumM*.

11.2.1.5 Industrial Production of Xanthan

Xanthan gum is produced by fermentation based on different carbohydrates as carbon source. In Europe and the United States, mostly, glucose syrup typically made from corn starch is used, while in China, starch is supplied directly. *X. campestris* is able to grow on starch as it produces its own amylase. Addition of sucrose to the medium enhances productivity. As a nitrogen source, either organic constituents such as corn steep liquor are used or, alternatively, inorganic ammonia. The latter will lead to clearer products. Phosphate, sulfate, and magnesium salts are also supplemented. The submerged fermentation is operated in a sterile aerobic environment, monitoring the temperature and the pH by adding alkaline solution. Depending on the production strain, the temperature is controlled between 28 and 32 °C and the pH between 6.5 and 7.5. The final concentration of xanthan strongly depends on the oxygen supply. In general, the higher the oxygen transfer rate, the better the productivity and final titer. Industrial batch fermentations achieve productivities between 0.4 and 0.7 $g\,l^{-1}\,h^{-1}$ and concentrations of 30–50 g/l xanthan gum.

The major challenge during the fermentation is the high viscosity and limited mass transfer caused by xanthan at concentrations above 2%. Only by high power steering, the viscosity remains manageable; however, as soon as stirring is stopped, the fermentation broth becomes solid because of the rheological properties of xanthan gum. This stirring is very energy demanding and such efficient cooling is essential.

Different fermenter designs and fermentation concepts have been evaluated over the last decades to cope with this challenge. One suggestion was to use emulsion fermentation in which the cells are kept within small droplets and secreted xanthan remains in these droplets. Using such a method, the viscosity can be reduced substantially and accordingly the cost of agitation and problems with aeration are diminished. Halogenated aliphatic compounds (perfluoro-1-nonene), hexadecane, and plant oil have been tested as organic phase. The emulsions were indeed beneficial for the oxygen transfer and very high titers (up to 65 g/l) could be achieved. However, recovery became a major problem and increased titers as well as energy input could not balance out the higher purification costs. Other attempts to increase productivity and titers include the immobilization of cells on solid substrates such as synthetic polymers or inorganic surfaces. Again, problems with xanthan recovery make this approach inferior to simple submersed fermentation. Different reactor concepts have also been analyzed to alleviate the problems caused by the high viscosity. Bubble column reactors, airlift reactors, loop reactors, foam reactors, and jet reactors partially showed better productivity or yield or reduced energy consumption than the conventional stirred tank reactor. Nevertheless, none was sufficiently superior so that stirred tank reactors are still the equipment of choice. Fed-batch processes that are typical for the

production of amino acids and other industrially important molecules are not so relevant for xanthan production. With a final titer of 50 g/l of xanthan, the major part of substrate can be provided at the very beginning of the process without causing osmotic problems and thus minimizing the supplementation steps.

After glucose exhaustion, the culture medium is heat-sterilized to kill the Xanthomonas cells. Moreover, the heating step is necessary to inactivate enzymes produced by the bacterium to degrade plant polysaccharides, such as endoglucanases, mannanases, amylases, and pectinases. In many applications, xanthan is used together with other hydrocolloids, such as cellulose gum, guar or locust bean gum, or succinoglycan. Residual enzyme activities would therefore degrade other polysaccharides and negatively influence the rheological properties of the product.

Scientific papers often report that it is common to remove the cells either by using filtration or centrifugation (after massive dilution). Both treatments would lack economic sense; rather 80–90% of xanthan gum is simply precipitated with isopropanol or ethanol after cooling the culture broth, including a considerable amount of biomass (5–7%). If a highly transparent xanthan gum is required, proteases are added to the culture broth before the pasteurization step in order to lyse the cells and to degrade proteins. Cell debris is not enclosed by the precipitated xanthan fibers but washed out by the alcohol. Treatment with sodium hydroxide leads to partial deacetylation, which improves the rheological properties for some applications (see Section 11.2.1.6). Alternatively, for the production of deacetylated xanthan, genetically modified strains could be used that lack the enzymes for acetyl transfer (see Section 11.2.1.4). However, as xanthan is heavily used in the food industry, such Genetically modified organism (GMO) are not well accepted.

Following precipitation, the xanthan fibers are separated by filtration or centrifugation, dried, milled, and packed (Figure 11.4). Used ethanol or isopropanol, which is collected from the solid–liquid separation step and from the drying process, is distilled in a rectification column and reused. Xanthan production is an energy-intensive process, especially power for agitation, cooling, and rectification contribute to the production costs.

11.2.1.6 Physicochemical Properties

Xanthan is a tasteless, white to cream-colored free flowing powder soluble in hot and cold water, but insoluble in most organic solvents. Its industrial importance is based on its ability to control the rheology of water-based systems. The unique combination of valuable properties of xanthan compared to other hydrocolloids is summarized as follows:

- High viscosities at low concentrations
- Highly pseudoplastic over a broad shear range
- Good thermal stability
- Good stability over a broad pH spectrum
- Resistant to degradation by most enzymes
- Synergistic behavior with galactomannans.

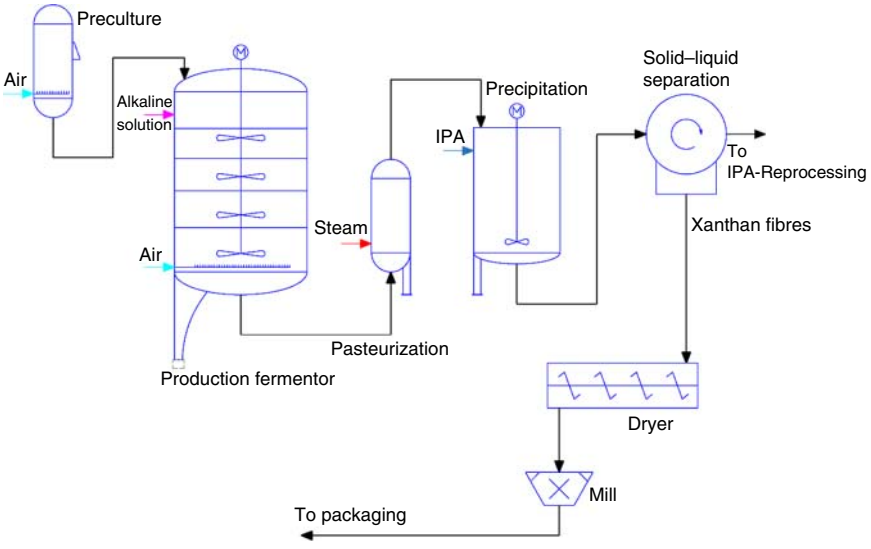


Figure 11.4 Flow chart of the xanthan production process. From left to right, the cultivation of the preculture and the main fermentation step on the bioreactor are depicted. Typical volumes are 6 m^3 for the preculture and 150 m^3 for the main bioreactor. In the next step, the pasteurization is applied by harvesting the fermentation broth and heating it to around 85 °C for around 20 minutes by use of steam. Following this, the polymer is precipitated by use of isopropanol alcohol or ethanol and the fibrous precipitate is separated, dried, and milled for packaging. IPA = isopropanol.

Xanthan is highly pseudoplastic, more than most other common hydrocolloids, and therefore, the viscosity of its solutions decreases with increasing shear rate (Figure 11.5).

It is only to a low-degree thixotropic, which means that the initial viscosity of xanthan recovers very fast after shear thinning. This is highly important for many applications of xanthan, where the immediate regain of high viscosity is required, once the shear stress has stopped.

The usage of hydrocolloids comprises applications with a broad pH spectrum. Generally, xanthan solutions maintain a uniform viscosity from pH 2 to 12 at ambient conditions. Higher temperatures (>40 °C) cause an accelerated decrease in viscosity of very acidic or alkaline xanthan solutions during longer storage. If solutions with extreme acidic pH values (1.5–2.5) and longer shelf lives are required, acetate-free xanthan gum rather than standard xanthan should be used as it is more acid stable.

Xanthan retains its viscosity until 70–80 °C depending on polymer and salt concentration in the solution. This property has practical utility for the use in food products, which are often pasteurized to prevent microbial growth or consumed while hot. Temperature stability of xanthan increases with increasing ionic strength caused by the shift of the melting point of the helix strand. Oxidants such as peroxides, persulfates, and hypochlorites cause rapid degradation of xanthan and depolymerization of the molecule is accelerated at higher

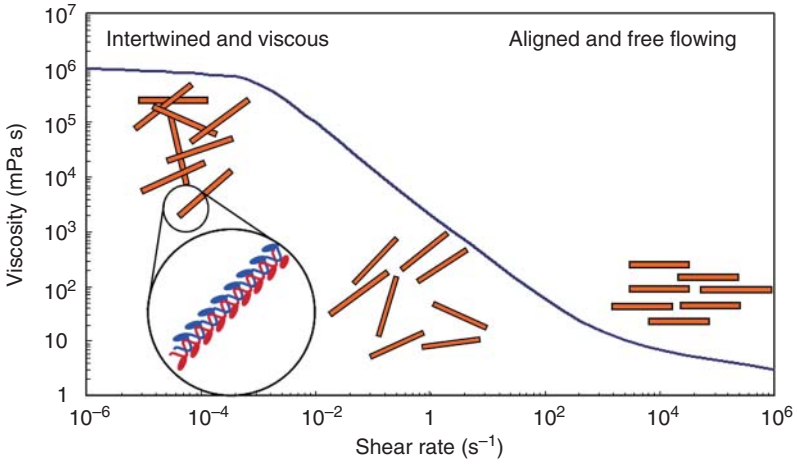


Figure 11.5 Viscoelastic behavior of xanthan. The double helices of xanthan gum (see also Figure 11.2) in aqueous solutions form relatively stiff rod-like structures, which are intertwined and cause a high viscosity under low shear stress. When higher shear rates are applied, the helices get ordered in parallel structures and can flow freely, thus reducing the viscosity. This behavior is reversible, and the viscosity suddenly reappears when shear stress is stopped. Therefore, xanthan gum is a shear-thinning polysaccharide.

temperatures. Reducing agents do not significantly influence the viscosity of xanthan in solution.

11.2.1.7 Applications

Food Applications A classic application is the use in pourable salad dressings where xanthan has proven to be an ideal stabilizer. Its rheological properties prevent oil separation and sedimentation of solid particles. The shear-thinning flow behavior (pseudoplasticity) contributes to mixability, pumpability, and pourability of industrially produced dressings. Finally, the tolerance of xanthan toward acids and salts guarantees a long shelf life for salad dressings or similar products.

In the bakery industry, xanthan is used to increase water binding during baking and storage and extends the shelf life of baked goods and refrigerated doughs. Gluten-free bread can be made with xanthan gum while still providing a fine-pored structure and elastic surface. Mixtures of xanthan and galactomannans are used in ice cream, for example, to realize a good mouthfeel with a pleasant consistency at low temperatures.

A rather new application is the use of xanthan as a thickener for people with swallowing difficulties. Many elderly people, patients with dysphagia or neurological problems, and patients after a stroke have problems with swallowing food and liquids. To reduce the risk of aspiration and choking, liquids need to be thickened to control the swallowing process.

Personal Care Applications Xanthan gum is an ideal stabilizer for various types of toothpastes. It provides uniformity and stability to the toothpaste, makes it easy

to be pumped or squeezed and gives a consistent and smooth string. Xanthan can be used as an emulsion stabilizer and water-binder in creams and lotions. It imparts high viscosity at low concentrations and enables pastes and creams to hold their shape. In shampoos, xanthan gum is used to adjust the viscosity and flow properties and suspends insoluble particles such as pigments or other active ingredients in medicated shampoos.

Pharmaceutical Applications Xanthan gum is an excellent stabilizer for pharmaceutical formulations. It uniformly suspends water-insoluble ingredients, e.g. barium sulfate in X-ray contrast media, and perfectly stabilizes all kinds of pharmaceutical emulsions. In lozenges, xanthan prolongs the contact time of the active ingredient and in tablets xanthan gum can be used to create a retarded drug release effect based on its viscosity and stability.

Industrial Applications The agrochemical industry uses xanthan as a stabilizer in concentrated suspensions of pesticides and fertilizers. It extends the contact time with the crops by reducing the drift of the active ingredients. A similar effect is exploited in domestic cleaning agents such as toilet purifiers, where the pseudoplasticity of xanthan increases the cling to vertical surfaces and extends contact time, thus enhancing the cleaning effect. Additionally, the high pH stability of xanthan enables its addition to acid and alkaline cleaners, especially in the form of deacetylated xanthan for acidic cleaners to prolong the shelf life. Xanthan gum is also useful for suspending abrasive components in metal polishes and to stabilize polish emulsions and is an excellent suspending aid for ceramic glazes and many other hard-to-suspend mixtures.

Additionally, xanthan is used in water-based printing inks to provide the proper viscosity during application, to control penetration, and to increase stability. In water-based paints, xanthan stabilizes the pigments and controls the flow properties of the paint. In textile and carpet printing pastes, xanthan gum is applied to control fine-line and color migration. Its temperature stability and pseudoplastic properties improve space printing and dyeing processes. Xanthan is also added to fire-retardant compositions that are applied in liquids containing fire-suppressing salts to suppress the spread of wildfires. It improves stability and adhesion of the composition and reduces its corrosivity.

The oil industry uses xanthan gum in oil well drilling fluids, well completion, and fracturing fluids and as a mobility control agent in flooding operations for enhanced oil recovery. In drilling operations, the drilling fluid is circulated downward through the drill string to cool and lubricate the string and the bit as well as suspend and transport the cuttings out of the borehole. The shear-thinning behavior of xanthan is very useful for water-based drilling fluids because low viscosities are advantageous at the high shearing drill bit to avoid additional drag. However, high viscosities at low shear rates are required when transporting the cuttings and to avoid sedimentation. The compatibility of xanthan with monovalent salts is especially useful in offshore drilling where seawater is used for drilling fluids.

Next to the main commercial polysaccharide xanthan, there exist several microbial polysaccharides with a different chemical structure and thus

properties differing from xanthan. In general, these polysaccharides can be produced like xanthan gum and can be applied in a similar manner and applications. The following section will present these alternatives by mainly focusing on the differences in chemical structure, properties, and applications.

11.2.2 Sphingans: Polysaccharides from *Sphingomonas* sp.

The term "sphingan" refers to a group of structurally related heteropolysaccharides, which are produced by different *Sphingomonas* sp., Gram-negative bacteria that get their name from glycosphingolipids they carry in the cell envelope. Four members of this group are of industrial importance: gellan, welan, sanxan, and diutan.

Gellan, isolated and characterized by Kang and Veeder in the late 1970s (US 4,326,053), is the commercially most important polysaccharide of the sphingan family. It is biotechnically produced by the strain *Sphingomonas* sp. ATCC 31461 with an estimated volume of 4000 tons in 2016 and is the only sphingan with food approval in the United States and the European Union. The other sphingans are not food approved, but diutan was regarded as safe in agricultural applications in the year 2016.

The **chemical structure** of gellan as presented in Figure 11.6a consists of a repeating tetrasaccharide unit, composed of two molecules of D-glucose, one D-glucuronic acid and one L-rhamnose, linked via β-(1,4) and β-(1,3) bonds. In native gellan, the β-(1,3)-linked glucose is esterified with acetate and L-glycerate in a ratio of 0.5 acetate and 1 glycerate per repeating unit. Diutan (Figure 11.6b) and welan (Figure 11.6c) have the same chemical structure in the backbone but differ by carrying additional side chains at the first glucose of the repeat unit as well as in the grade and type of decorations. Sanxan in contrast has a different pattern of the backbone and carries no additional branched sugars (Figure 11.6d).

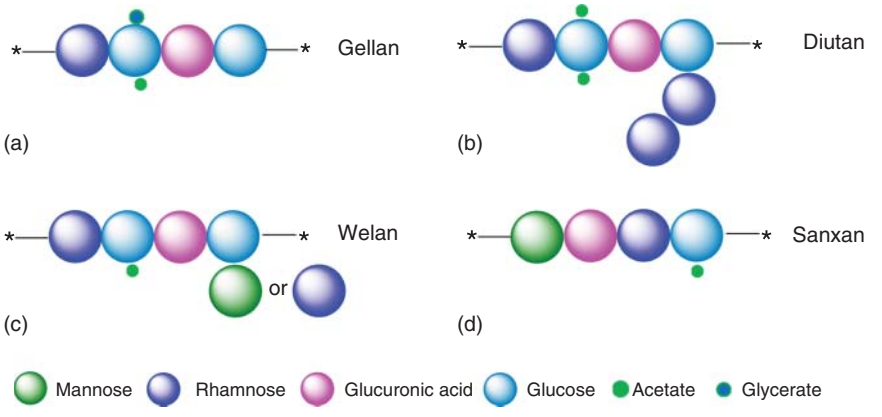


Figure 11.6 Schematic chemical structures of the four sphingans, (a) gellan, (b) welan, (c) diutan, and (d) sanxan, including the monomeric mannose, rhamnose, glucose, glucuronic acid, and the decorations by acetyl and glycerate groups. It is obvious that gellan, diutan, and welan have a very similar backbone, with only variations in the branches as well as substituents. Sanxan shows a different backbone without any side branches.

The **biosynthesis** of gellan and all sphingans follows the same schema as for xanthan, by assembly of the repeating unit, flipping the completed repeating unit toward the periplasm, where the polymer is finally polymerized and secreted into the environment. As for xanthan, the complete biosynthesis is clustered in nearly the same order in one operon in the case of gellan, welan, and diutan, and the genes for the rhamnose precursor production are also located on the sphingan operon. In contrast, the genes encoding the biosynthesis of sanxan are not in the same order and seem to result from an evolutionary rearrangement.

Similar to xanthan production, the carbon source for the fermentation medium usually consists of glucose syrup or liquefied starch and inorganic or organic nitrogen sources in combination with further macro- and micronutrients (K, Mg, and PO_4). The temperature is maintained between 28 and 32 °C and the pH can vary from pH 6 to 8. Most reported yields of gellan are in the range of 12–20 g/l after 30–50 hours, with a conversion efficiency of around 50% based on the carbohydrate content. After fermentation, the broth is heated to kill the cells and, if necessary, filtered to obtain a clear solution. After cooling down to around 60 °C, the polysaccharide is precipitated with isopropanol, separated, dried, and milled. Because gellan production gives a high-viscosity fermentation medium with strong gel character, filtration of the cells is in most cases not an economic option. Sanxan can be precipitated under acidic conditions (pH of 3), which is linked to its slightly different chemical structure. It can be postulated that a linear polysaccharide structure with a decoration far away from the glucuronyl residue might enable a staggered and therefore more crystalline form of the polymer, which can be easily precipitated in a positively charged environment. In contrast to sanxan, the glycerol group close to the glucuronic acid might shield the charge and therefore prevent the precipitation.

In the year 1993, there was patented a specific strain to obtain transparent gellan. The wild-type strain (ATCC 31461) produces large amounts of poly-hydroxy-butyrate (15–25% of dry biomass). This strain was mutated on the poly-hydroxy-butyrate biosynthesis and the poly-β-hydroxybutyrate (PHB)-free strain LPG-2 was selected. The cells of this strain can be lysed with lysozyme and proteins are subsequently degraded with proteinase. The cells are not enclosed in the precipitated fibers anymore and the cell components are washed out into the isopropanol/water fraction. The same procedure was recently patented for the diutan producing strain and also reported for sanxan.

The molecular mass of gellan is around 500 kDa and adopts a double-helical structure in aqueous solution, resulting in a strong gel character of the polymer solutions. Deacetylated gellan is obtained when pasteurizing the fermentation broth at pH 10. High acyl and low acyl gellan vary significantly in their gel characteristics. The high acyl form produces soft, elastic, nonbrittle gels, whereas the low acyl form produces firm, nonelastic, brittle gels. Contrary to gellan, the two related sphingans diutan (produced by *Sphingomonas* sp. ATCC 53159) and welan (produced by *Sphingomonas* sp. ATCC 31555) are more similar to xanthan in their viscoelastic properties most probably because of their additional side chains. Sanxan (produced by *Sphingomonas sanxanigenes*) has a strong network character and forms transparent gels.

In food applications, gellan is used as a gelling agent for desserts and confections, such as jellies, gummy candies, and marshmallows. Gellan can partly or completely substitute plant-based pectin, animal-based gelatin, and seaweed-based agar that are commonly used. Especially, the substitution of gelatin, the denaturated collagen originating from animals, mainly from bovine that can contain viruses or prions, is of highest interest for the food industry. Other typical food applications involve fruit fillings in bakery products and usage in dairy products such as ice cream, milkshakes, and sour cream. Low acyl, transparent gellan can replace low acyl pectin in low carb food. In 1988, Japan was the first country that approved the use of gellan for food applications. In the Asian kitchen, gellan is mainly used to adjust the texture of different food products. Later approval was obtained in other regions as well, and today, gellan, besides xanthan, is the only microbial polysaccharide to be used as a thickener that has been approved for food applications in the European Union (E418), the United States, Japan, and many other countries. In addition, it can replace agar-agar gelling agent in Petri dishes for culturing microorganisms and plant tissues. Because of its thermal stability, it is particularly useful when culturing thermophilic microorganisms. In personal care products, gellan is used as a structuring agent rather than a gelling agent, providing body, stabilizing oil-in-water emulsions, and pleasing skin feel in sunscreens, lotions, and hair conditioners. In toothpastes, gellan produces a reversible gel-like structure with excellent stand-up and flavor release. Other sphingans such as diutan and welan show a high tolerance of alkaline conditions; combined with excellent yield value makes them preferred additives for concrete in the construction industry and sanxan is used in oil drilling mainly in China.

11.2.3 Hyaluronic Acid: A High-Value Polysaccharide for Cosmetic Applications

The special property of this carbohydrate polymer is its extremely high water binding capacity. One gram of the polymer can bind several liters of water. Hyaluronic acid is a major component of human skin and therefore is a valuable ingredient in cosmetics products (Figure 11.7). The current world market for hyaluronic acid is estimated to be over $1 billion with a permanent increase because of its cosmetic and medical applications. For many years, hyaluronic

Figure 11.7 Effect of an O/W emulsion containing 0.1% hyaluronic acid (50 kDa) on wrinkles. (a) Before start of application; (b) after four weeks of application; and (c) after eight weeks of application. Source: Courtesy from Evonik Nutrition & Care GmbH.

acid has been produced from cock's comb but a nonanimal source was desired to realize a "green" and vegetarian source of higher selling power for this ingredient.

Microbiology brought about the solution as a number of bacteria also produce hyaluronic acid. Unfortunately, all of these are human pathogens (e.g. *Streptomyces pyogenes*), which neither facilitate easy production nor marketing of the product.

Hyaluronic acid can be considered as a minimal heteropolysaccharide because its subunit consists of only two sugar motifs, *N*-acetylmannosamine and glucuronic acid, regularly repeated along the linear chain. The high water binding capacity is due to the presence of a high density of electron pair donors (negatively charged carboxylates and amide bonds). Biosynthesis of hyaluronic acid is slightly different and less complex than that of xanthan or related polysaccharides, which allowed the design of recombinant harmless microbial strains that produce high amounts of this polymer. The biosynthesis comprises a hyaluronate synthase, which assembles UDP-glucuronic acid and UDP-N-acetylglucosamine under release of two molecules of UDP.

By applying high shear rates, the dynamic viscosity drops quickly and recovers very fast when reducing the shear rate. This behavior makes the polymer highly interesting for medical applications such as eye surgery. Hyaluronic acid is a relatively unstable polysaccharide, which quickly degrades under acidic or alkaline conditions, which makes the downstream processing more complex and expensive. In most cases, ultrafiltration is used to purify the polymer and concentrate it before its precipitation.

11.2.4 Alginate: Alternatives to Plant-Based Products by *Pseudomonas* and *Azotobacter* sp.

Alginate is a heteropolysaccharide, which consists of mannuronic and guluronic acid (C5 epimers) monomers, which are ordered in so-called G or M blocks or are randomly spread over the polymer. Bacterial alginate is mainly produced by *Azotobacter vinelandii* and some *Pseudomonas* strains. The biosynthesis follows the synthase-dependent pathway and starts with a pure mannuronic acid polymer (Figure 11.8a). Several epimerases are involved in the biosynthesis and specifically transform mannuronic acid into guluronic acid. Depending on the epimerase equipment of the strain, or the culture conditions (mainly oxygen content), highly ordered G:M ratios and distributions can be achieved (MG, GGM, MMG, etc.). Based on the G:M ratio and distribution, alginates show amorphous and crystalline regions. The most brittle gels can be realized by G-rich alginates in solutions with divalent ions such as Ca^{2+}. These divalent ions form a so-called egg-box structure (Figure 11.8b), which has a crystalline character. In contrast, M-rich alginates form smooth gels with a high number of amorphous regions.

11.2.5 Succinoglycan: Acidic Polysaccharide from *Rhizobium* sp.

Main production strains are *Sinorhizobium meliloti*, *Alcaligenes faecalis* var. *myxogenes*, and *Agrobacterium tumefaciens*. Succinoglycan consists of an

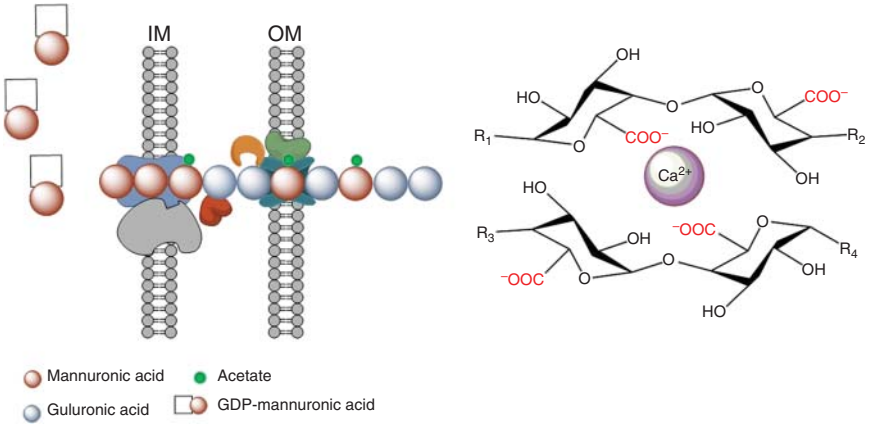


Figure 11.8 (a) Schematic presentation of the biosynthesis of alginate. The intracellular precursor GDP-mannuronic acid is assembled at the inner membrane (IM) via the alginate synthase toward polymannuronic acid. Within the transport to the outer environment passing the outer membrane (OM), the different epimerases realize the final ratio of M : G specific for the different strains or process conditions. (b) Egg-box structure of highly ordered G-blocks in alginate as coordinated by the negative charges of the carboxylic groups facing to the cation. R1–R4 represents the next M and G monomers of the alginate strands.

octasaccharide-repeating unit with four monomers in the backbone and four in the side branch, what represents one of the largest repeating unit described. The backbone consists of one galactose linked via a (1,3)-bond to the three (1,4)-linked glucose monomers. The β-(1,3)-linked trisaccharide side branch is linked via a (1,6)-bond to the backbone. Additional substituents are a terminal pyruvate ketal in the side branch and one acetyl group in the backbone. Additionally, the glucose in the neighborhood to the terminal glucose of the side chain carries a succinyl group as a rare substituent in microbial polysaccharides. Succinoglycan forms double-stranded helices as described for xanthan and gellan and shows a very high viscosity, which decreases remarkably, and nonreversible (only after a long time) at higher temperatures. The fermentation process is highly dependent on high oxygen levels and results in lower molecular mass succinoglycan when limited. The applications of succinoglycan are in oil drilling, stabilization of emulsions, and as viscosifier in acidic cleaners. Several current patents claim the utilization in food applications.

11.3 Homopolysaccharides

Besides heteropolysaccharides, some homopolysaccharides also find industrial applications. The only microbially produced homopolysaccharides of industrial importance are glucans, i.e. polymers that only contain glucose as a monomer. In spite of this uniformity, there are some differences caused by the possibility of different bonding with respect to the position on the hexose ring (C3, C4, or C6)

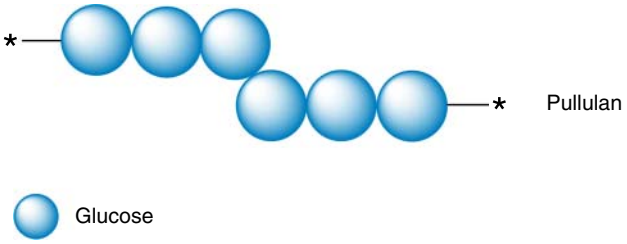


Figure 11.9 Schematic drawing of the chemical structure of pullulan. The monomers of the trisaccharide units are connected via α-(1,4)-bonds, and the kink is caused by the (1,6)-bond of the third glucose unit.

and the conformation of the anomeric carbon (α or β, Figure 11.9). This is the basis for the differentiation between α-glucan and β-glucan.

11.3.1 α-Glucans

11.3.1.1 Pullulan

Of the α-glucans only pullulan is a large-scale product by microbial fermentation. It is produced by *Aureobasidium pullulans*, a mold belonging to the Ascomycota. As its hyphae form chlamydospores, a yeast-like phenotype is described as a most promising morphology in submerged fermentation. Naturally, this strain forms a dark pigment, which is hard to be removed from the polysaccharide. Mutant strains, which lost the ability for pigment formation, are now used for industrial pullulan production. Biosynthesis of pullulan starts from Glc-1P, which is activated as UDP-glucose, forming isomaltosyl groups, which are extended toward isopanosyl groups and then assembled by a glycosyltransferase toward the final linear pullulan polymer. Enzymes involved in the biosynthesis are α-phosphoglucose mutase, UDPG-phosphorylase, and glycosyltransferase.

In pullulan, the glucose residues are linked via α-(1,4)-bonds with a (1,6)-bond after every third residue, leading to kinks in the linear chain (Figure 11.9). Application of pullulan is twofold. The major part of the production goes into the food industry because pullulan can form rather stable films that can act as an oxygen barrier or carrier for flavor compounds. The films are edible as pullulan is quickly dissolving in water and easily degraded by amylases. The second application is in many pharmaceutical applications because of the high amount of hydroxyl groups, which exerts inherent physiological properties and where it can be used as a drug carrier.

11.3.1.2 Dextran

Dextran consists of α-(1,6) and α-(1,3)-bonds, with many branches of α-(1,3)- and α-(1,4)-linkages and is produced mainly by *Leuconostoc mesenteroides* on sucrose. The biosynthesis is based on the extracellular enzyme dextransucrase (transglucosidase), which cleaves sucrose and catalyzes the transfers of nascent glucose onto the growing chain of α-glycosidic linked oligo- and polysaccharides. Dextran is used in pharmaceutical applications as antithrombotic and to reduce blood viscosity. The molecular mass of natural dextran can reach up to 5×10^8 Da,

which is not suitable for medical applications. Medical dextran has a molecular mass of around 75 kDa and the commercial dextran 70 is on the WHO Model List of Essential Medicines, the most important medication needed in health systems. These highly defined dextran fractions are obtained by hydrolysis in diluted acid and following precipitation of the targeted fraction. The world production of clinical dextrans is estimated to be >500 mt with a steadily growing volume. Additionally, many food applications are described for dextran because of its safe status. Further commercial applications are the different types of Sephadex, which is a trademark for cross-linked dextran gels, mainly applied in size exclusion chromatography. The main producers for commercial dextran are Tate & Lyle, DSM, Pharmacosmos, and Hebron.

11.3.2 β-Glucans

11.3.2.1 Linear β-glucans like cellulose and curdlan

Bacterial cellulose is a pure linear β-(1,4)-glucan of high crystallinity, nonwater solubility. Many microorganisms producing β-(1,4)-glucan such as *Gluconacetobacter* are forming a solid biofilm. Submersed fermentation is not possible, and production of microbial cellulose is not easy. Nevertheless, because of the highly promising properties of cellulose as material (e.g. acoustics) and especially in medical applications for wound healing and wound dressing, the industrial interest in bacterial cellulose is permanently increasing and some companies produce it on larger amounts.

Also, the linear β-(1,3)-glucans such as curdlan, as produced by various Agrobacterium strains are not water soluble but can be dissolved under alkaline conditions or in dimethyl sulfoxide (DSMO). They are of main interest for the food industry and provide the attractiveness of being precipitated with the cells by simple centrifugation and following alkaline treatment of the cell pellet. The polymer is then precipitated by acidification of the solution. This minimizes the organic solvents for product recovery extremely.

11.3.2.2 Branched β-Glucans Like Scleroglucan and Schizophyllan

Another interesting class of glucans are the β-(1,3)-glucans with single β-(1,6)-linked glucopyranosyl branches on every third glucose unit of the backbone, which are produced mainly by filamentous fungi, such as *Sclerotium rolfsii* or *Schizophyllum commune*. By the monomeric side chains, these polymers get water soluble compared to pure β-(1,3)-glucans such as curdlan or cellulose. Further, β-(1,3)-(1,6)-glucans with a similar structure are produced by *Botrytis cinerea* (cinerean) and the edible higher fungi *Lentinula edodes* (lentinan) also known as shiitake.

Based on the chemical structure, these homopolymeric glucans form triple helices, which are stabilized by intra – and intermolecular hydrogen bounds in aqueous solutions. The biosynthesis has not clearly been identified up to now but seems to be performed by a specific glucan synthase in combination with a glycosyltransferase for the attachment of the side branches. The production differs from xanthan by removal of the fungal biomass in the form of mycelia or pellets, including some washing steps to enhance the yield.

These polymers show a remarkable tolerance of their viscosity against extreme pH values (2–12) and the viscosity is stable until a temperature of 120–130 °C. Below 7 °C, these polymers form thermoreversible gels. Based on these superior characteristics compared to xanthan, scleroglucan and schizophyllan are heavily applied in enhanced oil recovery at high salinities and harsh reservoir conditions (80–120 °C) as present mainly in offshore applications. By use of these β-glucans, the yield of the wells can be enhanced by 10–15%. They are also applied as additives in drilling fluids by their good characteristics of hole cleaning, rate of penetration, and mud tolerance. Scleroglucan is commercially sold by Cargill in Europe and BASF started the large-scale production of schizophyllan by its subsidiary Wintershall for enhanced oil recovery. Interestingly, Wintershall uses the filtrated and concentrated fermentation broth directly for the enhanced oil recovery, without a precipitation and drying step. Additionally, scleroglucan is used in many food applications, mainly in Asia, and β-glucans also show antiviral and antitumor effect in human cell culture experiments.

Lentinan is used as a general immune response modifier and applied in cancer therapy. The lentinan-specific receptor (dectin-1) was identified in macrophages and displays its role in cancer patients. Lentinan as sold by the Japanese company Ajinomoto was probably the most expensive microbial polysaccharide on the market. However in June 2017, EA Pharma, the subsidiary of Ajinomoto, decided to withdraw lentinan injection for the treatment of gastric cancer in Japan as the demand for the product has dropped.

11.3.3 Fructosylpolymers like Levan

Levans are widespread microbial fructosyl homopolymers and are synthesized from sucrose by action of the secreted or membrane membrane-bound levansucrase enzymes. Main producing strains are *Bacillus* sp., *Zymomonas* sp., or *Halomonas* strains. Levans consist of β-(2,6)- and β-(2,1) -linked D-fructofuranosyl units with varying ratios within the different levan types. Levan can be easily degraded at higher temperatures and low pH and is highly soluble in water and oil as well as most organic solvents and has a relative low viscosity. Levans are used in bio-based glues or food applications, such as thickener of prebiotics in the form of oligomers but is approved for food applications just in some countries.

11.4 Perspectives

The tremendous success of microbial polysaccharides started in the 1960s with xanthan gum. Since then, more than hundred different polysaccharides from bacteria and fungi were isolated and characterized and a dozen are produced on an industrial scale. The advantage of biotechnological production is manifold: Fermentation and downstream processing can be controlled in a defined environment and is not influenced by weather extremes, climate change, or pollution. Scientists and engineers can improve the production process with strain improvement methods and better process design, resulting in a more efficient

production, being more flexible toward the demand of customers and being more competitive to hydrocolloids, which are derived from plants or seaweed.

The applications of microbial polysaccharides grow vastly, and patents issued especially for food and personal care applications surpass the patents for production processes or microorganisms by far. There is also an increasing trend to use sustainable hydrocolloids in industry. Especially manufacturers of food and personal care products to replace chemically modified polysaccharides like modified starch or modified cellulose with natural derived hydrocolloids. This trend for using sustainable products also becomes evident in other industries. Research is going on to use microbial polysaccharides for bio glues, replacing at least partially the classic organic chemicals. In some regions, the oil industry is not allowed to use chemically derived polymers such as polyacrylamide or polyacrylates for oil drilling and enhanced oil recovery anymore because they are declared as "red" chemicals in contrast to biodegradable microbial polysaccharides that are declared as "green" chemicals.

Concerning the biotechnological production process of microbial polysaccharides, the big question will be whether recombinant raw material (starch and glucose syrup) and recombinant production organisms will be accepted by consumers. European consumers do not accept GMO material for many years and more and more North American people refuse GMO-derived ingredients in their food. Food retailers and in consequence food producers already react to this trend and require non-GMO certification from the microbial polysaccharide manufacturers.

Further Reading

Ates, O. (2015). Systems biology of microbial exopolysaccharides production. *Front. Bioeng. Biotechnol.* 3: 200. https://doi.org/10.3389/fbioe.2015.00200.

Freitas, F., Torres, C.A.V., and Reis, M.A.M. (2017). Engineering aspects of microbial exopolysaccharide production. *Bioresour. Technol.* 245 (Pt. B): 1674–1683.

Hublik, G. (2012). Xanthan. In: *Polymer Science: A Comprehensive Reference* (eds. K. Matyjaszewski and M. Möller), 221–229. Elsevier.

Kreyenschulte, D., Krull, R., and Margaritis, A. (2014). Recent advances in microbial biopolymer production and purification. *Crit. Rev. Biotechnol.* 34 (1): 1–15.

Schatschneider, S., Schneider, J., Blom, J. et al. (2017). Systems and synthetic biology perspective of the versatile plant-pathogenic and polysaccharide-producing bacterium *Xanthomonas campestris*. *Microbiology* 163 (8): 1117–1144.

Schmid, J., Sieber, V., and Rehm, B. (2015). Bacterial exopolysaccharides: biosynthesis pathways and engineering strategies. *Front. Microbiol.* 6: 496.

Seviour, R.J., McNeil, B., Fazenda, M.L., and Harvey, L.M. (2011). Operating bioreactors for microbial exopolysaccharide production. *Crit. Rev. Biotechnol.* 31 (2): 170–185.

Ullrich, M.S. (2009). *Bacterial Polysaccharides – Current Innovations and Future Trends*. Caister Academic Press https://doi.org/10.13140/2.1.1992.0321.

12

Steroids

Shuvendu Das[1,2] and Sridhar Gopishetty[2]

[1] The University of Iowa, Department of Chemistry, E331 Chemistry Building, Iowa City, Iowa, 52242-1294, USA
[2] The University of Iowa, Center for Biocatalysis and Bioprocessing, 2501 Crosspark Road, Suite C100, Coralville, Iowa, 52241-3210, USA

CHAPTER MENU

12.1 Fields of Applications and Economic Importance

Steroids are specific structure terpenoid lipids that occur widely in living systems. Over 250 sterols and related compounds have been reported in plants, insects, vertebrates, and lower eukaryotes such as yeasts. Steroid hormones control cell proliferation and tissue differentiation and modulate gene expression. Hence, steroid-based pharmaceuticals are important for human and animal health in (i) disease prevention (antitumor and antiestrogenic), (ii) disease therapy (cancer and obesity), and (iii) lifestyle choices (menopause and human fertility). Steroids constitute a particularly influential class of hormones because of their range of action. With receptors in almost every nucleated cell in the body, they affect growth, metabolism, immune function, mood, memory, cognition, and

Industrial Microbiology, First Edition.
Edited by David B. Wilson, Hermann Sahm, Klaus-Peter Stahmann, and Mattheos Koffas.

Table 12.1 Annual market values of key steroids in the year 2016.

Steroids	Market value (US$ billions)
Testosterone	2
Estradiol	2.08
4-Androstene-3,17-dione (AD)	0.75
1,4-Androstadiene-3,17-dione (ADD)	
Other steroids	5
Total steroids market	10

behavior. Steroids are of special interest for the study of emotions and economic behavior because they help coordinate body and brain in archetypical situations, such as fight, flight, mating, feeding, search, and struggle for status. Because they are known to respond powerfully to such behavioral and social situations, steroid hormones may provide an important missing link in the emerging field of neuroeconomics between economic events and brain processes. As a result, about 300 approved steroid drugs exist to date and the numbers are growing. Indeed, the market for steroid drugs lies second only to that for antibiotics; the global market for steroids is in excess of $10 billion and more than one million tons annually (Table 12.1). Forecasts of the global pharmaceutical industry indicate that many drugs based on steroid compounds retain market leadership in future perspectives.

Among the steroids, androstenedione (AD; Figure 12.1), and 1,4-androstadiene-3, 17-dione (ADD) have the special status of being the starting compounds for the synthesis of main steroidal drugs. AD and ADD are key pharmaceutical steroid intermediates and their yearly reported market is 1000 tons. The combined worldwide market for AD and ADD, according to Forbes MediTech Inc. (FMT; Vancouver, BC, Canada), is approximately 750 million

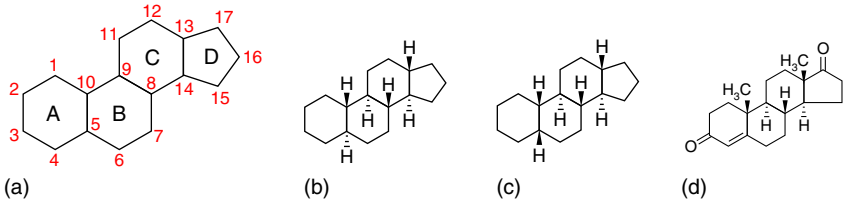


Figure 12.1 Chemical structure of steroids. (a) Gonane is representing the steroid nucleus. It consists of a phenanthrene (rings A, B, C) fused with cyclopentane (ring D). Unlike steroid hormones, gonane has no methyl groups at C10 and C13. Because of six chiral carbons (C5, C10, C9, C8, C14, and C13), 64 stereoisomers are possible, e.g. (b) 5α-gonane and (c) 5β-gonane. (d) 4-Androstene-3, 17-dione (AD) is a key intermediate for synthesis of steroid hormones such as testosterone.

US$ annually, but that is just for raw materials. AD is a key intermediate in the synthesis of steroids such as testosterone, estradiol, progesterone, cortisone, prednisone, or prednisolone, which have great interest for the pharmaceutical companies. Products derived from these starting materials are valued at nearly $4 billion per year (Table 12.1). These steroids form an integral part of the mammalian endocrinal system. As the major estrogen, estradiol serves as a growth hormone for tissues of reproductive organs, being coresponsible for the development of secondary sex characteristics in females, and has an important effect of maintaining bone structure. Progesterone as the major progestogen is involved in the female menstrual cycle. It supports pregnancies and is involved in embryogenesis. Testosterone as the major androgen is coresponsible for the development of secondary sex characteristics in males and helps regulate sperm production. Also, it has anabolic properties. Lastly, cortisol as a glucocorticoid regulates blood sugar and aids fat, carbohydrate, and protein metabolism.

12.2 Advantages of Biotransformations During Production of Steroids

Steroid biotransformation is a multimillion dollar industry, and pharmaceutical uses of steroids are numerous. Specific microbial transformation steps have been incorporated for synthesis of new steroids and their evaluation as drugs and hormones. Biotransformations have provided adequate tools for the large-scale production of natural or modified steroid analogs. Highly complex structure of steroids molecules renders the use of biocatalysts for the production of pharmacologically important steroid drug intermediates.

The production processes of steroid hormones may be classified as (i) extraction from plants or animal tissues, (ii) full organic synthesis, (iii) combination of chemical and enzymatic syntheses, or (iv) extraction from plants, or animal tissues followed by chemical and/or enzymatic conversion to the desired steroid. Evidently, with several exceptions, the first one can hardly be applied for large-scale production. Full chemical or chemical enzymatic syntheses are often multistage; time-, labor-, and energy consuming; environmentally risky; and in some cases are even impossible. More and more bioconversions became the methods of choice for industrial production, and growing demand for steroid pharmaceuticals stimulates the development of new cost-effective and environmentally friendly biotechnologies.

The production of steroid drug hormones is a good example of the successful application of microbial technology in large-scale industrial processes. Natural sources of pharmacologically active steroidal compounds are scarce, uneconomical to isolate and nonfeasible as stereochemical considerations limit the use of compounds derived from one animal as drugs in another. A large number of naturally occurring, extractable steroidal compounds have complex side chains and cannot be used as drugs. Among the abundantly available steroid precursors are cholesterol, steroidal sapogenins, steroidal alkaloids, and phytosterols.

The complex structure of the steroid molecule requires complicated, multistep schemes for the chemical synthesis of steroid compounds. It often involves

the preparation of intermediate derivatives with protected groups and their subsequent regeneration, once the intended reaction has occurred, limiting the overall process yield and making it expensive and time-consuming. Furthermore, the basic ring structure of some steroid derivatives is sensitive to cleavage by a wide variety of chemicals. Chemical synthesis also requires the use of reagents such as pyridine, sulfur trioxide, or selenium dioxide, which are hazardous to the health of production staff and constitute a serious environmental disposal problem.

The conversion of precursor steroids through microbes as compared to chemical process is less expensive, nontoxic, and less time-consuming. During bioconversion, microbes provide enzymes, which act upon and convert organic compounds or modify them. One of the major biotechnological aspects in microbial transformation is the application of a wide range of the microorganisms including bacteria, fungi, and microalgae in converting steroid substances into the pharmacologically active compounds or other useful intermediates and can provide an efficient alternative to chemical synthesis for the development of manufacturing processes, once the limitations often encountered of unsatisfactory productivity and/or purity levels of the conversion products are overcome. The steroid industry thus couples the chemical and biological approaches taking advantage of the best aspects of each. However, the question is not always if the chemical or the biocatalytic approach in the synthesis of a target molecule is better suited. Several examples of the combination of chemo- and biocatalytic steps in the synthesis of fine chemicals and pharmaceuticals exist already.

Generally, as already mentioned, whole cell catalysis is the method of choice for the enzymatic steps in industrial steroid synthesis. For the first time, the 7α-hydroxylation of cholesterol by *Proactinomyces roseus* was reported. In the 1950s, Upjohn Company and Squibb reported at the same time the 11α-hydroxylation of progesterone by *Rhizopus arrhizus* and *Aspergillus niger*, respectively, although the 11β-hydroxy configuration is required for the biological activity of corticosteroids. Thus, inversion of the configuration was necessary, which was successfully achieved by chemical oxidation and further reduction. The introduction of this enzymatic step reduced the number of required steps in the synthesis of corticosteroid hormones derivatives by 10 in contrast to the synthetic chemical route developed by Merck (Germany), where 31 steps were necessary to obtain 1 g of cortisone from 615 kg of deoxycholic acid. Thus, the microbial hydroxylation reduced the price of cortisone from US$200 to US$6 per gram. A well-established commercial biocatalytic process is the oxyfunctionalization of steroid hormones such as 11β-hydroxylation of 11-deoxycortisol with *Curvularia* sp. at a scale of ~100 t/yr by Bayer Pharmaceuticals Aktiengesellschaft (AG).

A current improvement in biocatalyst is recombinant expression of 17-ketosteroid reductase hydroxysteroid dehydrogenase (17β-HSD) from *Mycobacterium smegmatis* in *Escherichia coli*. Either natural sterols (e.g. phytosterols or cholesterol) or androstenedione (AD) conversion into testosterone successfully compete with the chemical synthesis of testosterone, Figure 12.2a,b.

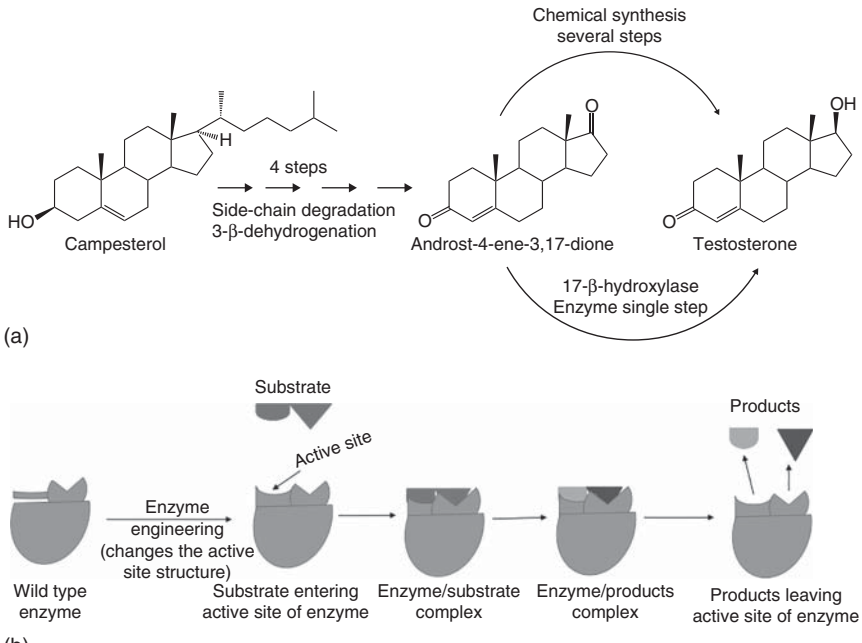


Figure 12.2 Current improvement in biocatalysis reduces the number of steps in steroid synthesis (a). Regioselectivity is caused by highly specific enzymes (b).

12.3 Development of Production Strains and Production Processes

Screening and isolation of active microbial strains for steroid bioconversion are presently an important part of the research and development effort in the steroid drug industry. The microbial metabolism of sterols has already been shown to be a promising means of preparing valuable steroids. During the second half of the twentieth century, many microbial strains were developed and tailored to synthesize many kinds of C19 and C22 steroids using phytosterols as substrates. So far, however, the production of steroids from phytosterols by microbial transformation is still not widely used in the pharmaceutical industry. This may be because of two inherent problems in the phytosterol biotransformation process: (i) The low water solubility of phytosterols can lead to poor bioavailability. (ii) The final products are toxic to microbial cells. Many technological strategies have been proposed to overcome these problems.

Microbial transformations expand the toolbox of organic synthesis, thus enabling obtaining of both well-established and new steroid derivatives of potential biological and pharmacological activity inaccessible otherwise. The similarity of some microbial enzymes to mammalian ones may be used as the so-called microbial models of mammalian metabolism in order to predict the transformation routes of the tested compounds (mainly, new bioactive

molecules) in the preclinical investigations. Steroid substrate spectrum for microorganisms is rather broad: along with plenty natural steroids that are synthesized in plants, insects, vertebrates, and lower eukaryotes, it includes industrially relevant steroids discharged into the environment. Diverse bacteria are capable of catabolizing steroids as carbon and energy sources, while fungi mainly catabolize steroids via derivatives that are more toxic than the original molecule. However, nonsufficient selectivity of steroid transformation reactions by wild-type strains often results in a mixture of products, and along with steroid core, degradation by wild type strains may restrict their industrial application. Innovative solutions in the field of steroid biotechnology are based on the application of genetically engineered microorganisms. An outstanding achievement is the development of a *Saccharomyces cerevisiae* strain enabling production of hydrocortisone from glucose by reconstructing the fully self-sufficient biosynthetic pathway involving 13-engineered genes encoding several mammalian steroid hydroxylases. More than 10 years were required for the development of the production bioprocess by Sanofi-Aventis Deutschland GmbH, but it is a unique and impressive experience showing a revolutionary era in the steroid field. Significant progress in the understanding of steroid catabolism pathways promoted generation of effective biocatalysts capable of producing valuable steroids. Different tools are applied for the deletion of specific genes, or their augmentation, thus enabling enhanced production of valuable steroids. The mutations in the sterol catabolic pathways also allow obtaining other useful intermediates for the synthesis of steroid therapeutic drugs such as those derived from the partial oxidation of the sitosterol side chain. There are just few examples illustrating the construction of effective biocatalysts for production of valuable steroids on the basis of nonpathogenic species. Generation of engineered strains capable of single-step selective bioconversions of phytosterol and other available and low-cost starting materials to valuable steroid compounds remains one of the most important tasks in the field of steroid biotechnology. With deep understanding of systematic cellular bioinformatics, the exploration of state-of-the-art genome editing tools for targeted gene knockout and knock-in would play a vital role in microbial cell engineering for steroid production.

Methodical and well-rounded process development, considering upstream and downstream processing simultaneously, is crucial for designing effective and economical production processes. Here is an example on downstream process development of androstenedione (AD). Based on a multiphasic fermentation process, here in this process, oil is added to improve substrate solubility. The design methodology incorporates expert knowledge in the form of heuristics to generate different downstream processing alternatives for processing the complex, multiphasic fermentation broth and recovering the target steroid precursor AD. Purification steps such as extraction, adsorption–desorption, and precipitation seem most promising and have been investigated in detail. Each process step selected is optimized and connected to a process route for recovering AD. Extraction plays a major role as every phase of the fermentation broth can be processed with this technique. The selection of adequate solvents is crucial for an efficient downstream process and mass determination. Different approaches exist in literature to determine a good extraction solvent. Especially

in early stages of process development, where thermodynamic data and information about impurities are limited, a target-oriented method for solvent selection is necessary. Robust methodologies for the solubility estimation are given by using the 1-octanol–water partition coefficient or the Hildebrand and Hansen solubility parameter. However, the validity and performance of the prediction methods remains to be investigated by experiments. Typically, next step in the downstream process is adsorption–desorption. During an adsorption–desorption process in capture mode, the target molecules located in a fluid mixture selectively bind to a solid phase (adsorbent) and are selectively desorbed with a solvent. Suitable adsorbents are characterized by a large surface area and high capacity and selectivity toward the target compound. In addition, stability against chemical, physical, or thermal stress is advantageous. From economic point of view, the adsorbent material shall be easily regenerable for multiple use and cheap. A variety of adsorbent materials are available on the market, which can differentiate between carbonaceous-, acidic-, and polymer-based adsorbents.

The final step in the process is precipitation and/or crystallization. Precipitation can be induced by addition of organic solvents, acids, bases, salts, or antisolvents. Considering the nature of the compounds under investigation, namely neutral, hydrophobic and low molecular weight, the addition of an antisolvent seems most appropriate to initiate a precipitation process. Requirements to an antisolvent are a low solubility of either the target or the impurity molecules depending on which molecule(s) shall be separated and the complete solubility in the molecules containing solution (here extract or desorption solvent). The products of a precipitation are mostly amorphous or semicrystalline, and the purity is probably lower compared to crystallization processes. However, the precipitation is faster and often easier to perform. Crystallization is a powerful separation technique and is induced by the supersaturation of the target molecule by cooling or evaporating the surrounding solution or adding an antisolvent. After crystallization, the product is present in crystalline form of different habitus (needle and plates). To exactly design crystallization processes, the solubility line and the metastable area of the solute in the target containing solution is to be determined. Crystallization processes are preferred, where no additives are used, as no subsequent solvent recovery and recycle are necessary (Figure 12.3).

12.4 Applied Types of Biotransformation

Biotransformations (bioconversions or microbial transformations) are chemical reactions that are catalyzed by microorganisms in terms of growing or resting cells or that are catalyzed by isolated enzymes. There is only a slight difference between a biotransformation and a bioconversion. A bioconversion utilizes the catalytic activity of living organisms and hence can involve several chemical reaction steps. A living microorganism will be continuously producing enzymes and hence bioconversions often involve enzymes, which are quite unstable for used substrates. The properties of biotransformations and bioconversions are very similar, and in many cases, the terms are cited as interchangeable.

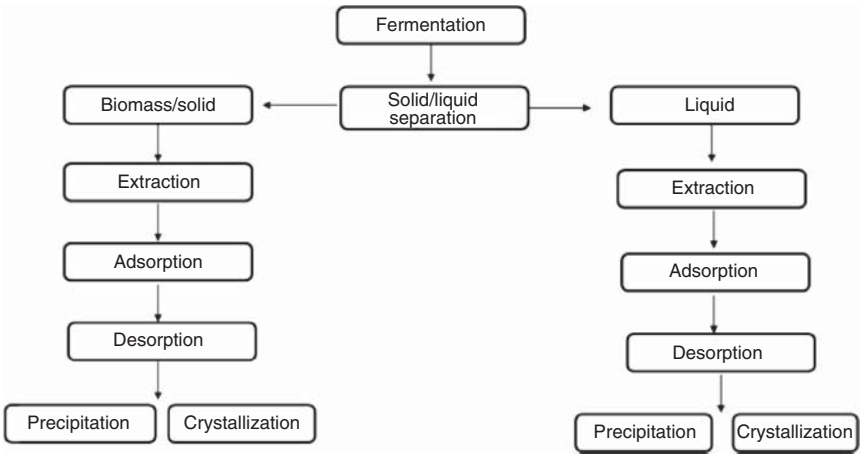


Figure 12.3 Important steps in downstream processing after biotransformation.

Before getting into details of biotransformation, it will be beneficial to explain the terms regioselectivity and stereoselectivity. A regioselective reaction is one in which one direction of bond making or breaking occurs preferentially over all other possible directions. Reactions are termed completely regioselective if the discrimination is 100%, or partially (x%), if the product of reaction at one site predominates over the product of reaction at other sites. In the past, the term "regiospecificity" was proposed for 100% regioselectivity. This terminology is not recommended owing to inconsistency with the term stereoselectivity, whereas stereoselectivity is the preferential formation in a chemical reaction of one stereoisomer over another.

Although there are hundreds of biotransformations known, only a few of them are useful for the synthesis of commercially important products. The significance of bioconversion reactions becomes obvious when the production of a particular compound is either difficult or costly by chemical methods. Further, biotransformations are generally preferred to chemical reactions because of substrate specificity, stereo specificity ecologically harmless reaction conditions (normal pressure, low temperature, and neutral pH), which are all important requirements. If these features can be combined with economic benefits, biotransformations become the functional part of new chemical processes for organic synthesis. The same is true for all other natural and most human-engineered chemicals. These multistep transformations are catalyzed by enzymes that are mostly specific for the reaction type and starting compound. Yet, very frequently, enzymes catalyze not only the reaction they were evolved for by nature over thousands or millions of years but also the conversion of structurally and/or electronically similar derivatives. This feature of enzymes can be used for numerous technical purposes like the cleanup of contaminated environments (bioremediation) or the production of high-value compounds for chemical, agricultural, and pharmaceutical industries.

A wide variety of biological catalysts can be used for biotransformation reactions. These include growing cells, resting cells, killed cells, immobilized

Table 12.2 List of different types of biocatalysts and biotransformations.

Type of biocatalyst	Type of biotransformation
Growing cells	Dehydrogenation/reduction
Resting cells	Hydroxylation
Cell lysate	Esterification
Free enzyme	Halogenation
Immobilized cells	Methoxylation
Immobilized enzyme	Isomerization
	Acylation
	Hydrolyzation/side-chain cleavage

cells, cell-free extracts, enzymes, and immobilized enzymes. The most important sources of biocatalysts and the procedures employed for biotransformation are briefly described (Table 12.2).

Growing cells: The desired cells are cultivated in a suitable medium. As the growth of the cells occurs (6–24 hours), a concentrated substrate is added to the culture. Sometimes, addition of emulsifiers (Tween, organic solvents) is required to solubilize substrates and/or products, e.g. steroid biotransformation. The substrate conversion to product can be monitored by spectroscopic or chromatographic techniques. Biotransformation can be terminated when the product formation is optimum.

Resting cells: The use of resting cells may be a good alternative when the best pH value, temperature, or media composition for the bioconversion is different from the values allowing the best growth conditions. In this situation, the cells are grown until enough biomass has been accumulated, they are harvested and washed with water or a buffered solution, and resuspended in the desired buffer for biocatalysis. As the cells are washed, unconsumed growth substrates and nutrients, as well as undesired growth metabolites, are removed from the system allowing better product recoveries and downstream processing. Besides, resting cells can show high product yields on carbon and energy sources as they are not used for biomass production, and the cells may be recycled and reused. However, these cells should be able to maintain high activities and cofactor regeneration over extended periods to be used in biocatalytic systems.

Many microbial biotransformations of steroidal compounds have been reported over the past half century, including (i) dehydrogenation/reduction, (ii) hydroxylation, (iii) esterification, (iv) halogenation, (v) methoxylation, (vi) isomerization, (vii) acylation, and (viii) hydrolyzation/side-chain cleavage. For these reactions, microorganisms are known to target selectively only one of all carbon atoms except C10 and C13. The degradation pathway of steroidal compounds from the A ring was first presented by Talalay in 1952. The microbial transformations of steroidal products has become one of the most successful examples of large-scale industrial processes because of the advantages such as

higher specificity, higher conversion rate, moderate reaction conditions, and lower pollution, compared to chemical synthesis.

12.5 Synthesis of Steroids in Organic – Aqueous Biphasic System

Biotransformations in biphasic systems have been steadily growing in recent decades. One of the main difficulties in the bioconversion of steroids is the weak substrate solubility in aqueous medium solution, which renders extremely poor productivity. Especially for steroids, the use of organic solvent in the medium is one approach that has been suggested for biotransformations. In the organic phase, the steroids have much greater solubility than in the aqueous phase. There are four advantages of biphasic systems over aqueous phase. Substrates and/or products can be dissolved with high concentrations; thermodynamically unfavorable reactions become possible; in the presence of organic solvent, microbiological contamination can be avoided; and product recovery becomes easier. In biphasic system, the organic phase contains dissolved steroid and the cells are present in the aqueous phase, and this is thought to be an ideal setup (this is done in aqueous organic biphasic system). In whole-cell bioconversion, for overcoming the problem of poor product and substrate solubility in water and also to eliminate their inhibitory effects on the biocatalyst, biphasic processes are used. Steroid biotransformation in two-phase systems is more frequently employed for bacteria as well as the microalgae, then by fungi. There are different types of biphasic systems including the aqueous organic biphasic system, for example, 1-decanol, water-miscible organic cosolvents such as dimethyl sulfoxide, and dimethyl formamide and ionic liquids such as 1-butyl-3-methylimidazolium hexafluorophosphate. Ionic liquid/aqueous biphasic systems as well as some unique biphasic systems are applied.

However, the major disadvantage is inhibition or denaturation of biocatalyst in the presence of organic solvents and reuse of enzyme is very important to save cost in industrial production. On the one side, the contact of the enzyme molecules with the bulk organic liquid at the phase interface results in inactivation of the enzyme; on the other side, in order to increase the interfacial area and enhance the mass transfer rate across the interface, the system has to be physically stirred, but stirring can also cause the denaturation and inactivation of enzyme molecules absorbed at the interface, as a result of interfacial tension.

Here is an example of steroid synthesis in organic–aqueous biphasic system. Cholest-4-en-3-one is an important synthetic intermediate in many steroid transformations. Previous studies have shown that it is effective against obesity, liver disease, and keratinization. Moreover, cholest-4-en-3-one can serve as a precursor for the synthesis of other drug intermediates, such as androst-4-ene-3,17-dione and androsta-1,4-diene-3,17-dione, which are major starting materials for the synthesis of anabolic drugs and contraceptive hormones. Its derivative olesoxime (cholest-4-en-3-one), a newly developed drug, displays neuroprotective properties and medicinal properties in the treatment of spinal muscular atrophy.

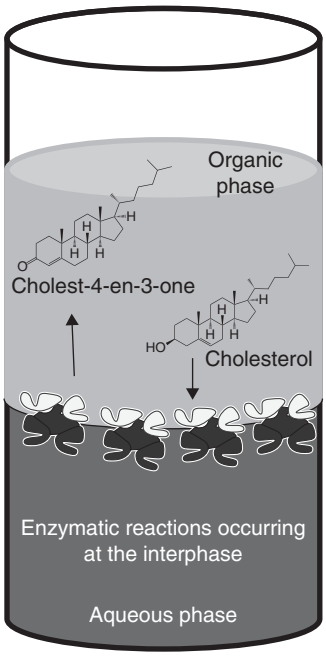


Figure 12.4 Synthesis of cholest-4-en-3-one in biphasic system. Active sites of the oxidase take the substrate from the organic phase.

Cholest-4-en-3-one can be synthesized by chemical methods, such as Oppenauer oxidation of cholesterol, acid-catalyzed isomerization of cholest-5-en-3-one, and by using pyridinium chlorochromate to convert cholesterol. However, these methods involve harmful solvents, such as chloroform, methanol, and benzene. Meanwhile, some of these methods need multistep reactions to yield cholest-4-en-3-one and the processes take a long time. Alternatively, cholest-4-en-3-one can be prepared using Cholesterol oxidase in one-step reaction (Figure 12.4).

12.6 Side-chain Degradation of Phytosterols by *Mycobacterium* to Gain Steroid Intermediates

Phytosterols are the naturally occurring sterols found in plants, such as, rice bran, corn, soybean, Brussels sprout, cauliflower, onion, orange, banana, and apple. These sterols are chemical compounds, structurally similar to cholesterol, which is obtained from the animal source. Phytosterols have a similar function like cholesterol in our body, but there is no side effect, rather it reduces the serum cholesterol and low-density lipoprotein. Phytosterols are the precursors of many key intermediates for the production of steroidal hormones and drugs. Stigmasterol, β-sitosterols, ergosterol, and campesterols are the most widely used phytosterols for making these intermediates. Use of different microorganisms has been attempted to synthesize these steroidal hormones and intermediates, but *Mycobacterium* species is the most successful one.

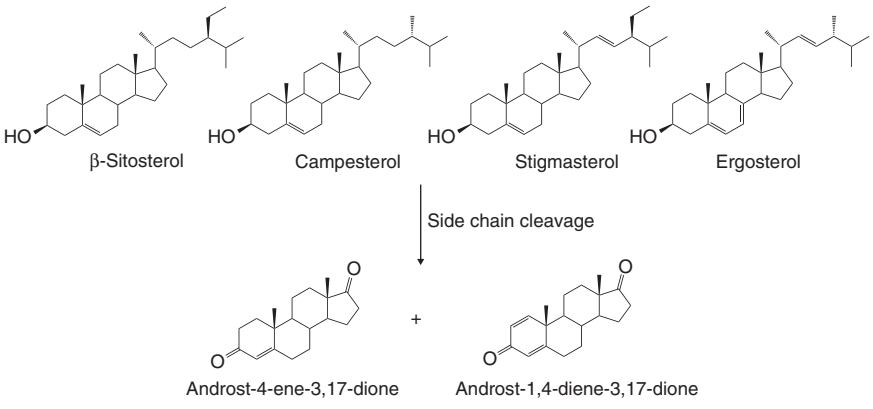


Figure 12.5 *Mycobacterium* strains degrade the side chain of phytosterols. Additionally, they can insert a second double bond in the A ring. The result is a mixture of two products.

Table 12.3 Percent conversion of side-chain degradation products of phytosterols by *Mycobacterium* sp.

Strains	Substrates	Conversion (%) after 14 days of incubation	
		AD	ADD
NRRL B-3683	β-Sitosterol	63	13
	Stigmasterol	29	2
	Ergosterol	32	1
NRRL B-3805	β-Sitosterol	33	51
	Stigmasterol	4	10
	Ergosterol	8	25

Mycobacterium sp. such as NRRL B-3805 and NRRL B-3683 [NRRL, Northern Regional Research Lab is a culture collection lab that is now referred to as American Research Service (ARC) culture collection; B-3805 and B-3683 are the strain numbers] cleave the phytosterol side chains and produce androstenedione (AD) and 1,4-androstadiene-3, 17-dione (ADD) as principal products (Figure 12.5). The side-chain cleavage of these sterols by microorganisms involves more than 10 consecutive enzymatic steps. The androst-1-ene-3,17-dione is formed first, and then 1,4-androstadiene-3, 17-dione is formed by an enzyme called dehydrogenase. The amount of AD and ADD formed depends on the nature of the strains and growth conditions used. In a specific study with substrates stigmasterols, β-sitosterols, and ergosterol (conducted by P. Sripalakit et al. (2006)), growing culture of strain NRRL B-3805 produced more ADD compared to AD, whereas strain NRRL B-3683 produced more AD compared to very little amount of ADD. The detailed results of this study are summarized in Table 12.3.

In another study, Wang et al. (2006) used resting cells for the biotransformation of phytosterols in cloud point system. They used strain NRRL B-3683 for

a mixture of sterols (β-sitosterols, stigmasterols, and campesterols, 45 : 30 : 25). They grew the cells seven days, then harvested the cells by centrifugation, and conducted the biotransformation with resting cells. In four to five days, up to 12 g/l of ADD can be produced with formation of AD as a minor product. They recycled the biocatalyst at least four times to increase the substrate-to-enzyme ratio. Enzyme was active to carry out further biotransformations keeping the high yield of ADD in the next cycles. Thus, this process can be used for large-scale production of ADD.

12.7 Biotransformation of Cholesterol to Gain Key Steroid Intermediates

Cholesterol is a steroid commonly found in nature with a great relevance in biology, medicine, and chemistry, playing an essential role as a structural component of animal cell membranes. The ubiquity of cholesterol in the environment has made it a reference biomarker for environmental pollution analysis and a common carbon source for different microorganisms, such as *Mycobacterium* sp. The search for microorganisms capable of degrading cholesterol was started over 70 years ago. In the early twentieth century, it was observed that several species of *Mycobacterium* could use cholesterol as the sole source of carbon and energy. The metabolic products of cholesterol are the precursors of calciferols, steroid hormones, and bile acids. Low-cost natural steroids, such as cholesterol, can be used as starting materials for synthesizing many bioactive steroids, and retail price of cholesterol per kilogram is about US$250. Therefore, an understanding of the bacterial metabolism of cholesterol could be useful in the development of biotechnological tools for the transformation of steroid components via metabolic engineering.

Significant progress in the understanding of steroid catabolism pathways promoted generation of effective biocatalysts capable of producing valuable steroids. Different tools are applied for the deletion of specific genes, or their augmentation, thus enabling enhanced production of valuable steroids derived from cholesterol catabolic pathways. Most attention was paid to the generation of effective producers of key androstane steroids, including androstenedione (AD), androstadienedione (ADD), and 9α-hydroxyandrostenedione (9-OH-AD) from cholesterol, which is a well-recognized cheap and available raw material for steroid production, Figure 12.6.

12.8 11-Hydroxylation by Fungi During Synthesis of Corticosteroids

Hydroxylation of steroids at the inactivated carbon atom is the most significant and useful process in the history of steroids. The presence of oxygen atom at the C-11 in corticosteroids is very important as most of the 11-hydroxylated corticosteroids are pharmacologically active. Mainly, they are used for antiallergic

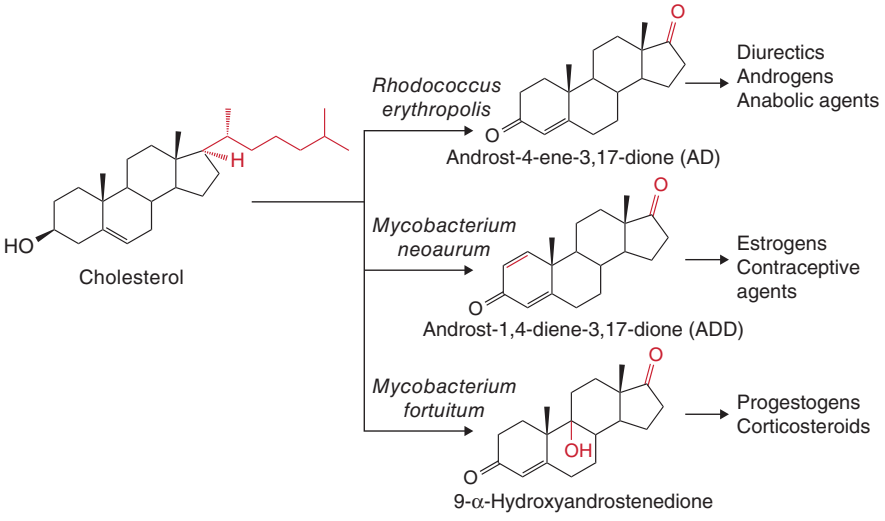


Figure 12.6 Biotransformation of cholesterol to key steroid intermediates.

and anti-inflammatory drugs. Several 11-hydroxylations have been reported by a wide variety of microorganisms. Hydroxylation at 11-position is mainly carried out by fungi. Among them, *Curvularia lunata*, *Rhizopus nigricans*, and *Aspergillus ochraceus* are significantly used.

Yaderetes et al. (2007) synthesized hydrocortisone via 11β-hydroxylation of cortexolone by using *C. lunata* (Figure 12.7). They grew the cells in the defined media at 28 °C, pH 6.0–6.2, and 220 rpm for 24–56 hours, depending on the requirements. After harvesting the mycelium, it was suspended in phosphate buffer (pH 6.1) to carry out the biotransformation at the same temperature and rpm of growth phase. They added 4–6 g/l of cortexolone as a microcrystalline suspension in phosphate buffer to the mycelium suspension for the transformation. After 40 hours of reaction, 4.0 g/l of cortexolone provided 68% (c/c) hydrocortisone, whereas 6.0 g/l of cortexolone gave only 30% (c/c) hydrocortisone. Lower yield with 6.0 g/l of cortexolone could be attributed because of the substrate inhibition.

In another study, immobilized *C. lunata* mycelium was used for 11β-hydroxylation of cortexolone to produce hydrocortisone. They used a photo-cross-linkable resin prepolymer called Entrapment (ENT-4000) to immobilize the *C. lunata* mycelium so that it can be reused. They recycled the immobilized biocatalyst 50 times in 100 operational days, and the overall yield of hydrocortisone in all cycles was 60–70% (w/w). They first grew the cultures 40–120 hours to find out the optimal cultivation and induction time period for the optimal 11β-hydroxylase activity. The maximum activity was reached after 50–60 hours of cultivation. Then, the yield of hydrocortisone was maximized after 30–40 hours of reaction. The initial concentration of cortexolone is very important for the better yield. They carried out the biotransformation in five different concentrations of cortexolone (1.5–7.2 mM) at the same conditions

(reaction volume 20 ml, 2.5% dimethyl sulfoxide, and 48 hours reaction time). The reaction mixture containing 2.9 mM cortexolone showed the best yield of hydrocortisone (54%, c/c); beyond that concentration, the yield of hydrocortisone decreased. In addition, they reported the optimal pH 6.5, and temperature 30 °C for the biocatalysis.

Solubility is a major issue for most of the steroids to increase the bioavailability and the rate of reaction. Different approaches have been adopted to increase the solubility of the steroids in the reaction mixture, such as the use of organic solvents and different additives, e.g. β-cyclodextrin. Roglic et al. (2005) studied 11α-hydroxylation of progesterone by *R. nigricans* with and without the addition of β-cyclodextrin. Cyclodextrins are cyclic oligosaccharides containing α-(1,4)-linked glucopyranose units. There are three types of basic cyclodextrins based on the number of glucopyranose units: α-Cyclodextrin (6 units), β-cyclodextrin (7 units), and γ-cyclodextrin (8 units). β-Cyclodextrins are mostly used because of their cheaper price and availability. Cyclodextrin makes complexes with the low aqueous soluble steroids by hydrophobic interactions, and more steroid–β-cyclodextrin complex dissolves in water. As a result, the substrate concentration is increased and consequently the reaction rate increases. Cyclodextrins as a host take the steroid molecules as a guest in its hydrophobic cavity for the complex formation (host–guest interaction). The hydrophobic guest steroid molecules replace the enthalpy-rich water molecules from the cavity and create a more favorable apolar–apolar interaction. It reduces the cyclodextrin ring strain and consequently possesses a more stable lower energy state. This is the main driving force to make the complex. Cyclodextrins can be reused after the completion of the reaction as there is no formation and breaking of covalent bonds during cyclodextrin–steroid complex formation. Recycling of the cyclodextrin makes the process economic. 11α-Hydroxyprogesterone is used for the treatment of androgen-dependent skin conditions and most importantly, an inhibitor of isoforms of 11-β-hydroxysteroid dehydrogenase. Addition of β-cyclodextrin had increased the rate of transformation, when the initial progesterone concentration was above its water solubility (11.5 ± 1.1 mg/l at 23 °C). The concentration ratio of progesterone and β-cyclodextrin used was 1 : 1.385 and the initial concentration of progesterone was 0.05 g/l. As shown in Figure 12.8, the biotransformation of progesterone completes faster in progesterone-β-cyclodextrin complex compared to the sole progesterone. Conversion of progesterone-β-cyclodextrin reaction mixture proceeds up to around 75 minutes while the conversion of reaction mixture without β-cyclodextrin proceeds more slowly, till 150 minutes. The larger concentration slopes of the complex indicate that progesterone is consumed rapidly and consequently 11α-hydroxyprogesterone is produced faster. Moreover, the yield of the 11α-hydroxyprogesterone was not affected much by the addition of β-cyclodextrin. Progesterone-β-cyclodextrin complex produced 40 mg/l of 11α-hydroxyprogesterone, whereas progesterone without β-cyclodextrin produced 42 mg/l of 11α-hydroxyprogesterone. Thus, β-cyclodextrin can be used as an external additive to increase the reaction rate of steroid hydroxylation.

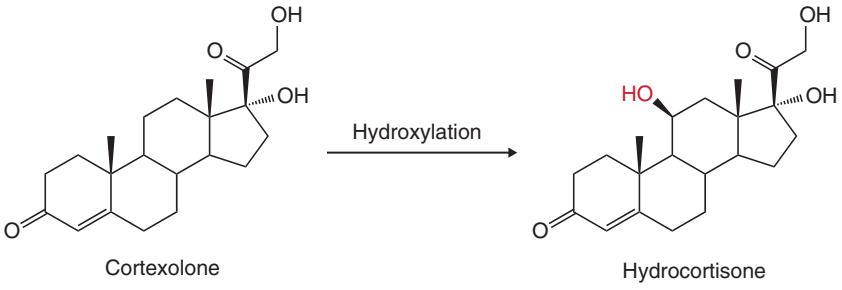


Figure 12.7 Synthesis of hydrocortisone from cortexolone by *Curvularia lunata*.

12.9 Δ^1-Dehydrogenation by *Arthrobacter* for the Production of Prednisolone

As the demands of pharmaceutical steroids continuously increase, the highly efficient methods to produce prednisolone are described here. Prednisolone is one of the most essential medicines used for several treatments including allergies, inflammation, arthritis, asthma, and cancers. One easy way to produce prednisolone is Δ^1-dehydrogenation of cortisol (Figure 12.9).

Ohlson et al. (1978) synthesized prednisolone from cortisol by using immobilized *Arthrobacter simplex* cells. Immobilized enzyme technology has several advantages, such as convenience, stabilization, and easy separation of the product from the catalyst. The cells were grown in the fermentor at 28 °C, and the culture was induced with 0.2 mM cortisol in 6% methanol after 12 hours of elapsed fermentation time. Then, fermentation continued another 12 hours before harvesting the cells. The resting cells were suspended in 0.05 M Tris–HCl, pH 7.5, and immobilized with polyacrylamide. The entrapped bacteria (>10%, w/v) showed 40% of the original free-cell activity. They concluded the optimum pH range 6–9,

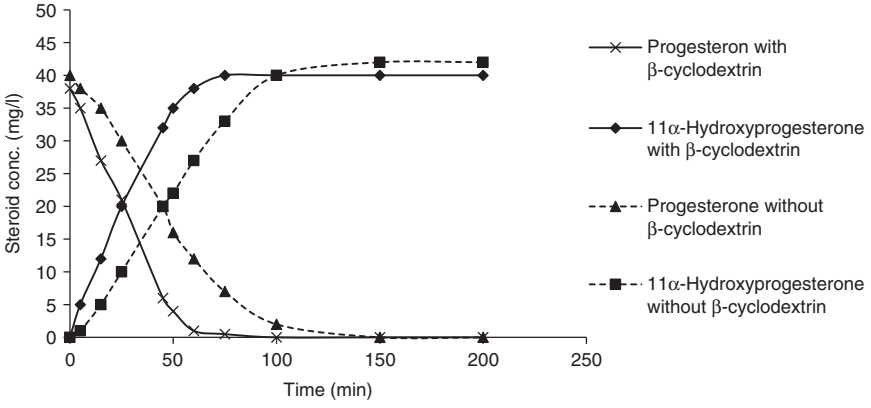


Figure 12.8 Kinetics of *Rhizopus nigricans* catalyzed conversion of progesterone to 11α-hydroxyprogesterone with or without *β*-cyclodextrin.

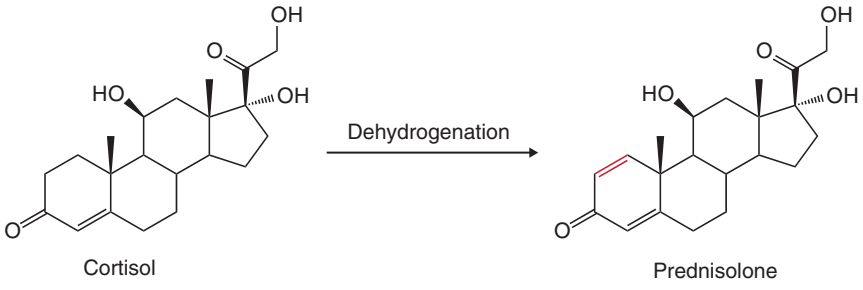


Figure 12.9 Enzymatic synthesis of prednisolone via dehydrogenation of cortisol.

temperature 34 °C, and the substrate (cortisol) concentration 1 mM. For a continuous production of prednisolone, they performed the reaction in a column. They improved the Δ^1-dehydrogenase activity of *A. simplex* 7–10 times compared to the original activity, by incubating the entrapped cells in various nutrients media, e.g. peptone (Figure 12.9). The increased activity can be attributed due to the possible growth of the microorganisms within the entrapped gel matrix. Final yield of prednisolone was achieved as 0.5 g/g of gel/day. Frozen immobilized cells did not lose any significant amount of activity over the four-month period of storage.

We have described the low aqueous solubility issue of the steroids in Section 12.8. It impacts the efficiency of the Δ^1-dehydrogenation of hydrocortisone for the production of prednisolone too. Silbiger and Freeman (1988) improved the yield of prednisolone by using the water-miscible organic solvents and polyacrylamide-hydrazide (PAAH) as an immobilized matrix. They used 10% (v/v) ethylene glycol as the cosolvent to transport the hydrocortisone through the column containing the *A. simplex* immobilized cells. Generally, this dehydrogenation mechanism needs molecular oxygen as the final electron and hydrogen acceptor to carry out this transformation. However, the solubility of the oxygen is very low in the water and the volume of oxygen in the immobilized system is very limited. Therefore, menadione sodium bisulfate (MBS), an artificial electron acceptor, can be used to eliminate the requirement of oxygen. After adopting the ethyleneglycol, PAAH, and MBS, they optimized the process for the production of prednisolone. The 2.5 mM hydrocortisone mixture input in the column produced stable and high yield (average 90%; c/c) of prednisolone over the one-month period, without any side reactions observed. Thus, this process can be used industrially for the continuous production of prednisolone.

12.10 17-Keto Reduction by *Saccharomyces* in Testosterone Production

Testosterone is a vital hormone medicine and precursor for the synthesis of other steroids. However, traditional methods of synthesizing testosterone cost too much and cause severe pollution. Alternatively, baker's yeast can be used for the production of testosterone. *S. cerevisiae* is easily available, very cheap, and provides a more efficient greener route to produce testosterone from the

Androst-4-ene-3,17-dione Testosterone

Figure 12.10 Enzymatic reduction of androst-4-ene-3,17-dione (17-ketotestosterone) for the production of testosterone.

androst-4-ene-3,17-dione (AD). The 17-keto group of AD is stereospecifically reduced to the β-hydroxyl group to provide testosterone (Figure 12.10).

Singer et al. (1991) used *S.cerevisiae* for the enzymatic transformation of AD to testosterone in the presence of a solubilizer, cyclodextrin. With the help of cyclodextrin, this synthesis has been improved and satisfied the industrial requirements. They tested different cyclodextrins, but the γ-cyclodextrin was the best cyclodextrin to carry out the reduction of AD. γ-Cyclodextrin and β-cyclodextrin containing AD (2 g/l) produced 90% (c/c) and 85% (c/c) testosterone, respectively, after 120 hours of reaction at 30 °C. Slight higher yield with γ-cyclodextrin could be attributed because of the bigger hydrophobic core size of γ-cyclodextrin compared to the β-cyclodextrin. The bigger cavity can adopt more substrate molecules and the availability of the AD might be higher for the γ-cyclodextrin. Surprisingly, AD without cyclodextrin produced only 30% (c/c) testosterone under identical reaction conditions. Hence, cyclodextrin can be used for increasing the productivity of testosterone from AD.

12.11 Double-Bond Isomerization of Steroids

The isomerase is an enzyme that catalyzes the conversion between isomers. Among different isomerizations, double-bond isomerization of steroids is very important for the pharmaceutical industry. In the double-bond isomerization, the position of the double bond changes, sometimes from one ring to another ring, resulting in the formation of new steroids. The biological activity such as anti-inflammatory activity of the steroid molecules depends on its structure. Therefore, by using this double-bond isomerase, we can efficiently make very important new steroidal drugs. The steroid isomerase has the highest turnover number, known in enzymology. 3-Keto steroid Δ^4-Δ^5 isomerase (EC 5.3.3.1) is most widely used isomerase for the study of steroid metabolism, specifically from the mechanistic viewpoint. Steroid Δ^4-Δ^5 isomerase changes the position of the double bond between C5 and C6 to C4 and C5 and vice versa, for the isomerization of 3-ketosteroids (Figure 12.11). During the double-bond isomerization, the electrons of the π-bond that is two carbons away from the carbonyl group can move to the adjacent σ-bond that is near to the carbonyl group. The key mechanism of the isomerization is the involvement of the bifunctional acidic and basic groups of the active site of isomerase that can protonate and

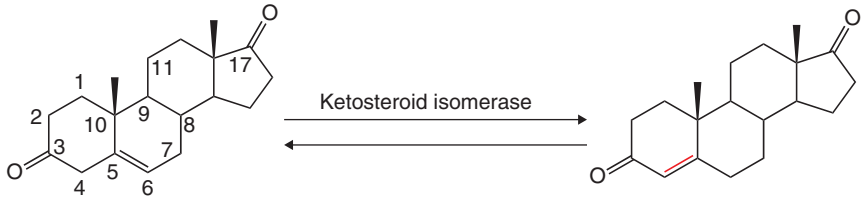


Figure 12.11 Conversion of androst-5-ene-3,17-dione to androst-4-ene-3,17-dione by a ketosteroid isomerase.

deprotonate. Tyrosine and histidine are the two principal amino acids present in the active site that are responsible for this catalytic process.

Weintraub et al. (1980) extensively studied the proton transfer mechanism for double-bond isomerization of 3-keto steroids. They explained in their study, $\Delta^{5(6)}$-substrates such as androst-5-ene-3,17-dione are perfect to undergo the proton transfer reaction for the isomerization process. Androst-5-ene-3,17-dione isomerizes to androst-4-ene-3,17-dione by a *Pseudomonas testosteroni* 3-keto steroid Δ^4-Δ^5 isomerase (Figure 12.11). Four main steps are involved for this isomerization process: (i) protonation of the carbonyl group, (ii) loss of C4 β-proton, (iii) reprotonation at C6 β-position, and (iv) deprotonation of carbonyl group. Protonation and deprotonation occurs by involving tyrosine phenolic group and histidine imidazole group, respectively. As the isomerization process is an equilibrium process, theoretical yield of the product of interest should be 50%, considering that both sides of the reactions are equally favorable. By changing the pH of the reaction mixture, the yield can be controlled by shifting the equilibrium to the favorite side. When the pH changes, concentrations of phenolate ions and imidazole ions also change, resulting in the equilibrium shift. In the industry, the important practice is removal of the target steroid from the reaction mixture, after its immediate formation. Thus, equilibrium shifts toward the choice of your steroid.

12.12 Perspectives

Remarkable progress in microbial transformation of steroids has been made since 1950. Several new steroids and their secondary metabolites have been discovered. Different immobilized techniques (for recycling of the biocatalysts) are adopted to increase the enzyme-to-substrate ratio. Rational design of the fermentation media and optimization of the bioreactor conditions are successfully introduced to improve the productivity of the steroids. Most of the steroid biotransformations have been reported either with fungus or bacteria. Steroid biotransformations with microalgae are very limited. Although, the use of microalgae has some limitations, such as penetration of the light inside the culture bed, this area may be the interest of future research for steroid biotransformations because of some advantages, such as availability in large scale and consumption of carbon dioxide.

Solubility of hydrophobic steroids is still a major problem, but the research is going on, such as use of different cyclodextrins, nonaqueous solvents, and biphasic organic–aqueous mixture. Our focus should shift from the use of organic solvents to the more green solvents like ionic liquid, which are more eco-friendly.

After discovery of the recombinant DNA technology including the whole genome sequencing, the area of steroid biotransformation has been broadened and diversified. Low expression enzyme can be overexpressed by cloning to increase the productivity of the steroids. Cloned enzymes and/or metabolic engineering (including knocking out of the undesired genes) in the organisms minimize the formation of side reaction products. Generation of novel biocatalysts either to accept the new steroid substrates or increase the catalytic efficiency of the existing substrates is now underway. Research to alter the position of the introduced functional group and the stereospecificity in the transformed steroids is also being carried out. Often this area of research is called "Enzyme Engineering." Advancements in research including metabolic engineering and enzyme engineering could be able to produce all steroid hormones and drugs in "Microbial Cells" in future.

References

Ohlson, S., Larsson, P., and Mosbach, K. (1978). Steroid transformation by activated living immobilized *Arthrobacter simplex* cells. *Biotechnol. Bioeng.* 20 (8): 1267–1284.

Roglic, U., Plazl, P., and Plazl, I. (2005). The influence of β-cyclodextrin on the kinetics of progesterone transformation by *Rhizopus* nigricans. *Biocatal. Biotransform.* 23 (5): 299–305.

Silbiger, E. and Freeman, A. (1988). Continuous non-aerated Δ^1-dehydrogenation of hydrocortisone by PAHH-bead entrapped *Arthrobacter simplex*. *Appl. Microbiol. Biotechnol.* 29: 413–418.

Singer, Y., Shity, H., and Bar, R. (1991). Microbial transformations in a cyclodextrin medium (part 2. reduction of androstenedione to testosterone by *Saccharomyces cerevisiae*). *Appl. Microbiol. Biotechnol.* 35: 731–737.

Sripalakit, P., Wichai, U., and Saraphanchotiwitthaya, A. (2006). Biotransformation of various natural sterols to androstenones by *Mycobacterium* sp. and some steroid-converting microbial strains. *J. Mol. Catal. B: Enzym.* 41 (1-2): 49–54.

Wang, Z., Zhao, F., Chen, D., and Li, D. (2006). Biotransformation of phytosterol to produce androsta-diene-dione by resting cells of *Mycobacterium* in cloud point system. *Process Biochem.* 41: 557–561.

Weintraub, H., Baulieu, E.E., and Alfsen, A. (1980). Influence of the position of the double bond in steroid substrates on the efficiency of the proton-transfer reaction by *Pseudomonas testosteroni* 3-oxo steroid Δ^4-Δ^5-isomerase. *Biochem. J.* 185: 723–732.

Yaderetes, V., Andryushina, V., Bartoshevich, Y. et al. (2007). A study of steroid hydroxylation activity of *Curvularia lunata* mycelium. *Prikladnaya Biokhimiya I Mikrobiologiya* 43 (6): 697–702.

Further Reading

Barredo, J.L. and Herráiz, I. (2017). *Microbial Steroids: Methods and Protocols*. New York: Humana Press.

Bhatti, H. and Khera, R. (2012). Biological transformations of steroidal compounds: a review. *Steroids* 77 (12): 1267–1290.

Carvalho, C. (2016). Whole cell biocatalysts: essential workers from nature to the industry. *Microb. Biotechnol.* 10: 250–263.

Donova, M.V. (2017). Steroid bioconversions. *Microb. Steroids* 1645: 1–13.

Jabbar, Z.A., Maryam, H., Maqsood, S., and Ali, S. (2017). Microbial transformation of steroids: a focus on types and techniques. *World J. Pharm. Life Sci.* 3 (5): 193–203.

Jones, J. and Wigfield, D. (1969). Steroids and steroidases. IX. Activation parameters and the mechanism of base-and enzyme-catalyzed isomerizations of androst-5-ene-3, 17-dione. The nature of the active center of the $\Delta^5 \rightarrow \Delta^4$-3-ketosteroid isomerase of *Pseudomonas testosterone*. *Can. J. Chem.* 47: 4459–4466.

13

Bioleaching

Sören Bellenberg[1], *Mario Vera Véliz*[2,3], *and Wolfgang Sand*[4,5]

[1] *Linnaeus University, Centre for Ecology and Evolution in Microbial Model Systems, Kalmar, 392 31, Sweden*
[2] *Pontificia Universidad Católica de Chile, Institute for Biological and Medical Engineering, Schools of Engineering, Medicine and Biological Sciences, Santiago, 8331150, Chile*
[3] *Pontificia Universidad Católica de Chile, School of Engineering, Department of Hydraulic and Environmental Engineering, Santiago, 8331150, Chile*
[4] *Technische Universität Bergakademie Freiberg, Insitute of Biosciences, Leipziger Str.29, 09599 Freiberg, Germany*
[5] *Donghua University, College of Environmental Engineering, Songjiang, Shanghai, 200333, PR China*

CHAPTER MENU

13.1 Acidophilic Microorganisms Dissolve Metals from Sulfide Ores

Biomining technologies are becoming more established as experience in industrial scale has been obtained within the past 50 years. It describes the biological processing of ores for metal recovery using microorganisms for bioleaching or biooxidation processes. Biomining is currently the only relevant application of acidophilic, chemolithoautotrophic microorganisms. However, heterotrophic microorganisms also play an important role in commercially applied consortia of acidophilic bioleaching microorganisms. Bioleaching is the term used for the process where the target metals, such as copper (Cu), nickel (Ni), cobalt (Co), uranium (U), or zinc (Zn), are solubilized into the leach solution. Metal sulfides that encase insoluble target metals, such as gold (Au), can be oxidized and dissolved by biologically catalyzed chemical reactions, in order to increase the accessibility of gold for subsequent cyanide leaching. Consequently, in that case, the term biooxidation is used.

The target metals can be further recovered as valuable resources after the biological processing either from the leachate (e.g. Cu, Ni, Zn, Co, U, and other

Industrial Microbiology, First Edition.
Edited by David B. Wilson, Hermann Sahm, Klaus-Peter Stahmann, and Mattheos Koffas.

metals) or from the solid mineral residue (e.g. gold [Au], silver [Ag], and lead [Pb]). Advantages of biomining technologies compared to smelting and roasting of metal-bearing ores include environmental, energetic, and, because of the ever-decreasing metal grade of available ores, economic aspects. The operation of biomining processes is performed at lower temperatures and pressures than pyrometallurgical and hydrometallurgical processing options, such as smelting or pressure autoclave leaching. In comparison to chemical leaching, biomining technologies demand low additions of chemicals. Furthermore, despite that solvent extraction and electrochemical winning of, e.g. copper cathodes are energy-intensive processes, biomining poses a reduced energy footprint also at the extraction and refining stage. Biohydrometallurgical processing of low-grade ores is an economical and environmentally friendly alternative to established chemical and physical mineral processing strategies, as bioleaching takes place in a controlled environment, preventing seepage of heavy metal containing acid solutions into the environment. Biomining has considerable potential as a leading metal recovery strategy in the future. Considerable driving forces for this development are, in addition to those factors mentioned above, environmental liabilities and more strict legislations aiming at lowering the environmental impact, land use, and, especially, CO_2 emissions of mining and metal recovery technologies. Challenges for application of biomining technologies include their slow kinetics, resulting in long reaction times and, in some cases, unpredictable robustness and reliability of the bioleaching processes with respect to target metal production. This main disadvantage has caused biomining to remain rather a niche than a mainstream technology within the mining industry. Nevertheless, estimates of 15–20% of copper (Cu), 3–5% of gold (Au), and smaller percentages of cobalt (Co), nickel (Ni), and zinc (Zn) confirm that this biotechnology has been established in the mining industry.

In the past, ore bodies were mined if they contained significantly higher target metal grades than those available today, and the exploitation of ever lower-grade resources leads to innovations in technologies for separation of gangue minerals from valuable metal resources. Regarding copper mining, ore grades are today typically in the range between 0.25% and 1% copper. The low concentration of the target metal may not allow for an economical pyrometallurgical process, as energy costs for beneficiation processes increase extraordinarily with ever-decreasing ore grades. Hence, pyrometallurgy has its limitations. That applies especially for low-grade metal sulfide ores, when after grinding metal-bearing ore particles may not be efficiently separated from target metal-deficient minerals with similar density, flotation, or magnetic properties or simply when the energy costs for separation processes exceed critical margins. In addition, polymetallic ores and ores that contain contaminants such as arsenic (As), bismuth (Bi), lead (Pb), and antimony (Sb) have become interesting resources for biomining operations. These metal contaminants pose problems when processed by traditional pyrometallurgy, as environmental issues may arise because of emission of pollutants.

At the commercial scale, biomining biotechnologies are currently restricted to the recovery of metals from metal sulfides. This chapter gives a brief excerpt of the most important microbial species present in bioleaching habitats. Leaching

mechanisms as well as the relevance of biofilm formation by mineral-oxidizing microorganisms, such as *Acidithiobacillus ferrooxidans* on the metal sulfide ores, are summarized. The economical impact of the biomining technology is outlined for copper and gold production. In addition, perspectives of future biomining technologies such as reductive leaching and *in situ* leaching are discussed.

13.1.1 Brief Overview on the Diversity of Acidophilic Mineral-Oxidizing Microorganisms

Microorganisms used in biomining technologies are truly acid-loving bacteria and consequently termed "acidophiles", which have their optimum growth pH below 3. These include chemolithotrophic bacteria and archaea capable to use reduced inorganic sulfur compounds (RISC) or iron(II) ions as their energy source (Table 13.1). Mesophilic and moderately thermophilic bacterial and archaeal species have been described.

Those acidophilic microorganisms dissolve metal sulfides by generating sulfuric acid or iron(III) ions. Acid-soluble metal sulfides dissolve by acid attack, while both acid-soluble and acid-insoluble metal sulfides are dissolved due to chemical oxidation of the sulfide ions in the mineral structure by the oxidative agent iron(III) ions. Several mineral-oxidizing species are strict autotrophs, meaning that they obtain their carbon only by CO_2 fixation. However, heterotrophic and mixotrophic microorganisms coexist in these habitats. Heterotrophic and mixotrophic acidophiles have important ecological functions concerning utilization of organic carbon in acidic environments. Several species possess the ability to grow under anaerobic conditions, by using iron(III) ions as a terminal electron acceptor for oxidation of RISCs.

13.1.2 Natural and Man-Made Habitats of Mineral-oxidizing Microorganisms

Mineral-oxidizing acidophiles occur ubiquitously in soils concomitantly with the presence of their energetic substrates, depending on local geological and environmental characteristics. Sulfur-oxidizing acidophilic bacteria actively produce sulfuric acid by oxidation of RISCs and, consequently, acidify their environment. Under acidic conditions, metal cations are solubilized. In addition, at pH below 4, the oxidation of iron(II) ions by oxygen does not occur spontaneously, as it does at neutral pH. Consequently, iron(II) ions are chemically stable and readily available as electron donor for microbial life under acidic conditions. Iron(II) ion oxidation is biologically accelerated by acidophilic iron-oxidizing microorganisms by a factor of 10^6. The iron(II) ions in turn are provided due to the chemical oxidation of sulfide minerals by iron(III) ions. Consequently, an iron cycle that is largely accelerated by the metabolism of iron-oxidizing acidophiles drives oxidation and dissolution of metal sulfides. At the same time, microbial sulfur oxidation degrades poorly soluble intermediate sulfur compounds providing sulfuric acid. Hence, it maintains low pH and ensures solubility of metal ions.

Natural acidic environments that harbor acidophilic iron- and/or sulfur-oxidizing microorganisms include geothermal sites, deep sea hydrothermal

Table 13.1 Selection of acidophilic mineral-oxidizing microorganisms.

Species	Optimum of growth		Oxidation of		Utilization of C_{org}	Reduction of Iron(III)
	pH	Temperature (°C)	Iron(II)	RISC[a)]		
Mineral-oxidizing bacteria						
Acidiferrobacter thiooxydans	2.0	38	+	+		+
Acidimicrobium ferrooxidans	2.0	45–50	+		+	+
Acidithiobacillus caldus	2–2.5	45		+		+
Acidithiobacillus ferridurans	2.0	30	+	+		+
Acidithiobacillus ferriphilus	2.0	30	+	+		+
Acidithiobacillus ferrivorans	2.5	27–32	+	+		+
Acidithiobacillus ferrooxidans	2.5	30–35	+	+		+
Acidithiobacillus thiooxidans	2.0–3.0	28–30		+		+
Ferrimicrobium acidiphilum	2.0	35	+		+	+
Leptospirillum ferriphilum	1.5–3.0	30–37	+			
Leptospirillum ferrooxidans	1.3–1.8	28–30	+			
Sulfobacillus thermosulfidooxidans	2.0	45–48	+	+	+	+
Mineral-oxidizing archaea						
Acidianus brierleyi	1.5–2.0	70	+	+	+	
Ferroplasma acidiphilum	1.7	35	+		+	
Metallosphaera hakonensis	2.0–3.0	70	+	+	+	
Sulfolobus metallicus	2.0–3.0	65	+	+	+	

a) Reduced Inorganic Sulfur Compounds

Source: Data from Schippers et al. (2014), with permission of Springer Nature.

vents, soils rich in sulfide sediments, such as acid sulfate soils, and naturally surface-exposed sulfide ore deposits. The most studied example of a natural habitat of mineral-oxidizing microorganisms is Rio Tinto, Spain (Figure 13.1). This river of about 100 km in length drains the Iberian pyrite belt and flows into the Mediterranean Sea near the city of Huelva. Activity of leaching bacteria in the pyrite-rich subsurface is reported to form the heavy metal and sulfate laden stream that is entirely characterized by pH values of 1.5–3.1. Its characteristic red color indicates a high load of iron(III) ions. Other natural habitats occur due to geothermal and volcanic activity as the source for RISCs and iron(II) ions that serve as electron donors for polyextremophilic microorganisms, which thrive at high temperatures and low pH. Consequently, acidic streams, which harbor acidophilic microorganisms, are associated with geothermal and volcanic activity all over the world.

However, in most perceived cases, human activity has created habitats for leaching microorganisms. Mining activities in historical times, and especially the technological advances in mining efforts over the past 200 years, have increased the amount of habitats for mineral-oxidizing microorganisms. Unwanted bioleaching causes acid mine drainage (AMD)/acid rock drainage (ARD), which

Figure 13.1 Rio Tinto, Spain, is an example for a natural habitat of mineral-oxidizing microorganisms. The river is characterized by low pH values (1.5–3.1) and high loads of sulfate, iron, and other heavy metals over its entire length of about 100 km. Its source is in the Sierra Morena in the Iberian pyrite belt, where the geological situation represents a natural exposure of metal sulfides to oxygen and water. The microbial oxidation is not restricted to the immediate earths' surface. Bacteria have been found in the deeper subsurface aquifers and pores of the pyrite-containing rocks. Source: Photograph by Ricardo Amils.

is generated, because of the hauling of metal sulfide-rich overburden and mining waste rock material enabling contact with oxygen and water and its microbial oxidation. AMD is characterized by low pH and elevated concentrations of sulfate and heavy metals. As such, not only iron, copper, and zinc but also arsenic, cadmium, and other hazardous elements are mobilized. These processes may result in major environmental damages and health threats. In some cases, water resources for local human populations are affected. AMD may especially become a problem because of low environmental standards and weak law enforcement, causing health hazards and social problems, especially in developing countries. Mine closure, reclamation, remediation, and water management nowadays must be considered at the planning stage of mining projects. As such, water treatment systems for mine waters are mandatory.

Often AMD is treated by addition of lime (CaO, $Ca(OH)_2$, and $CaCO_3$) to neutralize effluents and to precipitate metals as hydroxides and sulfate as gypsum. The carbon footprint of this technology is evident due to emissions for transport of limestone, CO_2 release during the production of lime from limestone, and its release due to the dissolution of carbonate minerals in the acidic effluents. Novel mine water treatment systems for efficient removal of metals and sulfate have been described and applied. These treatment plants include a series of compartments, comprising aerobic wetlands for iron oxidation and precipitation, followed by an anaerobic compost bioreactor for generation of alkalinity and removal of sulfate as sulfides that precipitate with chalcophilic metals. The latter can be achieved in a single step due to the activity of acid-tolerant sulfate-reducing bacteria. In a last step, non-chalcophilic metals and organic carbon are eliminated by filtration and activity of chemoorganotrophic microorganisms in sand filtration systems. However, another approach for the removal of sulfate and iron(II) ions from mine waters in the Lusatian lignite mining district has been described. The effluent with a pH of 4.0–4.5 is treated by the activity of biofilms of acidophilic and/or acid-tolerant microorganisms for iron oxidation and subsequent precipitation of iron as schwertmannite coming along with coprecipitation of other metals.

In the future, instead of remediation, the most effective measures against AMD will target the inhibition of its generation. Such technologies employing carbonaceous phosphate mining waste for inhibition of acidophilic mineral-oxidizing microorganisms and proliferating growth of neutrophilic heterotrophs have been successfully tested in laboratory and field experiments but are not yet established in large-scale industrial application.

13.1.3 Biological Catalysis of Metal Sulfide Oxidation

Based on the acid solubility of metal sulfides, there are two different bioleaching pathways relevant for acid-soluble or acid-insoluble metal sulfides (Figure 13.2). The thiosulfate pathway is involved in the dissolution of acid-insoluble metal sulfides. Acid-insoluble minerals, such as pyrite (FeS_2) and molybdenite (MoS_2), are oxidized by iron(III) ions through several oxidation steps to release thiosulfate (Eq. (13.1)). Thiosulfate is oxidized biologically to sulfate (Eq. (13.2)). In the

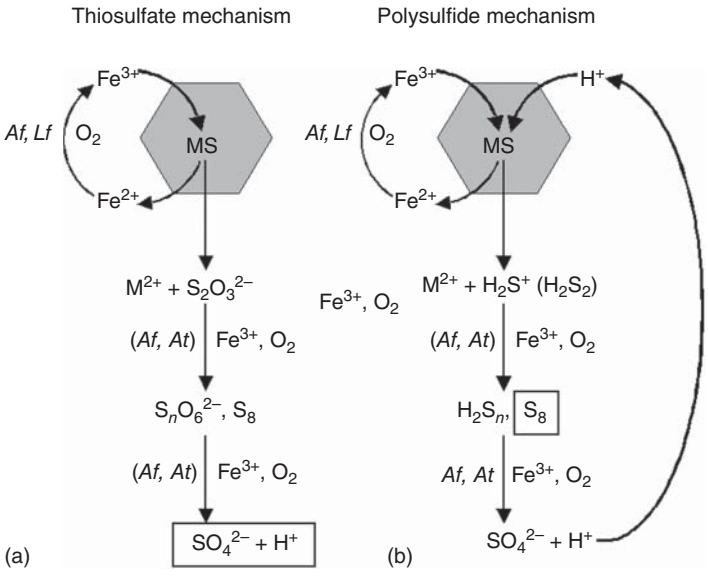


Figure 13.2 Schematic comparison of the thiosulfate (a) and polysulfide (b) mechanisms in the (bio)leaching of metal sulfides. Iron(III) ions attack metal sulfides (MS) by electron extraction and are thereby reduced to iron(II) ions. As a result, the metal sulfide crystal releases metal cations (M^{2+}) and water-soluble intermediary sulfur compounds. Iron(II)-oxidizing bacteria such as *A. ferrooxidans* (*Af*) and *L. ferrooxidans* (*Lf*) catalyze the recycling of iron(III) ions in acidic solutions. In the case of acid-soluble metal sulfides (b), an additional attack is performed by protons, which can bind the valence band electrons of these metal sulfides. The liberated sulfur compounds are oxidized abiotically and by sulfur-oxidizing bacteria such as *A. ferrooxidans* and *A. thiooxidans*. In the case of mainly abiotically driven reactions, the contribution of sulfur oxidizers is indicated in brackets. The main reaction products that accumulate in the absence of sulfur compound oxidizers are boxed, i.e. sulfuric acid and elemental sulfur (b), in the case of acid-insoluble and acid-soluble metal sulfides, respectively. Source: Vera et al. (2013). Reproduced with permission of Springer.

presence of iron-oxidizing microorganisms and absence of sulfur-oxidizing acidophiles, iron(III) ions are available for the chemical oxidation of thiosulfate to sulfate (Eq. (13.3)). In addition, in the absence of sulfur-oxidizing microorganisms, significant amounts of elemental sulfur accumulate. It is formed due to chemical cleavage of thiosulfate according to Eq. (13.4):

$$FeS_2 + 6\,Fe^{3+} + 3\,H_2O \rightarrow {S_2O_3}^{2-} + 7\,Fe^{2+} + 6\,H^+ \tag{13.1}$$

$${S_2O_3}^{2-} + 2\,O_2 + H_2O \rightarrow 2\,{SO_4}^{2-} + 2\,H^+ \tag{13.2}$$

$${S_2O_3}^{2-} + 8\,Fe^{3+} + 5\,H_2O \rightarrow 2\,{SO_4}^{2-} + 8\,Fe^{2+} + 10\,H^+ \tag{13.3}$$

$$8\,{S_2O_3}^{2-} \rightarrow S_8 + 8\,{SO_3}^{2-} \tag{13.4}$$

The polysulfide pathway is relevant for dissolution of acid-soluble metal sulfides, such as galena (PbS), arsenopyrite (FeAsS), sphalerite (ZnS), alabandite (MnS), and chalcopyrite ($CuFeS_2$). These minerals are solubilized through the

combined action of acid (H^+) and iron(III) ions, with the release of hydrogen sulfide (H_2S). Under acidic conditions, the latter undergoes spontaneous rearrangement to polysulfide (Eq. (13.5)), chemical oxidation to elemental sulfur (Eq. (13.6)), and, in the presence of sulfur-oxidizing microorganisms, biological oxidation to sulfate (Eq. (13.7)):

$$PbS + Fe^{3+} + H^+ \rightarrow Pb^{2+} + 0.5\ H_2S_n + Fe^{2+} \quad (n \geq 2) \tag{13.5}$$

$$H_2S_n + 2\,Fe^{3+} \rightarrow 0.25\,S_8 + 2\,Fe^{2+} + 2\,H^+ \tag{13.6}$$

$$0.25\,S_8 + 3\,O_2 + 2\,H_2O \rightarrow 2\,SO_4{}^{2-} + 4\,H^+ \tag{13.7}$$

These two pathways are termed indirect mechanisms, as in both cases, microorganisms do not catalyze a direct enzymatic oxidation of metal sulfides. In acidic solution, the metal sulfides are solely oxidized chemically by iron(III) ions. The oxidized iron(III) ions are supplied by the microbial oxidation of iron(II) ions. As their name implies, acid-soluble metal sulfides are in addition to oxidative attack by iron(III) ions, also dissolved by proton attack.

13.1.4 Importance of Biofilm Formation and Extracellular Polymeric Substances for Bioleaching by *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*

In general, for biologically accelerated metal sulfide oxidation, two leaching modes have been proposed, which refer to the perspective of the microbial cells involved: contact and noncontact leaching. Noncontact leaching is carried out by free-swimming, planktonic cells that provide iron(III) ions by oxidation of iron(II) ions. The iron(III) ions are reduced at the mineral surface and thereby the sulfide moiety of the sulfide mineral is oxidized. Consequently, iron(II) ions are regenerated and enter the catalytic cycle again by serving as energy source for the iron-oxidizing microorganisms. In contrast to that concept, contact leaching takes into account the biofilm-forming cells, which are attached on the surface of metal sulfides. In this case, the electrochemical processes resulting in metal sulfide dissolution are enhanced in the interface between the microbial cells and the metal sulfide surface (Figure 13.3).

Some cells of motile leaching bacterial strains show chemotactic attraction toward iron(II) ions and/or RISC. These compounds may be released from metal sulfides that undergo dissolution. Local anodes are chemically reactive mineral sites at which the chemical metal sulfide oxidation and subsequent dissolution occurs preferentially due to low crystallinity, crystal lattice defect sites, or presence of heteroatoms in the crystal structure. Cells may be often found attached, preferentially at those sites also due to enhanced contact area at amorphous mineral surfaces, corrosion pits, and crystal defect sites. In addition, cells remain attached to the mineral especially when protected from potential shear forces in corrosion pits and in mineral pores (Figure 13.4).

The most common and widespread lifestyle of microbes on earth is in the form of biofilms. They are communities of different species of microorganisms on surfaces of materials, which are embedded in a self-produced matrix of

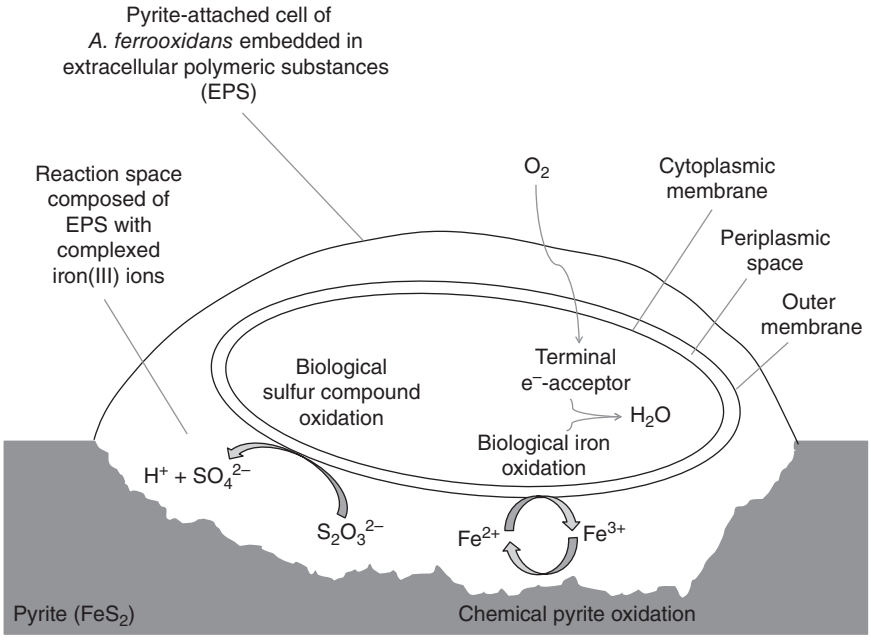


Figure 13.3 Mechanism of pyrite oxidation by a single cell of *A. ferrooxidans*. The scheme shows its embedment in extracellular polymeric substances (EPS). The bacteria produce micrometer-size corrosion pits after attachment on a plain surface of pyrite. Cytoplasmic membrane, periplasmic space, and outer membrane are indicated. Dimensions are not in scale as the rod-shaped bacterial cell measures 1–2 μm in length and approximately 0.5 μm in diameter, while the distance between bacterial cell and the mineral measures up to 100 nm at the interface. Here, the EPS are forming a reaction space due to functional glucuronic acid residues that enrich Fe^{3+} by complex binding. The chemical oxidation of pyrite by Fe^{3+} releases $S_2O_3^{2-}$ and Fe^{2+}. Biological Fe^{2+} oxidation with O_2 as terminal electron acceptor regenerates Fe^{3+}. Biological and chemical oxidation of $S_2O_3^{2-}$ produces SO_4^{2-} and H^+. Source: modified from Rohwerder and Sand (2007). Modified with permission of Springer.

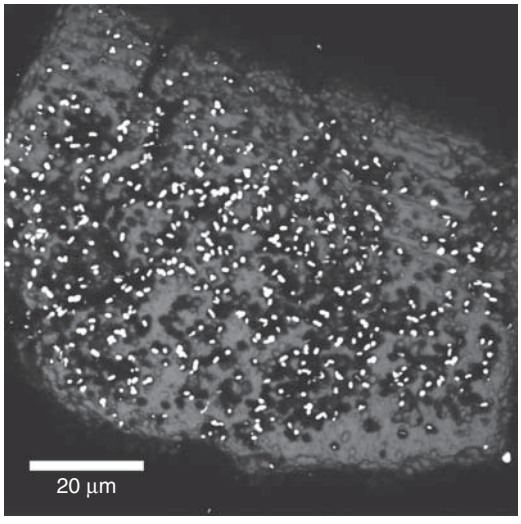


Figure 13.4 Bacterial cells attached on a pyrite particle. A 3D projection after confocal laser scanning microscopy of a pyrite grain colonized with *A. ferrooxidans* is shown. The grain was incubated with the bacteria for 18 days. Nucleic acid-staining (white) visualized mineral-oxidizing microorganisms on the surface (gray). Cell attachment often correlates with corrosion pits or surface imperfections. Source: Photograph by Sören Bellenberg.

extracellular polymeric substances (EPS). This slime matrix mainly consists of polysaccharides, proteins, nucleic acids, and lipophilic compounds. Biofilms can be found as surface-associated cell agglomerates, occurring at solid–liquid, air–liquid interfaces or filamentous streamers or mats of cells embedded in EPS. The biofilm lifestyle protects cells from environmental stresses such as desiccation, nutrient starvation, radiation, and/or oxidative stress.

In general, biofilms in both engineered bioleaching systems and environmental acidophilic AMD habitats are characterized by a relative low species abundance, which is restricted by the extremely acidic pH, high concentration of heavy metals, and the narrow range of electron donors and acceptors available. In general, these biofilms are dominated by iron-oxidizing chemolithoautotrophs such as *Leptospirillum* spp. and mixed with populations of heterotrophic or mixotrophic bacteria, archaea, and eukaryotes.

In bioleaching, consideration of the contact between microorganisms and the mineral surface is of high importance. Cell attachment and subsequent biofilm formation on metal sulfides increase leaching activity. A microenvironment is formed between the cells and the mineral (Figure 13.3). The metal sulfide dissolution occurs at this interface, as it is a reaction space that is composed of EPS, which are functionalized for bioleaching activity (Figure 13.4). Biofilms formed by autotrophic acidophiles on pyrite differ significantly from biofilms formed by neutrophilic and heterotrophic microorganisms on other surfaces. On pyrite, only very thin biofilms of a single cell layer are formed, compared to several hundreds of micrometer to millimeter range three-dimensional biofilm architectures known from neutrophilic species on various substrata. The thin biofilm structure in combination with the never fully covered mineral surface allows efficient bioleaching, while massive biofilm growth would inhibit pyrite oxidation due to diffusion-limited transport processes causing subsequent starvation of chemolithotrophic microorganisms. In addition, lithotrophic growth with iron(II) ions and RISCs for CO_2 fixation and limited carbon availability explain the fact that mineral-oxidizing acidophiles produce significantly lower amounts of EPS than heterotrophic, neutrophilic microorganisms. In *A. ferrooxidans*, EPS are mainly composed of polysaccharides, lipids, and uronic acids. The composition of EPS from pyrite-grown cells is comparable to those of iron(II)-grown cells. They consist of the monosaccharides glucose, rhamnose, fucose, xylose, mannose, fatty acids with saturated alkyl chains of 12–20 carbon atoms, glucuronic acid, and iron(III) ions. However, pyrite-grown cells roughly possess 10-fold higher amounts of EPS compared to those of iron(II)-grown cells, and this difference can be explained by the presence of additional metabolic energy from sulfur oxidation and also underlines their importance for pyrite oxidation. The EPS of *A. ferrooxidans* cells, which grew in the presence of iron, binds iron(III) ions by uronic acid residues in their polysaccharide matrix, which act as ligands.

The first function of these complexed iron(III) cations is the mediation of cell attachment by electrostatic attraction of cells to the negatively charged pyrite surface. The iron ions provide a net positive surface potential to the bacterial cells, which binds them electrostatically to sulfide minerals. They are characterized by negative surface potentials under acidic conditions. The second function

of complexed iron(III) ions is to mediate the oxidative dissolution of the metal sulfide, equally to dissolved iron(III) ions in noncontact leaching. The EPS of pyrite attached cells is consequently of fundamental importance for attachment and bioleaching activity. Especially when total iron ion concentrations are low in the bulk leach solution (<200 mg/l), the biofilm cell population has been demonstrated to be exclusively relevant for the initiation of the dissolution of pyrite. This is related to the attachment of microbial cells to the metal sulfide mineral and the accumulation of iron(III) ions in EPS of attached cells, which cause and facilitate the initiation of the bioleaching process. EPS can be visualized directly using fluorescently labeled lectins, i.e. proteins that bind carbohydrate moieties (Figure 13.5).

In general, the majority of bioleaching microorganisms can form biofilms on metal sulfides. Assuming a nonlimiting metal sulfide surface area, up to 80–90% of a certain inoculum may attach to metal sulfides within 24 hours. However, some cells always remain in planktonic state. It is important to remark that the initial cell attachment ratios of different microbial species strongly depend on the nature of the species as well as their precultivation conditions. In general, *Leptospirillum* spp. show higher attachment rates to pyrite than iron-oxidizing *Acidithiobacillus* spp., and for *A. ferrooxidans*, iron(II)- or pyrite-grown cells show higher attachment rates to pyrite than sulfur-grown cells. This observation is explained by changes in EPS composition. Sulfur-grown cells lack glucuronic acid residues and consequently also complexed iron(III) ions in their EPS. In addition to electrostatic interactions, hydrophobic interactions also contribute to the attachment of cells to metal sulfide surfaces. This applies especially to very hydrophobic surfaces, e.g. of elemental sulfur, which may form on acid-soluble metal sulfides. EPS of *A. ferrooxidans* cells grown on elemental sulfur contain considerably more lipids compared to those of pyrite-grown cells. Biofilm formation is also important for the persistence of the microorganisms in bioleaching reactors as cell attachment prevents washout of cells with the leach liquor and ensures that biomass will be retained. Consequently, improvement of

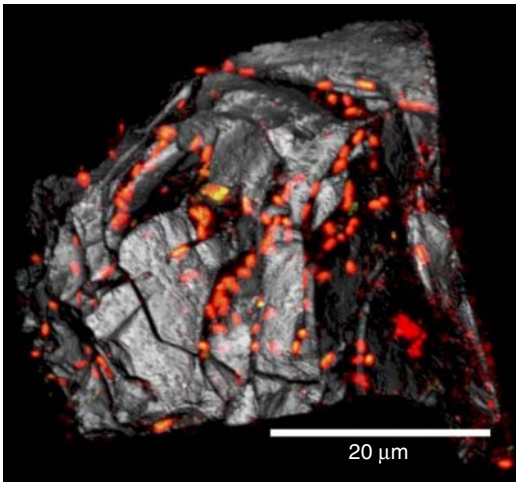


Figure 13.5 Capsular polysaccharides of pyrite-attached *A. ferrooxidans* cells. Confocal laser scanning microscopy image showing a 3D projection of a pyrite grain (50 µm) colonized with cells of *A. ferrooxidans* after one week of incubation. Polysaccharides (red) and intracellular nucleic acid (green and yellow) are visualized. From Vera et al. (2013), with permission of Springer Nature.

biofilm formation on metal sulfides bears potential for further improvement of bioleaching technologies.

Biofilm formation and EPS biosynthesis in *A. ferrooxidans* are regulated at different levels, comprising among others, energy substrates, inorganic phosphate (P_i) limitation, and cell–cell communication mechanisms of quorum sensing (QS). Among many other factors, in industrial processes, cell attachment is primarily influenced by the accessibility of the metal sulfide ore in the gangue mineral. Leaching efficiency is therefore enhanced by crushing and milling of the ore to increase the available metal sulfide surface area that is in contact with the leach liquor and available for surface reactions, cell attachment, and biofilm formation. Different approaches allow contact of the leaching agent and the microbial cells with the metal sulfide ore. These approaches are, for example, tank-, heap-, dump-, and *in situ* bioleaching systems.

13.2 Bioleaching of Copper, Nickel, Zinc, and Cobalt

Ores of the metals copper, zinc, and nickel are predominantly metal sulfides. The largest remaining copper deposits exist in the form of the primary copper sulfide mineral chalcopyrite ($CuFeS_2$). Even though copper sulfides are more refractory than the copper oxide ores, they are suitable for biological leaching. Therefore, research in biomining is currently focused on increasing chalcopyrite bioleaching efficiency. The use of bioleaching for the treatment of pure chalcopyrite concentrates has not found commercial application because of its slow bioleaching kinetics and mainly due to established, trusted, and competitive smelter technologies. As mentioned before, application of bioleaching operations may be suitable when ore grades do not justify the effort for beneficiation and smelting processes or when contaminant elements are abundant in ore concentrates. As an example for the latter, polymetallic copper concentrates were the subject of a biotechnical process that was progressed to demonstration in 2001 and further into successful commission by Mintek and partners, Industrias Peñoles S.A. de C.V. of Mexico and BacTech. Copper, zinc, and silver were recovered from the leachate for the revenue of that project.

13.2.1 Economic Impact

Established (bio)-hydrometallurgical processes include chemical and/or biological heap leaching of copper oxide ores and secondary copper sulfide ores. About 80% of the copper obtained by biomining originates from the secondary copper sulfides chalcocite (Cu_2S) and covellite (CuS). The remaining 20% accounts for the contribution of copper oxides that co-occur in copper sulfide ores and for bioleaching of the most abundant but also most refractory copper sulfide ore, chalcopyrite ($CuFeS_2$). A strong increase in hydrometallurgical winning of copper by chemical and biological leaching from 10% to 20% of total copper production occurred during the 1990s until the beginning of the twenty-first century. The proportion of copper produced by leaching remained until 2010 at around

21%. The overall mine copper production had increased by 62% in the previous 15 years. Consequently, despite the fact that absolute numbers of copper production by (bio)-hydrometallurgical processes have grown, a significant percentage increase of the share of copper from bioleaching operations of the total copper production has not been observed.

The respective copper projects are often open-pit mines, such as the one shown in Figure 13.6, illustrating the dimensions of such open-pit mining sites in northern Chile.

In 1981, copper production from (bio)-leaching processes accounted to around 0.6 Mt and increased to around 3.3 Mt in 2010. In the same time, the total production of copper increased from about 8 Mt to about 16 Mt. Data from 2010 show that copper production by biomining represented at least 8% (1.25 Mt) of the total copper production of 15.7 Mt. These values do not include production from dump copper leaching. However, in dump copper mining, bioleaching is the main driving force for copper solubilization that it is not engineered to be optimized. More optimistic estimates suggest that bioleaching nowadays accounts for 15–20% of the world copper production. Biomining operations in Chile represented 72.9% of the global bioleaching copper production (1.25 Mt) in 2010. In the same year, bioleaching plants in the United States, Peru, China, Myanmar, and Australia contributed 18.2%, 5.2%, 2.6%, 0.7%, and 0.3%, respectively. A selection of current copper mining operations in Chile that apply bioleaching in parts of their mineral processing solutions are shown in Table 13.2. Consequently, the economical impact of biological copper mining is significant, and it is expected to increase in the future. Among the reasons for that is the increasing shortage of high-grade copper ores, which make low-grade and often polymetallic ores the remaining choice for exploitation by the mining industry.

Figure 13.6 Open-pit copper mine in northern Chile. The terrace height is 20–25 m. Also note the haul truck in the middle as a size reference. Source: Photograph by Sören Bellenberg.

Table 13.2 Copper mining operations with bioleaching processes in 2010 in Chile.

Copper mine	Ore reserves (Mt)	Copper grade (%)	Copper production (kt copper/yr)
Lomas Bayas	73	0.36	75
Escondida Norte	1701	0.55	180
Zaldivar	235	0.91	150
Cerro Colorado	204	1.02	100
Spence	370	1.06	200
Quebrada Blanca	130	1.15	75

Source: Neale et al. (2011). Data with permission of The Southern African Institute of Mining & Metallurgy.

Compared to copper production, bioleaching of other metals such as nickel, cobalt, and zinc is not as well established. However, technologies have been developed and are in industrial use. One example is the heap bioleaching of a polymetallic low-grade ore (Talvivaara, Finland). In 2011, about 16 000 tons of nickel and nearly 32 000 tons of zinc were recovered while in the case of full production estimated for 2015, 50 000 tons nickel (Ni), 90 000 tons zinc (Zn), 15 000 tons of copper (Cu), and 1800 tons of cobalt (Co) per year were targeted. Consequently, this heap bioleaching project could have yielded about 3% of the world's primary nickel production. However, this project is a marked step backward for the implementation of biohydrometallurgical mining technologies. Presumably, because of irresponsible water management and unrealistic legal boundaries concerning environmental liabilities, the needed capacities of the project for water retention and on-site treatment for a safe discharge to the environment were not met. It finally resulted in an environmental disaster caused by leakages of heavy metal containing slurries from tailing ponds into the environment.

In a stirred-tank bioleaching extraction plant near Kasese, Uganda, pyrite concentrate containing cobalt, copper, nickel, and zinc has been processed, producing about 1100 tons cobalt/year. These are about 1.3% of world production of cobalt, which amounted to around 88 000 tons in 2010. The operation ceased in 2013 as the ore resource available on-site was depleted.

An opportunity for biological recovery of nickel was identified for the treatment of a nickel sulfide concentrate produced as a by-product of Mondo Mineral's talc mining operations in Vuonos and Sotkamo in Finland. The high-grade nickel concentrate that was generated at both sites by magnetic and flotation separation techniques contains arsenic as impurities in metal sulfides such as pentlandite ($(Fe,Ni)_9S_8$), pyrrhotite ($Fe_{(1-x)}S$ [$x = 0$–0.2]), pyrite (FeS_2), and gersdorffite (NiAsS). Because of the content of arsenic, smelting was not an option. In addition, stockpiling of the value-bearing and otherwise waste material was not acceptable as it would also be an unacceptable environmental risk for the generation of AMD. Therefore, bioleaching was considered to produce a valuable nickel product and, at the same time, using the same process to allow arsenic separation and to avoid AMD from uncontrolled sulfide oxidation in the alternative

Figure 13.7 Mondo Minerals bioleach plant in Vuonos, Finland. (a) Concentrate is fed into four primary tanks operating in parallel. The secondary oxidation stage comprises another four tanks in series, which provide sufficient residence time for the target level of biologically catalyzed sulfide oxidation and sufficient reactor units to minimize short circuiting of the slurry; detail views on the stirred-tank impeller, cooling baffles (b) and the aeration ring (c). With permission from Neale et al. (2016).

waste stockpiles or tailings. It was demonstrated that bioleaching is an economically viable option for this particular case. The stirred-tank bioleaching plant was developed in close cooperation with Mintek (South Africa) and went into commission in 2016 (Figure 13.7). It is expected that this plant will treat approximately 12 000 tons of nickel ore concentrate/year and produce around 1000 tons of nickel annually.

13.2.2 Heap, Dump, or Stirred-tank Bioleaching of Copper, Nickel, Zinc, and Cobalt

The biohydrometallurgical processing of metal sulfide ores is currently distinguished by the following three processes:

- Heap or dump bioleaching of low-grade, secondary copper sulfide containing ores such as chalcocite (Cu_2S) and covellite (CuS)
- Heap or dump bioleaching of low-grade, primary copper sulfide ore, i.e. chalcopyrite ($CuFeS_2$)
- Stirred-tank bioleaching of metal sulfide concentrates.

Especially at smaller scales, stirred-tank biohydrometallurgical plants have potential for application and may prove as advantageous compared to other

competing technologies, in case the grade of the ore is sufficiently high. This statement may sound as conflicting with the general idea that biomining has its main justification because of energetic, environmental, and economic considerations that come along with the exploitation of large quantities of low-grade ores. However, the mineral characteristics, such as the target metal grade, the presence of potential additional target metals in polymetallic ores that are difficult to separate after pyrometallurgical processing or the presence of toxic elements such as arsenic (As) or bismuth (Bi) also impact the suitability of the different metal winning processes, as is illustrated in Figure 13.8. Consequently, biomining strategies are amenable for the recovery of a major target metal, which is in most cases copper (Cu), but also for the integrated recovery of metals such as nickel (Ni), zinc (Zn), cobalt (Co), and others.

In Figure 13.9, common copper ore grades of the most large-scale, recent heap leaching copper mining projects are plotted against their respective annual copper production volume. It becomes evident that copper ores with metal grades as low as 0.25–1% are currently mined and subjected to chemical and/or biological heap leaching. Regarding copper leaching, heap bioleaching projects that produce less than 50 000 tons copper/year are characterized by higher ore grades than projects that produce more than 50 000 tons copper/year. Those operations are usually characterized by larger quantities of ore reserves, higher ore throughput, longer mine life expectations, and copper grades as low as 0.3% are tolerated. In any case, low-grade ores demand low-cost metal recovery technologies, such as heap (bio)-leaching in order to generate revenue. At higher target metal grades, more sophisticated, effective, and costly processes, such as stirred-tank reactor bioleaching, may become cost-efficient in the future. For copper projects, a threshold ore grade of 0.5% has been suggested for tank

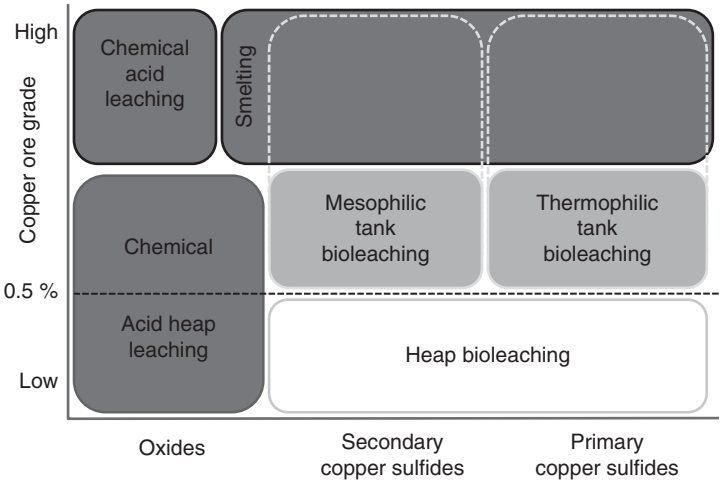


Figure 13.8 Copper extraction technologies. The type of the mineral and its copper grade influence the choice of the technology for metal extraction from a copper ore. Source: Clark et al. (2006). Reproduced with permission of Elsevier.

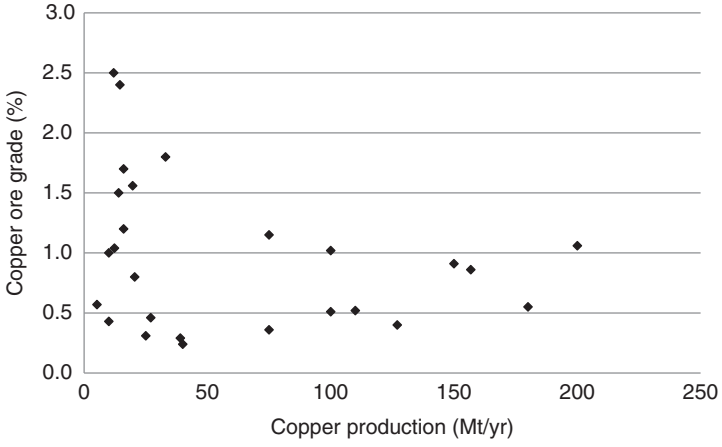


Figure 13.9 Copper ore grades (%) versus annual copper production (Mt/yr) of 12 Chilean and 12 other copper projects that involve chemical and biological heap leaching for copper recovery. Source: From Neale et al. (2011), with permission of The Southern African Institute of Mining & Metallurgy.

leaching (Figure 13.8). Higher target metal grades at current environmental legislation in most mining-relevant countries still justify the classical smelting processes. However, depending on environmental liabilities and legal boundaries, a future perspective for bioleaching technologies exists also due to the mandatory prevention of CO_2 emissions.

Economical relevance regarding copper winning is currently mainly observed for the heap- or dump-leaching processes that are characterized by lower capital and operational costs than those associated with stirred-tank bioleaching operations. Heaps used in hydrometallurgical metal winning processes are very large and, in some cases, several kilometers long, constructed from low-grade ores, which contain metal-bearing ores. For that, the ore is mined, broken to gravel, and piled up in at least 5 m high beds. Several beds may be piled up to heaps reaching 20 m or higher. These heaps are engineered with aeration systems that ensure air supply from the bottom and irrigation systems that distribute the leaching solution from the top onto the heap (Figure 13.10). In order to provide contact of the solution with the metal-bearing rock, while the leaching liquor flows through the heap, a homogeneous distribution of the liquid in an unsaturated system is desired. This allows for efficient oxygen (O_2) and carbon dioxide (CO_2) mass transfer and consequently provides the electron acceptor (O_2) and the carbon source (CO_2) for the chemolithoautotrophic, acidophilic mineral-oxidizing microorganisms. As already mentioned, the microorganisms provide the chemical leaching agents, iron(III) ions, and protons by oxidizing iron(II) ions and sulfur compounds, respectively. Consequently, to ensure efficient metal winning and to avoid product loss and contamination of groundwater systems, the heap is constructed on impermeable plastic bottom liners. Often that is practiced using reusable "on–off" dynamic leach pads (Figure 13.10). The metal-containing pregnant leach solution (PLS) is collected and recirculated. From a side-stream target

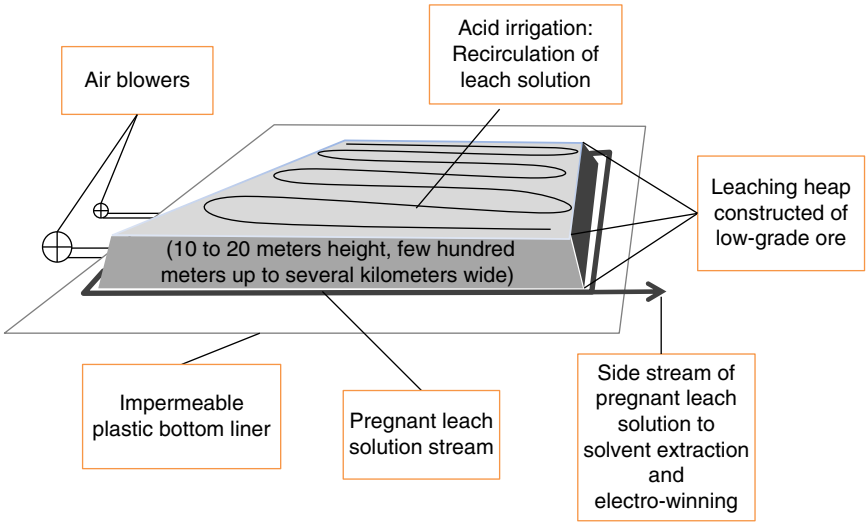


Figure 13.10 Schematic view on a heap bioleaching plant. Source: Drawn by Sören Bellenberg.

metals such as copper are usually recovered by solvent extraction followed by electrowinning, which results in highly pure copper cathodes. Typical copper recovery efficiencies of 80–90% are obtained after periods of 1.5–2 years of recirculation and aeration.

Dump biomining is done in a similar manner. It has been used extensively and for centuries for the recovery of copper from low-grade ores that were considered as waste rock materials. The Kennecott copper dump leaching operation was initiated in the 1950s. In contrast to engineered heaps for bioleaching, the particle size in dump leaching operations is typically much larger (e.g. ~150 mm in dumps compared to <15 mm in heaps) and very variable. High stacking heights, from 15 m to as much as 100 m, are typical and the dumps are leached by irrigation directly at the disposal site. Technical aeration systems are lacking and often inoculation is not done in dump leaching. The acidophilic microorganisms, which occur naturally on the ore, cause the biological oxidation of the metal sulfides. In some cases, microorganisms were added by irrigation in order to accelerate the dump leaching process. Examples of dump bioleaching operations are Rio Tinto (Spain), producing 8000 tons copper/year over the period from 1950 to 1980; Cananea (Mexico), producing 9000 tons copper/year over a similar period, and Duval (United States), producing 2500 tons copper/year from ores containing 0.15–0.20%. Even though productivities and recoveries are low in dump leaching, irrigation of the otherwise waste material is done using low investment and operating costs, which render it to be an economical process.

All copper ore categories, which comprise oxides, primary and secondary sulfides, are classified as acid-soluble. However, copper sulfide ores are more refractory than oxide ores and the biologically driven supply of iron(III) ions is necessary for their dissolution. Copper oxide ores are amenable to chemical leaching by sulfuric acid alone. Because of the absence of iron(II) ions or sulfide

moieties in copper oxides, in addition to the acid consumption of the oxide ores, they are not amenable for bioleaching using mineral-oxidizing, acidophilic microorganisms. Because of the known composition of ores in countries such as Chile and Peru, it is foreseeable that copper production by chemical or biological leaching processes is limited due to the refractory nature of sulfide ores. Copper sulfide dissolution in the case of conventional heap bioleaching at mesophilic temperatures (25–35 °C) is limited to the secondary copper sulfides chalcocite (Cu_2S) and covellite (CuS), which are efficiently bioleached already under mesophilic conditions. The refractory chalcopyrite ($CuFeS_2$) demands thermophilic bioleaching conditions for an efficient copper recovery as specified below. In pilot and demonstration plants, chalcopyrite could be dissolved at elevated temperatures, both in stirred-tank and in heap bioleaching processes. A high copper yield could be obtained with the use of thermophilic archaea (genera *Acidianus*, *Metallosphaera*, and *Sulfolobus*) at 65–75 °C. However, increased temperatures demand higher energy costs especially in cold climatic regions and require improved corrosion resistance of materials as well as increased process management. At the laboratory scale, promising approaches using electrochemically controlled tank bioleaching of chalcopyrite with high copper yields have been demonstrated at ambient temperatures. Chalcopyrite dissolution at low to moderate temperatures is highly depending on the redox potential. Copper extractions greater than 97% have been obtained when operating at low redox potential levels, in the range between 410 and 440 mV (versus Ag/AgCl). These strategies may represent viable process modifications to improve hydrometallurgical processes for processing of chalcopyrite. Furthermore, chemical leaching by a chloride-rich solution is applied for low-grade copper ores. Examples are projects in the north of Chile, where process water is scarce and seawater is the only available water source. However, a recent study reports significantly lower copper recovery from chalcopyrite ores using chemical chloride leaching as compared to bioleaching. Many mineral-oxidizing acidophiles are known to be sensitive toward elevated levels of chloride. Under acidic conditions, a small fraction of chloride ions may be protonated to form HCl. This compound is penetrating cell membranes and in the cytoplasm of the microbial cells HCl dissociates to release the proton, consuming the proton-motive force and acidifying the cytoplasm. Therefore, most mineral-oxidizing acidophilic bacteria are severely inhibited in the presence of chloride. Adaptation to elevated chloride concentrations and discovery of novel chloride-tolerant microorganisms will improve chloride bioleaching. As such, chloride leaching processes may select for chloride-tolerant strains able to oxidize iron(II) ions at elevated chloride concentrations under acidic conditions. However, marine chloride levels of 35 g/l and higher clearly exceed the levels to which some mineral-oxidizing acidophiles have until now been shown to adapt to.

Consequently, percentile increases in copper winning by bioleaching also depend on the development of improved biological leaching processes. In heap operations, the lag-time from start of irrigation and heap inoculation until reasonable metal contents in the PLS prevail may last one year or significantly longer at low temperatures. Consequently, improved inoculation strategies are currently under study in order to reduce the lag-time until metal recovery may

commence. Another aspect that may facilitate bioleaching is the availability of water treatment technologies. Current examples for mining operations in arid regions where seawater is the only available water resource can be found in northern Chile and regions in Australia. Also, evaporation-driven concentration of leach liquors in arid climate regions demands on-site water treatment. Water and its transport pose a significant ecological footprint and costs in the mining industry, especially in the often remote and arid mining areas, e.g. in Australia and South America. Green and sustainable energy supply in these areas enabling water treatment, carbon-neutral ore processing, and transport technologies are of importance for ecological considerations in future mining. Renewable energy supply in these regions would provide environmental benefits during operation of mining sites and could in addition already pave ways for beneficial post-mining infrastructure developments.

13.3 Gold

As for many resources, the easily accessible, leachable deposits that were deposited near surface have been preferentially exploited. In the case of gold, these resources are close to be depleted. Hence, it is consistent that the production of gold will in the future have to target low-grade sulfide ores, which are refractory in terms of gold recovery by cyanide leaching. Here, an oxidative pretreatment of the sulfide ores is necessary. Roasting of the sulfide ore has been the only available economical process for an oxidative pretreatment of gold-encasing sulfide ores preceding cyanide leaching. As already mentioned, low-grade ores in combination with impurities such as arsenic as well as energy costs and carbon footprints render smelting unattractive. Alternatively, a process that can be used is high-pressure oxidation, which is by the same abovementioned reasons also considered unadvantageous. In the 1980s, biooxidation, as an alternative pretreatment process using bioleaching for removal of gold-encasing metal sulfides, was developed and for the first time industrially applied. As a consequence, biomining technologies will become increasingly important, in order to prepare refractory gold-bearing sulfide ores for gold recovery by cyanide leaching.

Nowadays, biooxidation is the technology of choice for the treatment of gold-bearing metal sulfide concentrates. The BIOX™ process is the most successful commercial stirred-tank bioleaching technology established in industry. The first commercial application of bioleaching of refractory gold-bearing sulfide ores was a plant commissioned at the Fairview Gold Mine in 1986. Since then, 12 BIOX® plants using Goldfields technology and two Bacox™ plants using Bactech-Mintek technology have been installed. Table 13.3 shows biooxidation projects and their production data from 2010. As outlined above, the importance of biooxidation for gold production will increase in the future. As such, 13 new projects that include biooxidation processes, with an additional capacity of 30 tons gold/year production, were in 2017 reported to be in the planning phase or close to commissioning.

Table 13.3 Biooxidation gold mining operations and production data from 2010.

Country	Project name	Owner	Gold production (oz/yr)	Gold production (kg/yr)
South Africa	Barberton	Shanduka Resources	98 000	2 778
Ghana	Bogoso–Prestea	Government of Ghana	280 000	7 938
Peru	Coricancha		20 000	567
Australia	Fosterville	NuEnergy Capital Ltd.	112 000	3 175
China	Jiaojia		55 000	1 559
China	Jinfeng	China National Gold Group Corp	150 000	4 252
Ghana	Obuasi	Anglogold Ashanti	400 000	11 340
Russia	Olimpiada	Polyus gold	839 000	23 785
Greece	Olympias	Aktor SA	235 000	6 662
New Zealand	Reefton	Royalco Resources Ltd.	87 300	2 475
Australia	Tasmania	BCD Resources NL	80 000	2 268
Australia	Wiluna	Franco-Nevada Corp	120 000	3 402
Kazakhstan	Suzdal	Nord Gold (Severstal)	72 000	2 041
Uzbekistan	Kokpatas	Navoi mining	353 000	10 007
Total gold production using biooxidation in 2010			901 300	82 250

Source: Data with permission from Schippers et al. (2011).

13.3.1 Economic Impact

In 2010, the global gold mine production amounted to 2450 tons. This represents roughly 60% of the total gold production as the remaining share of roughly 40% can be attributed to the gold production from recycling. In 2010, data available from 444 gold projects indicated that gold was obtained as a primary or secondary product from refractory ores using hydrometallurgical technologies. In those, a total production capacity of gold of around 1950 tons was documented. From that amount, at least 80 tons of gold and additionally 160 tons of silver as a side product were produced in at least 16 active gold projects applying biooxidation. Consequently, the share of the gold obtained after biohydrometallurgical treatment of the gold-encasing sulfide ore corresponds to approximately 4.1% of the hydrometallurgical and 3.2% of the global gold mine production.

13.3.2 Unlocking Gold by Biooxidation of the Mineral Matrix

There are three technically different biooxidation processes distinguished:

- Biooxidation in heaps for low-grade, refractory gold ores
- Biooxidation in stirred tanks for refractory gold ore concentrates (known, e.g. as the BIOX or Bacox process)
- Coating of inert tailing material with sulfide ore gold concentrates and their biooxidation in heaps.

The reaction rates during biooxidation in heaps are remarkably slower than in stirred-tank bioreactors and it takes from months up to years until the treated ore can be subjected to cyanidation for the recovery of gold. However, this technology is obviously characterized by very low capital and operating costs. The gold extraction, in comparison to the BIOX process, is lower and typically between 50% and 75%. However, the process can be profitable especially for low-grade sulfide gold ores, which are available at existing mining sites. In general, transport costs easily render metal recovery technologies for low-grade ores uneconomical. Nevertheless, this technology can be advantageous, when established at existing mining sites. In addition, as gold-bearing metal sulfide rock is a potential source of AMD because of uncontrolled bioleaching, the intentional biooxidation of the metal sulfide matrix can be considered as a secondary benefit. It is more adequate to dispose mining waste material after lowering their sulfide mineral content by oxidation, as it occurs during bioleaching processes.

Biooxidation with stirred tanks, in comparison to the heap leaching technology, is characterized by rapid reaction rates. Obviously, stirring, cooling, supply of O_2 and CO_2, and process control render the capital and operating expenses substantially higher in comparison to heap bioleaching. This process becomes competitive with the traditional process consisting of roasting and cyanidation, despite yielding typically a slightly lower gold recovery. The chemolithoautotrophic microorganisms used in BIOX processes need nutrients such as nitrogen, phosphorus, potassium, and carbon, which are typically not present in sufficient amounts in the sulfide concentrates or in the case of carbon in the supplied air. Therefore, additional CO_2 is supplied with the aeration and the other nutrients must be present in the form of ammonium, sulfate, and phosphate salts. In addition, this technology requires very fine grinding of the gold-bearing metal sulfide concentrate in order to achieve a high mineral surface accessible for biooxidation via iron(III) ions. These disadvantages are largely offset by the fact that the process is designed to be emission-free, environmentally friendly, and less energy demanding than smelting processes.

The biooxidation of gold-bearing arsenopyrite concentrates in continuous-flow operation (Figure 13.11) takes place in large-scale stirred-tank reactors (100–1000 m^3), which are typically set up in parallel in a first stage and in series in a second stage to achieve sufficient residence times for the biooxidation. This is necessary as the relatively slow process kinetics demand a residence time of the ore in the bioreactor of four to seven days. For an efficient oxidation process, several process parameters such as temperature, E_H, pH, O_2, and CO_2 concentration among others are controlled, as optimum conditions for the metal sulfide-oxidizing bacteria are desired. The product of this process is decanted off, washed, and thickened for further cyanidation and gold recovery. The leachate is neutralized using limestone, for a safe disposal of arsenic containing iron hydroxide precipitates and process water recovery (Figure 13.11).

The elevated capital and operational costs of stirred-tank operations demand for sufficiently high-grade gold-bearing sulfide ore concentrates.

The residence time of four to seven days of the ore in the bioreactors is the result of a remarkable microbial adaptation process that is ongoing and shapes the strain fitness and microbial community composition. Continuous selection

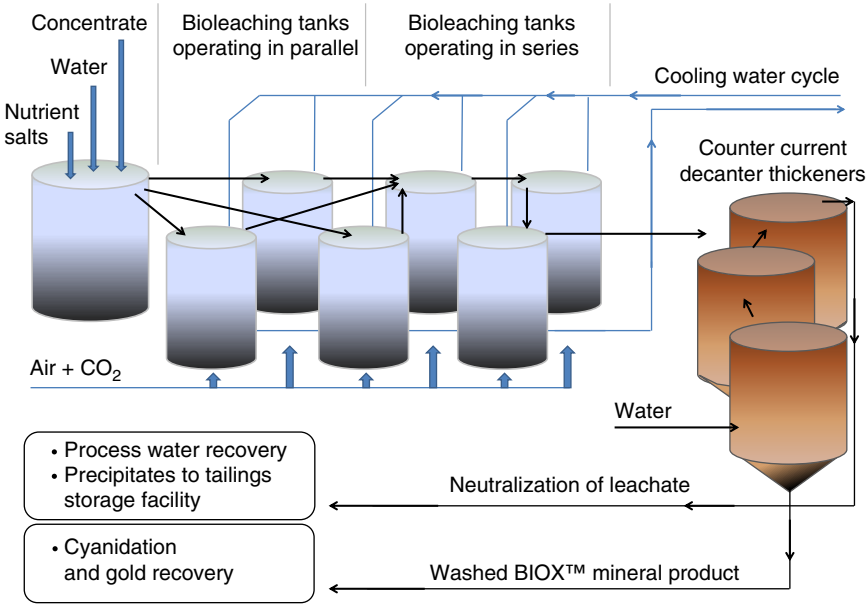


Figure 13.11 Simplified BIOX process scheme for biooxidation of gold-bearing arsenopyrite ores. Note that a cooling cycle is mandatory to prevent overheating of the reactors because of the exothermic microbial and chemical sulfide oxidation. Source: Drawn by Sören Bellenberg.

for the fastest growing strains under multiple stress influences occurs in the continuous reactors. Before 1990, the research on acidophilic metal-sulfide oxidizing bacteria focused on *A. ferrooxidans*. This species was preferably isolated because of selective cultivation conditions. Consequently, the importance of this species has been overestimated in many habitats and industrial bioleaching processes. *A. ferrooxidans* was believed to be most important not only for bioleaching also for the commercial BIOX process. However, the introduction of molecular techniques for the identification of microorganisms has surprisingly revealed that *A. ferrooxidans* did not occur in the running BIOX process at Fairview. Instead, it was found that species that were later named *Leptospirillum ferriphilum* and *Acidithiobacillus caldus* dominated the microbial population. These species grow optimally at temperatures around 40 °C, whereas *A. ferrooxidans* has an optimum growth temperature around 30 °C. Consequently, research in many laboratories changed focus on these and other new isolates from commercial bioleaching processes. Among other aspects of interest, their genetic systems for arsenic resistance were studied in the laboratory of Douglas Rawlings. A major finding was that arsenic-exposed *L. ferriphilum* and *A. caldus* strains from the Fairview BIOX process had acquired additional arsenic resistance systems by horizontal gene transfer. In that context, it shall be noted that methods for genetic manipulation of acidophilic mineral-oxidizing microorganisms are still not sufficiently efficient to use recombinant strains in process optimization. However, by natural adaptation of the consortium, a

reduced residence time of the ore in the reactors and a faster process has been established.

13.4 Uranium

13.4.1 Economic Impact

Because of the currently observed development of uranium prices, that in 2016 fell to the lowest level since 2005, cheap technologies for uranium recovery, such as *in situ* (bio)-leaching are competitive. In Europe and the United States, the demand for nuclear fuels is expected to continue to decrease, which is one of the reasons for the recent uranium price development. In 2017, however, more than 50 new nuclear power plants were under construction in China, India, Russia, Korea, and other countries. Proposals for new power plants exceeded 150 worldwide. The capacity of around 30 active *in situ* leaching projects for uranium worldwide was in 2010 reported to be 34 000 tons uranium/year, which represented one third of its world production. However, biological processes are not in all cases relevant for the dissolution of uranium from their source ores. With an extraction yield of 70–80%, the *in situ* bioleaching of uranium is considered to be very effective. In Germany, uranium was mined until 1990 by the SDAG Wismut using *in situ* bioleaching (Königstein) as well as heap bioleaching (Ronneburg). In Canada, commercial bioleaching of uranium from low-grade ores has been practiced from the 1960s until today. Historic examples are the Stanrock (1958–1985) and Milliken (1958–1964) mines with an annual production of 50–60 tons uranium. Other countries, in which uranium mining is conducted, are the United States, Brazil, South Africa, Australia, Russia, Finland, and Kazakhstan.

13.4.2 *In Situ* Biomining of Uranium

Today, uranium mining by *in situ* leaching is, due to the low capital and operational costs, a mining strategy. During the leaching process, insoluble uraninite (UO_2, U(IV)) is chemically oxidized to water-soluble uranyl cations ($(UO_2)^{2+}$, U(VI)). Iron(III) ions act as chemical oxidants and are, as in the case of metal sulfide oxidation, reduced to iron(II) ions. The iron(III) ions can be provided by microbial oxidation of iron(II) ions or using chemical oxidants for iron(II) ion oxidation. Acidophilic, iron(II)-oxidizing microorganisms such as *Leptospirillum ferrooxidans* or *A. ferrooxidans* can be used for the regeneration of the oxidant, which is done in reactors before injection into the accessed ore deposit. This iron(III) ion-containing solution is introduced in the fractured ore deposit and the uranium-enriched leachate solution is pumped to the surface for metal recovery by ion exchange. However, depending on the acid-consumption characteristics of the uranium-bearing minerals, biomining is not always suitable. As already mentioned, bioleaching is not applied at deposits that are characterized by a high acid consumption. In addition, some further basic requirements have to be considered for bioleaching of uranium ores. There are refractory nonsulfide uranium minerals unsuitable for bioleaching. In general, for bioleaching, the ore

deposits must be sufficiently fractured and accessible in order to permit sufficient contact of the leach liquor with the uranium ore. Next, the presence of pyrite has been shown to be beneficial, as its biological oxidation provides iron for chemical oxidation of the UO_2 or other uranium-bearing minerals and is an energy source for mineral-oxidizing microorganisms. Other types of uranium deposits may be suitable for chemical *in situ* leaching methods and those represent either acid or alkaline digestion in connection with chemical metal-complexing agents. These chemical agents are applied in combination with chemical oxidants for iron(II) ion oxidation. Costs for these chemicals may render these chemical leaching operations uneconomic compared to biological uranium leaching processes. Consequently, some projects employ combinations of chemical and biological replenishment of the oxidant iron(III) ions.

13.5 Perspectives

13.5.1 Urban Mining – Processing of Electronic Waste and Industrial Residues

The amounts of waste of electronic and electric equipment (WEEE) are increasing annually, mainly because of a decreased half-life time of ownership and use, fast economic growth, and consistent replacement of electronic equipment due to modern life standards. WEEE is being predicted to be some of the fastest growing waste streams within the next years. In general, WEEE is composed of a broad range of products. Concomitantly, with their diversity, the composition of WEEE may vary according to its type. In general, there are five major categories of components in WEEE: ferrous metals, nonferrous metals, glass, plastics, and some other materials; it also contains potentially hazardous substances such as mercury (Hg), chlorofluorocarbons (CFCs), and cadmium (Cd).

The recovery of valuable metals from WEEE, such as gold (Au), silver (Ag), copper (Cu), zinc (Zn), cobalt (Co), and other metals, represents a necessity to end practiced storage, export, or disposal of WEEE as landfill. This aspect is especially relevant, as most metals are present at higher concentration in WEEE than in ores. For example, the energy used to extract 1 kg of Al from WEEE is equal to less than one-tenth of the energy required for its production from bauxite. Printed circuit boards (PCBs) are particularly interesting to reuse because of their content of precious metals such as silver and gold. In addition, WEEE also contain rare earth elements (REEs). The demand of REEs increased during the technological revolution of the recent few decades, while their scarcity gives them a high market price. China currently dominates the REEs market with a share of around 90%. EU has considered REEs to be among the most critical resources, and current and future EU projects intend to reduce the dependency on China's supply.

Metal recovery from WEEE can be done chemically by acid extraction, assisted by physical pretreatment such as shredding as well as biological approaches. A great potential for recycling technologies is already represented by manual, mechanical, and magnetic sorting strategies. Bioleaching may be suitable for processing complex residues of small WEEE particles that have been preselected

or preclassified by sorting and disassembling processes. Burning of WEEE at temperatures close to 1000 °C eliminates plastic components, while the metals remaining in the ashes can be biologically leached and recovered by electrometallurgical processes such as electrowinning. However, the high energy amounts required as well as the generation of toxic gases may represent disadvantages. It is evident that not all the WEEE has the same potential to be processed by bioleaching. In addition, for successful biotechnical metal recovery using iron- and sulfur-oxidizing microorganisms, separation and pretreatment steps must ensure the removal of toxic organic substances, such as oils. The biological oxidation of iron(II) to iron(III) ions enhances chemical oxidation of Cu^0 to Cu^{2+}. The latter is soluble in the acidic leachate and can be recovered. Similar mechanisms have been demonstrated to enhance the solubilization of Ni, Zn, and Al from WEEE. The economic impact of bioleaching in WEEE processing is still marginal, presumably because of application of alternative physical and chemical processes and the necessity to further develop REE metal-selective recovery technologies for processing polymetallic solutions from bioleaching. However, this approach may improve recycling efficiency and resource supply in the near future targeting selected waste streams, ashes, slags, and further industrial residues.

13.5.2 Microbial Iron Reduction for Dissolution of Mineral Oxides

Several leaching microorganisms are able to grow using anaerobic respiration. Anaerobic growth can be driven by oxidation of sulfur compounds or hydrogen coupled to iron(III) ion reduction. The anaerobic biomining of metal sulfides at low pH has been demonstrated with *A. ferrooxidans* for bioleaching of Nickel limonites, in which nickel is associated with ferric oxo-hydroxides such as goethite (FeOOH). Reductive bioleaching has already been used for a process called Ferredox. *Acidithiobacillus thiooxidans* strains are also able to catalyze iron reduction under aerobic conditions at very low pH. It has been recently shown that iron(III) ion reduction by *A. thiooxidans* under aerobic conditions contributes to reductive dissolution of nickel laterite tailings. A consortium of *A. thiooxidans* and *A. ferrooxidans* extracted similar amounts of nickel and cobalt (~60%) from laterite ores. The economic and environmental advantages of aerobic reductive leaching for processing of laterite tailings comprise a 1.8-fold decreased acid consumption than for anaerobic reductive leaching, no requirement for establishment of an anoxic atmosphere, as well as efficient nickel and cobalt recovery in a ferrous-based leach solution, which facilitates the subsequent metal recovery.

13.5.3 Biomining Goes Underground – *In Situ* Leaching as a Green Mining Technology?

In situ leaching implies that ore bodies have been made accessible to leaching but they remain in place. In that way, it is possible to access kilometer deep located and fractured ore bodies while avoiding the energy-demanding steps of hauling overburden andore to the surface. Hydraulic fracturing is already being used to

access deeply buried shale deposits for extraction of their oil and gas contents. In a similar manner, uranium deposits have been accessed. For *in situ* metal mining from deeply buried metal sulfide ore bodies, oxygenated, iron(III) ion-rich sulfuric acid and mineral-oxidizing microorganisms are injected into the deposits after fracturing. The metals solubilized from the minerals can be recovered from the leach liquors that are extracted to the surface (Figure 13.12). *In situ* leaching research projects have already pointed out efficient recoveries of some target metals. However, efficient fracturing of the ore and contact with the leach liquor is a critical key parameter in conjunction with temperature and aeration. *In situ* biomining has been used to dissolve metals from ores, since the second century after Christ. It was a common practice of copper leaching in Cyprus and later by the Romans, which obtained copper at the site that later became the Rio Tinto mine in southern Spain. Copper was leached from the naturally exposed sulfide ore deposits and recovered from solution by metallurgical cementation using elemental iron.

Especially in the situation where near-surface resources have largely been exploited and deeper deposits may not be mined in an economically and ecologically feasible manner, novel mining strategies, such as *in situ* mining processes, are increasingly discussed and investigated. A great potential can be associated with this less invasive and less energy-intensive mining strategy. Mining today has severe consequences on land consumption, destroying landscapes and ecosystems. The selective removal of valuable metals from their ores in their natural deposit will drastically reduce the environmental impact and costs of current mining technologies.

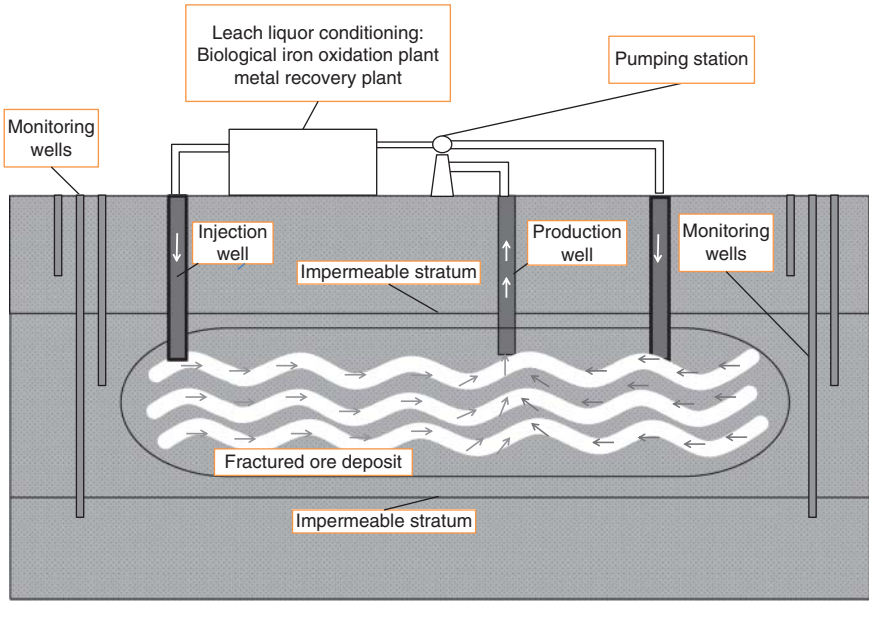


Figure 13.12 Schematic depiction of an *in situ* bioleaching plant. Source: Drawn by Sören Bellenberg.

Concerning energy use for transportation of material, which is avoided by *in situ* mining, a striking advantage can be attributed to this technique. Land use for waste rock storage and disposal is also strongly reduced as well as destruction of landscape on the surface, as it is common nowadays in open-pit mining projects (Figure 13.6). The technique is also suitable for treatment of low-grade ore deposits. *In situ* mining is practically dust-free, which also means a lowered health risk for employees. It also needs less water resources because of low evaporation and the intended efficient recovery of the metal-bearing leach liquor for its treatment, metal recovery, and reuse. In sum, these aspects suggest significant economical and ecological advantages compared to classical mining techniques. However, on the other hand, critical assessment of risks and possible disadvantages has to be incorporated during planning of potential *in situ* mining projects. As such, in general, lower metal recoveries can be expected due to lowered contact of leach liquor and the ore. As a consequence, more time may be necessary for efficient mineral degradation and metal recovery. Loss of the leach liquor would result in product loss and environmental damage. A further risk is that in the presence of sufficient substrates for bioleaching microorganisms such as pyrite (FeS_2) or iron(II) ions, bioleaching may proceed uncontrolled even decades after production and may be difficult to control if the oxygen supply cannot be controlled after the fracturing. Consequently, over long periods, a further risk for groundwater and surface water may potentially exist, and this demands monitoring measures to be implemented. This aspect and public availability of monitoring data will support public acceptance of such critically discussed technologies. *In situ* leaching mining projects using sulfuric acid have yet to be proven to be environmentally sound. Sulfuric acid *in situ* uranium leach projects have a poor track record in Eastern Germany, Eastern Europe, and Russia. This came about primarily because adequate safeguards were not designed into the projects from the start. Most successfully reclaimed *in situ* leach projects to date have been for uranium in sandstone-hosted deposits. Most of these have employed chemical leaching agents, such as sodium carbonate and oxygen or peroxide. These are believed to be easier and less costly to reclaim than *in situ* bioleaching projects employing sulfuric acid. The solution flow patterns through ore deposits are hard to predict, quantify, and control. This must be a requirement for efficient and sustainable *in situ* mining projects using monitoring well systems. When ore deposits above the ground water level are targeted, there will be a requirement to create engineered barriers to contain leach solutions within the ore deposit, accessible for extraction well systems. In general, *in situ* leaching may be favored in the situation when the ore deposit is located beneath the water table under impermeable layers. The control of the leach liquor flow is important for both protection of the environment, groundwater users, and for recovery of the leach solution and the valuable metal. Implementation of monitoring programs to prevent hazards will therefore be needed to control product recovery, cooperate with the general public, meet common interests, and comply with authorities' responsibilities to control groundwater quality. Costs for such programs are justified within license allocation and estimated to be significantly lower than current expenditures for traditional mining technologies. Consequently, biologically catalyzed *in situ* leaching

via iron(III) ions in combination with sulfuric acid could become a viable resource technology in the near future.

References

Clark, M.E., Batty, J.D., van Buuren, C.B. et al. (2006). Biotechnology in minerals processing: technological breakthroughs creating value. *Hydrometallurgy* 83 (1–4): 3–9.

Neale, J.W., Gericke, M., and Ramcharan, K. (2011). The application of bioleaching to base metal sulfides in southern Africa: prospects and opportunities. *Southern African Base Metals Conference*.

John Neale, Janne Seppälä, Arto Laukka, Pieter van Aswegen and Stephen Barnett (2016). The Mondo Minerals Nickel sulfide bioleach project: construction, commissioning and early plant operation. *ALTA 2016 Nickel-Cobalt-Copper Sessions 2016*, Perth, Australia.

Rohwerder, T. and Sand, W. (2007). Mechanisms and biochemical fundamentals of bacterial metal sulfide oxidation. In: *Microbial Processing of Metal Sulfides* (eds. E.R. Donati and W. Sand), 35–58. Dordrecht: Springer Netherlands.

Schippers, A., Hedrich, S., Vasters, J. et al. (2014). Biomining: metal recovery from ores with microorganisms. *Adv. Biochem. Eng. Biotechnol.* 141: 1–47.

Schippers, A., Vasters, J., and Drobe, M. (2011). Biomining – Entwicklung der Metallgewinnung mittels Mikroorganismen im Bergbau. Commodity Top News Nr. 39. Deutsche Rohstoffagentur (DERA) in der Bundesanstalt für Geowissenschaften und Rohstoffe (BGR).

Vera, M., Schippers, A., and Sand, W. (2013). Progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation-part A. *Appl. Microbiol. Biotechnol.* 97: 7529–7541.

Further Reading

Brierley, C.L. (2016). Biological processing: biological processing of sulfidic ores and concentrates—integrating innovations. In: *Innovative Process Development in Metallurgical Industry: Concept to Commission* (eds. V.I. Lakshmanan, R. Roy and V. Ramachandran), 109–135. Cham: Springer International Publishing.

Brierley, C.L. and Brierley, J.A. (2013). Progress in bioleaching: Part B: applications of microbial processes by the minerals industries. *Appl. Microbiol. Biotechnol.* 97 (17): 7543–7552.

Denef, V.J., Mueller, R.S., and Banfield, J.F. (2010). AMD biofilms: using model communities to study microbial evolution and ecological complexity in nature. *ISME J.* 4 (5): 599–610.

Quatrini, R. and Johnson, D.B. (eds.) (2016). *Acidophiles: Life in Extremely Acidic Environments*, 153–176. Caister: Academic Press.

14

Wastewater Treatment Processes

Claudia Gallert[1] *and Josef Winter*[2]

[1] *University of Applied Sciences Emden Leer, Division for Microbiology – Biotechnology, Faculty of Technology, Constantiaplatz 4, 26723 Emden, Germany*
[2] *Karlsruhe Institute of Technology, KIT, Faculty of Civil Engineering, Geo and Environmental Sciences, Am Fasanengarten, 76131 Karlsruhe, Germany*

CHAPTER MENU

Abbreviations used more often in different chapters:

AOB	ammonia oxidizing bacteria
AOX	absorbable organic halogens
ATP	adenosine triphosphate
$BOD_{(5)}$	biochemical oxygen demand (in 5 days)
COD	chemical oxygen demand
DOC	dissolved organic carbon
EPA	Environmental Protection Agency
EPS	extracellular polymeric substances
HRT	hydraulic retention time
MW	Molecular weight
N_{org}	organic nitrogen
NOB	nitrite oxidizing bacteria
PAO	polyphosphate accumulating bacteria
PE	population equivalent
PHB	poly-β-hydroxybutyrate
SRT	solid retention time
STP	sewage treatment plant
SVI	sludge volume index
TCA	tricarboxylic acid cycle

Industrial Microbiology, First Edition.
Edited by David B. Wilson, Hermann Sahm, Klaus-Peter Stahmann, and Mattheos Koffas.

TKN	total Kjeldahl nitrogen
TOC	total organic carbon
TS	total solids
TSS	total suspended solids
VS	volatile solids
WTP	wastewater treatment plant

14.1 Introduction

14.1.1 Historical Development of Sewage Treatment

In ancient Rome, open channels existed for draining night soil and urine out of the city, presumably for optical and olfactory reasons. Only in the second half of the nineteenth century was it recognized that sewage, in particular suspended feces in municipal wastewater (the "blackwater" fraction), is dangerous for health, leading to waterborne diseases such as typhoid fever, cholera, tuberculosis, or dysentery epidemics. After Robert Koch (1843–1910) had isolated *Mycobacterium tuberculosis* (1882) and *Vibrio cholerae* (1884), other bacteria in human and animal feces were recognized as health-endangering, pathogenic microorganisms (Imhoff and Imhoff 2007). The first precautionary measures to improve sanitation in human settlements were open sewers for rapid drainage of night soil and urine away from settlements into the next river, creek, or lake. Open sewers were then replaced by underground sewer pipes for drainage of sewage and rainwater, managing sanitation and hygiene within human settlements much better. At the beginning of the nineteenth century, the self-cleaning capacity of surface waters was still sufficient to take up all domestic effluents (Dunbar 1908). Increasing amounts of wastewater from cities with a rapidly increasing population, however, exceeded the self-cleaning capacity of surface waters by natural degradation processes in streams and lakes. This demanded some kind of sewage treatment. At first, only screens or sieves were installed for mechanical removal of floating and large suspended material. With time, sewage treatment improved by combinations of mechanical (sedimentation and flotation), chemical (precipitation), and biological (carbon, nitrogen, and phosphorus removal) treatment processes, leading to STPs (sewage treatment plants) for primary, secondary, and tertiary sewage treatment with a maximum of carbon, nitrogen, and phosphate removal. Latest attempts to improve sewage treatment further (fourth or advanced purification stage) are focusing on the removal or, at least, minimization of organic trace pollutants and of pathogenic bacteria in the treated sewage. Physical–chemical procedures such as chlorination (mainly United States), ozonation, or hydrogen peroxide oxidation ± UV irradiation, followed by adsorption of oxidation residues onto charcoal (United States and Europe), are installed for this purpose.

In Germany and in the United States, the first sewer systems were installed in 1842 in Hamburg and in the late 1850s in Chicago and Brooklyn, respectively, followed by sewer systems in other cities. Nowadays, more than 95% of the total sewage of Germany, including that of households in areas with a small population

or even of single houses, is drained in underground sewers to central STPs. Only about 5% of remote households still treat their sewage onsite in three-chamber septic tanks, followed by soil filtration. In the United States, the proportion of onsite small treatment units (mainly septic tanks) is much higher between 19% and 25% because of a different settlement structure with a large portion of single houses along roads and in remote areas (Center for Sustainable Systems, University of Michigan 2015).

Initially, STPs only consisted of screens and gravity sedimentation ponds or tanks, in which coarse material and small-particulate suspended solids, accounting for approximately 30% of the organic pollutants and most of the fecal bacteria of sewage, were separated by filtration and gravity sedimentation. Until about 1950 in small German communities and even longer in the United States, STPs only consisted of screens and sedimentation ponds to clarify the sewage. Progress in the microbiology of aerobic and anaerobic degradation of organic pollutants led to the development of combined mechanical and biological sewage treatment systems, such as the "Imhoff-tank" or "Emscher-Brunnen" in Germany and the Clarigester in the United States. In these treatment systems, aerobic degradation in the water body at the surface and anaerobic degradation in the water body about 0.5 m below the surface and in the sludge sediment were combined.

For rapid elimination of dissolved organic carbon (DOC) compounds from sewage in STPs, the clarified effluent of primary sedimentation tanks is treated aerobically in trickling filters or activated sludge tanks. To comply with legal limits for wastewater discharge into surface waters in Europe, trickling filters and activated sludge processes must be sized so that biodegradable, dissolved, or colloidal organic material is degraded to less than 15 mg/l BOD_5 (biochemical oxygen demand in 5 days). Furthermore, ammonia must be removed by nitrification and denitrification to maintain concentrations of e.g. $N_{total} \leq 13$ mg/l and of $N_{ammonia} \leq 10$ mg/l in the treated sewage of class 5 STPs (>100.000 PE [population equivalent = 60 g BOD_5], Table 14.1).

The situation in the United States is different and is regulated by the CWA (Clean Water Act), first issued in 1948 and significantly reorganized and expanded in 1972. Wastewater treatment must comply with federal and state environmental standards. United States EPA (Environmental Protection Agency) issues sewage discharge permits that take into consideration the location of the settlement and the freshwater availability. Big cities of the United States on the east coast, on the Northern section of the west coast, or in the Great Lakes district have plenty of fresh water, whereas big cities on the southern section of the west coast are located in the semiarid regions of California, e.g. San Francisco, Los Angeles or San Diego and suffer water scarcity. Thus, discharge permits depend on whether treated sewage can be disposed into the ocean (marine environment) after removal of only the biologically degradable part of the carbon freight (BOD_5), as in almost all settlements on the east coast (e.g. Boston, New York, and Miami) or on the Northern section of the west coast (e.g. Seattle) through ocean fallouts or whether the treated sewage must serve for water reclamation and reuse, as in Southern California (e.g. San Francisco, Los Angeles, Glendale, and San Diego). For reuse of sewage as purified recycled water, activated sludge treatment must remove BOD_5 quantitatively and nitrify all ammonia for

Table 14.1 Composition of municipal wastewater (sewage) in Germany and the United States and purification requirements.

Sum parameter	Specific pollutant load g/(PE d)	Concentration of raw sewage mg/l[a] Germany/United States[b]	Wastewater treatment requirements[c] (STP size category 5)[d] mg/l	Elimination (%)
BOD_5	60	500/ 150– 500	≤15	≤97
COD	120	1000/ 300–1000	≤75	≤92.5
TKN	11	92/ 22– 80		
NH_4^+-N			≤10	
N_{total}			≤13	≤85.9[e]
P_{total}	2.5	21/ 5– 20	≤ 1	≤95.2

PE = Population equivalent.

a) Based on a drinking water consumption of 120 l/(person d).

b) Values according to US EPA (1995).

c) Purification requirements based on the German wastewater treatment ordinance (AbwV 2004): The requirements are valid for ammonia-N and N_{total} for a water temperature of ≥12 °C in the effluent of a STP. Instead of ≥12 °C, purification standards may be required from May until October.

d) STP size category 5 means >6000 kg BOD_5/d.

e) Based on TKN in original sewage.

subsequent denitrification. Finally, for sanitation, the purified sewage must be disinfected by chlorination or treated otherwise (see Section 14.4.2).

For marine discharge of treated sewage on the east coast of the United States through marine outfalls e.g. into Massachusetts Bay by the Deer Island Sewage Treatment Plant of Boston, the second largest STP of the United States (after the Stickney Water Reclamation plant in Chicago with outfalls into Lake Michigan), the EPA permit requires >85% of BOD_5 removal. After mechanical and biological treatment of sewage in the Deer Island STP of Boston (see Figure 14.4B), clarified sewage is discharged offshore via 10 mile long outfall tunnels through diffuser pipes into the deep water of the Atlantic Ocean. However, when treated sewage must serve the purpose of recycled water for water reclamation as in the Hyperion Water Reclamation Plant of Los Angeles, removal of BOD_5 to a much higher extent and of nitrogen by nitrification and denitrification is necessary. Most of such purified wastewater then serves as an indirect potable water reuse by recharging groundwater to prevent salt water intrusion in respective coastal areas, where more groundwater is used for irrigation of food crops in agriculture than is recharged by rain fall. Besides groundwater recharge, recycle water may be directly used as irrigation water or as process water for industrial purposes.

In Europe, phosphate elimination from sewage by chemical precipitation with ferrous or aluminum salts or by biological means with enriched polyphosphate accumulating bacteria is required to comply with the boundary concentrations for discharge of wastewater that are mandatory by the European Union (EU) or German wastewater legal ordinances, thus preventing eutrophication of surface waters. In the United States, elimination of nitrogen and of phosphate may

not be necessary when treated sewage or wastewater from large settlements is discharged into the Atlantic or Pacific Ocean. The purified wastewater from trickling filters or activated sludge treatment, which still contains all the nitrogen and phosphate, is disinfected and discharged offshore into the ocean. Digested sewage sludge from primary and secondary treatment is dewatered and the biosolids are pelleted to be used as a natural organic fertilizer or soil conditioner. In small communities and in single households, septic tanks followed by slow sand filtration are required.

14.1.2 Resources from Wastewater Treatment

Clean fresh water is necessary to supply the world's population with sufficient potable water. When the natural freshwater resources in a certain region are less than 1000 m^3/person and year, the region is classified as suffering from water scarcity. Although many countries in the Middle East or Africa suffer from droughts and a severe water scarcity all the year, others may suffer water scarcity for instance only during dry summer periods. At the Millennium Summit in New York (September 2000), a "halving of the proportion of the worlds people without permanent access to clean, hygienically harmless drinking water from 65% to 32% by 2015" was considered necessary. In industrialized countries with enough clean water resources, the primary goal should be less potable water consumption by a more economic use of clean drinking water.

Clean water becomes wastewater when the physical, chemical, and biological properties are changing during human use. Thus, cooling water of power plants with an elevated temperature becomes wastewater. A large part of the daily, per-person consumed drinking water is, for instance, used – one might also say misused – as a means to sweep human feces and kitchen residues through underground sewers into STPs and is thus "converted" to a highly diluted wastewater. The specific, per-person daily consumed amount of drinking water might be significantly reduced if showering water or water from laundry (called "grey water") or rainwater from the roof of houses were collected separately in sedimentation tanks, sanitized, and used as less clean household water for flushing toilets or watering the garden. Wastewater treatment to the stage of drinking water quality in principle is possible in industrialized countries, but is not (yet) widely applied because of the very high treatment costs. In sparsely populated areas, a direct utilization of sanitized feces and urine as a natural fertilizer would be more sustainable and more economic than purification in an STP without nutrient recycling. This might save resources, in particular phosphorus, which is available on earth from primary resources for only a few more decades.

To reduce sewage treatment costs, its energy content should be recovered and its nutrient content should be extracted. For this purpose, suspended organic compounds (primary and secondary sewage sludge) must be separated during mechanical and after biological sewage treatment, respectively, and digested to energy-rich biogas, usable to drive gas engines and electricity generators or to replace natural gas after CO_2 removal. The digester effluent should be separated into nutrient-rich fractions of nondigestible solids and sludge water. The solids can be directly used as fertilizer if free of toxic compounds. They are applicable

in neighbouring agriculture or forestry or pelleted to biosolids (e.g. class A or B). Alternatively, phosphorus might be extracted chemically and processed to a mineral fertilizer. The sludge water also contains some phosphorus and most of the nitrogen as ammonia. It may be used directly as a fertilizer, economically only over short distances or it must be recirculated into the STP for further nutrient removal.

14.1.3 Wastewater and Storm Water Drainage

Sewage and storm water are discharged either together in combined, large "mixed" sewage system or separately in a large rainwater and a smaller sewage system. Mixed sewer systems for municipal wastewater and rainwater, as well as separate sewers for sewage alone, drain domestic or industrial wastewater and run off rainwater from settlements to STPs. In separated sewers, the smaller pipe drains sewage to the STP and the larger rainwater pipe collects rainwater and guides it to a mechanical treatment facility for screening and separation of suspended organics and for sand sedimentation by gravity. Oil and grease float on the surface and are skimmed off. The mechanically treated rainwater is pumped to a soil retention filter or discharged into the closest surface water. The amount of domestic wastewater that arrives at the STP follows diurnal variations with maximum amounts in the morning, around noon, and in the early evening, representing the water consumption habits of the population.

The DWA (Deutsche Vereinigung für Wasserwirtschaft, Abwasser und Abfall, German Association for Water, Wastewater and Waste, a nongovernmental organization) has estimated that in 2017, 8502 million m^3 of wastewater from inhabitants, industry, and commerce was treated in STPs with a specific energy consumption of 31.08 kWh per inhabitant and year (DWA 2018). Subtracting the PE equivalents of wastewater from small industries that are (co-)treated in municipal STPs, the specific wastewater amount generated per inhabitant per year accounts for 79 m^3 or 216 l/person/d, which is about double the amount of drinking water consumption. According to drinking water suppliers, the drinking water consumption in Germany is 120 l/person/d, which should result in maximally 44m^3 sewage/person/yr. Thus, 35 m^3 wastewater must come from other sources, such as storm water runoff from paved areas in mixed sewerage systems or infiltration of trickling water/groundwater water into leaky mixed or separated sewers. According to DWA, elder sewage pipes are often leaky. In Germany, most communities charge fees for treatment of sewage per household based on the consumption of drinking water plus a lump sum for sewer maintenance and rainwater treatment, where a separate storm water channel exists. Alternatively, a split fee is charged for treatment of sewage, based on freshwater consumption plus a rainwater lump fee based on the paved area of private properties.

14.1.4 Wastewater Characterization and Processes for Effective Wastewater Treatment

Pollution of municipal wastewater in the United States and in Germany is determined with similar parameters according either to APHA (2003) (Standard

Methods for the Examination of Water and Wastewater, American Public Health Association) in the United States or DEV (1983) ("Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung") in Germany, respectively. For this purpose, total solids (TS), total suspended solids (TSS), volatile solids (VS), biochemical oxygen demand in 5 days (BOD_5), chemical oxygen demand (COD – determined after dichromate or potassium permanganate oxidation of organic wastewater compounds), total nitrogen content according to Kjeldahl (TKN), organic nitrogen content (mainly from protein, N_{org}), and total phosphorus content must be analyzed. In Table 14.1, typical (average) concentrations of these pollutants in domestic German or European wastewater for a per-person daily water consumption of 120 l are compiled. Municipal wastewater in the United States is much more diluted (Table 14.1) because of the consumption of much more drinking water in the range of 200–365 l, depending on household size (Metcalf & Eddy, Inc. 2003). In addition to the sewage characterization, the cleaning targets according to annex 1 of the German sewage ordinance (AbwV 2004) are listed. It may be mentioned that the discharge limits for most of the parameters are much stricter in the EU and in Germany than in the United States not only for carbon removal but especially with respect to nitrogenous compounds and phosphorus removal. The per capita-related specific pollution freight PE is defined as 60 g BOD_5 in Germany and 85 g BOD_5 in the United States. A PE-unit is the amount of oxygen that is required for mineralization of the biologically degradable organic compounds in five days in an aqueous environment by a complex, adapted aerobic-mixed inoculum. The use of PE units allows the standardization of the biologically degradable pollution freight in domestic, commercial, and industrial wastewater that is treated in domestic STPs and thus comparable pricing for treatment at public STPs. PE units are independent of the amount of water that is used in households, by commerce or in industrial production processes, and are essentially required together with the amount of water consumption for sizing all components of an STP and in particular the aeration system, as well as for calculation of specific treatment costs. Average concentrations of the parameters of sewage in Table 14.1 result from an average daily per capita consumption of 120 l drinking water.

Whereas in the United States discharge limits for treated sewage depend on the receiving waters (marine or sweet water habitats) or on the necessity of water reuse (arid and semiarid land areas) in European countries, boundary values for purified sewage and industrial wastewater are overall more strict and depend on the size, meaning the amount of treated PE units of the STP. For instance, in a STP of size five in Germany that receives a daily BOD_5 freight of >6000 kg (equivalent to >100.000 PE or > 12 000 m^3/d), more than 97% of the BOD_5, 92.5% of the COD, 85.9% of the TKN, and 95.2% of the total phosphorus must be removed (Table 14.1).

Discussions on wastewater purification in an STP often focus only on the treatment of the polluted water phase and exclude sludge treatment, although sewage sludge from primary and secondary clarifiers usually is treated in anaerobic digesters in German STPs (Section 14.3.6). Alternatively, primary and secondary sewage sludge must be dewatered and incinerated. When sewage sludge is used as an organic fertilizer in agriculture after anaerobic digestion, sanitation and

dewatering, the values for the content of lead, cadmium, chromium, copper, nickel, mercury, and zinc, the sum of organic halogens (absorbable organic halogens, AOX), total and ammonia nitrogen, phosphate, potassium, magnesium as well as the dried residue, organic substances, basifying substances and the pH value must fall below boundary values of the sewage sludge ordinance (AbfKlärV 2017) and of the fertilizer regulation (DüMV 2012, Table 14.2). In the United States EPA handbook of biosolids management (EPA Region VIII 1995), the usage of biosolids (class A or B), a semisolid wastewater product that is stabilized biologically or chemically for land application and surface disposal, is regulated. Ceiling concentrations for heavy metals, organic residues, pathogenic bacteria, viruses and fungi are listed in Table 14.2.

14.1.5 Suspended or Immobilized Bacteria as Biocatalysts for Effective Sewage Treatment

Aerobic degradation of dissolved and colloidal sewage compounds in activated sludge systems is catalyzed by sludge flocs that consist of aggregated or agglomerated filamentous, EPS (*e*xtracellular *p*olymeric *s*ubstances) producing bacteria, belonging to the genera *Achromobacter*, *Aerobacter*, *Alcaligenes*, *Bacillus*, *Citromonas*, *Escherichia*, *Pseudomonas*, or *Zoogloea*. These bacterial agglomerates form either light flocs or more dense granules. When activated sludge is recirculated to increase the sludge age, the mixed microbial population in the flocs changes. The heterotrophic bacteria not only mineralize the easily degradable BOD_5 compounds but also some more recalcitrant carbon compounds to CO_2 and H_2O and slow-growing autotrophic nitrifiers are established and convert ammonia to nitrate. Nitrate is denitrified by denitrifying bacteria to N_2 gas. A low value of the SVI (*s*ludge *v*olume *i*ndex – sludge volume per gram TS after 30 minutes of gravity sedimentation) of 80–120 ml/g TS indicates a compact sludge and allows rapid settlement in sedimentation tanks (clarifier). The presence of surface-active substances such as laundry detergents, household cleaners, dish soap, or fat and oil decreases rapid sedimentation (Gerardi 2006).

Although bacteria form sludge flocs in activated sludge treatment processes (Section 14.3.2), in fixed-bed or fluidized bed reactors, they form biofilms on the surface of structured support materials or on small moving inert support materials, respectively. Classical examples of fixed-bed reactors are trickling filter systems (Section 14.3.3) containing porous volcanic stones or structured plastic cassettes as substrata for biofilm formation. Primary adhesion of microorganisms on the surface or – protected from shear forces caused by turbulent flow – in the pores of these carrier materials is mediated by electrostatic forces. After EPS excretion, which acts as a "biological" glue, multi-layer growth of the bacteria and formation of a stable biolfilm is possible. To avoid clogging of the filter bed, a balanced grazing of the biofilm by protozoa and macro invertebrates must be maintained.

In Table 14.3, free or syntrophic interactions of bacteria during wastewater purification and sludge digestion are listed. Disturbances of these interactions, e.g. by poisonous substances and detergents in the wastewater or by highly turbulent mixing, etc., may reduce degradation efficiency and in the worst case lead to failure of the reactors.

Table 14.2 Boundary values or ceiling concentration of treated sewage for use as organic fertilizer in gardening and agriculture according to the German sewage sludge ordinance (AbfKlärV 2017), the fertilizer regulation (DüMV 2012), or the US EPA biosolids management handbook (EPA Region VIII 1995).

Parameter	Boundary value according to AbfKlärV[a] and DüMV[b]	Ceiling concentration according to biosolids management handbook EPA[c]
mg/ kg dry solids		
Arsenic	40	75
Cadmium	1.5	85
Chromium (Cr^{VI})	2	
Copper	900	4 300
Lead	150	840
Mercury	1	57
Molybdenum		75
Nickel	80	420
Selenium		100
Thallium	1	
Zinc	4 000	7 500
AOX[d]	400	
Perfluorinated tensides	0.1	
Benzo[a]pyrene BaP	1	
PCB[e] (mg/kg TS)	0.1 for the congeners 28, 52, 101, 138, 153, 180	
PCDD/dl-PCB[f]	30 ng TEQ	
Salmonella sp[g]	0 per 50 g	<3 per 4 g (class A)
Pathogens listed in Directive 2000/29/EC[h] Thermoresistant viruses (mainly members of the genus Tobamovirus) Fungi with resistant dormant bodies	No	
Fecal coliforms[g]		< 1 000 per g (class A) < 2 000 000 per g (class B)

a) Sewage sludge ordinance (AbfKlärV).
b) Fertilizer regulation (DüMV).
c) Biosolids Management Handbook of US EPA and National Manual of Good Practice for Biosolids June 2012; https://www.wef.org/globalassets/assets-wef/3---resources/topics/a-n/biosolids/national-biosolids-partnership/manual-of-good-practice-for-biosolids-v2011.pdf.
d) AOX = Sum of absorbable halogen-organic compounds.
e) PCB = Polychlorinated biphenyl.
f) PCDD/dl-PCB = Polychlorinated Dibenzodioxine/Dioxine-like PCB TEQ according to WHO-TEQ 2005.
g) MPN = Most Probable Number per solid dry mass.
h) Pathogen removal according to 2000/29/EC on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community.

Table 14.3 Mode and significance of interactions of microorganisms during wastewater treatment.

Kind of interaction	Definition	Process	Task
Mutualistic symbiosis	Biocoenosis, advantageous for both partners	Nitrification	AOB produce nitrite, NOB oxidize the poisonous nitrite to nitrate
Syntrophy	Beneficial interdependence of two partners	Interspecies H_2-transfer during methanogenesis in anaerobic environment, e.g. during sewage digestion	The H_2-producer can only release H_2 if the H_2-consumer within a short diffusion stretch converts it to methane to keep the p_{H_2} low
Competition	Competition between species for a growth-limiting resource	C-degradation and nitrification in sludge flocs	Competition between heterotrophic carbon-degrading and autotrophic nitrifying bacteria for dissolved oxygen
Food chain, food net	Sequential, synergistic degradation with energy gain. Relatively balanced production of metabolites of one group of bacteria for consumption by a following group of bacteria	Anaerobic degradation of wastewater and wastes in digesters	Acidogenic bacteria hydrolyze biopolymers and ferment the monomers to fatty acids, CO_2, and H_2. The metabolites must be further converted by acetogenic and methanogenic *Archaea* to finally biogas
Predator–prey relationship	Food chain of organisms were living organisms are food for other (wolferine) organisms	Eat–die relationship	Protozoa, ciliates in trickling filters, grazing bacterial biofilms

AOB = ammonia-oxidizing bacteria; NOB = nitrite-oxidizing bacteria.

14.2 Biological Basics of Carbon, Nitrogen, and Phosphorus Removal from Sewage

Municipal sewage must be purified to meet the environmental standards listed in Table 14.1. The wastewater may not be toxic for microorganisms and the pollutants must be dissolved or rapidly dissolvable by bacterial hydrolysis with exoenzymes for bioavailability. Degradation rates are dependent on the type and concentration of the substrate, bacterial density, temperature, pH, and salinity.

14.2.1 Aerobic and Anaerobic Degradation of Carbon Compounds

For aerobic degradation of dissolved organic substances, oxygen is required, whereas for anaerobic degradation, oxygen must be strictly excluded. Aerobic

Table 14.4 Differences of aerobic and anaerobic degradation of biopolymers.

Parameter	Aerobic bacteria	Anaerobic bacteria
Way of life	Aerobic; facultatively anaerobic	Obligately/strictly anaerobic
Ecosystem	Well-aerated habitats, ubiquitous distribution	Sediments of fresh water/salt water, rumen, rice fields, anaerobic digesters
Oxidation	Substrate respiration with O_2 to $CO_2 + H_2O$ via glycolysis, TCA, and respiration chain (mineralization)	Complete/partial oxidation of metabolites of glycolysis to CO_2 (e.g. in the process of sulfate reduction) or disproportionation to $CO_2 + CH_4$ (e.g. methanation)
Terminal electron acceptors	O_2, NO_3^-, NO_2^-	SO_4^{2-}, CO_2, Fe^{3+}, Mn^{4+}, Cr^{6+}, Se^{6+}, As^{5+}, U^{6+}
Energy gain	High, 38 mol ATP/mol glucose	Low, 4 mol ATP/mol glucose
Biomass growth	High 25% of degraded COD[a)] 50% of degraded COD[b)]	Low 5% of degraded COD[a)] 10% of degraded COD[b)]
Turnover at surplus substrate supply	High with a high growth rate of microorganisms and high biomass production	High, unbalanced metabolism with decreasing pH, low growth rates, or even inhibition of microorganisms
Nutrient requirements COD:N:P	100 : 5 : 0.5–1	800 : 5 : 1
Biocatalysts	Single species of bacteria can catalyze the whole mineralization to CO_2	Sequential food chain catalyzed by different species of *Bacteria* and *Archaea*, mutualistic interdependence

a) At low load.
b) At high load.

degradation can be tracked by the decrease of BOD_5, whereas anaerobic degradation can be followed by the decrease of COD, TOC (*T*otal *O*rganic *C*arbon), or DOC. Aerobic and anaerobic bacteria metabolize organic compounds in a different way. The fundamental differences are listed in Table 14.4.

Aerobic mineralization of carbonaceous compounds proceeds via glycolysis and/or the tricarboxylic acid cycle (TCA) to carbon dioxide. Highly energized electrons that are generated during the oxidation of carbon sources lead to the formation of ATP when "flowing" through the respiratory chain and reacting with oxygen and protons to form water. Almost two-third of the energy content of respired organic compounds is conserved by oxidative phosphorylation, supplying the ATP for luxuriant growth of aerobic bacteria and only about one-third of the energy is lost as heat. In activated sludge systems with aeration by air, containing ~80% nitrogen, the heat is stripped out by the off-gas, while self-heating occurs in highly loaded activated sludge tanks, if pure oxygen at the minimally required flow rates for respiration is used for oxygen supply. Aerobic wastewater bacteria require a COD:N:P-ratio of about 100:5:1 in sewage for balanced growth,

which is fortunately existing in domestic sewage. For this reason, no nutrients have to be added for activated sludge or trickling filter treatment. If sludge bacteria are supplied with surplus substrates as in high-load activated sludge systems, about 50% of the carbonaceous intermediates from BOD_5 degradation are used for bacterial growth, whereas under conditions of limited substrate supply as in weak or low-load activated sludge systems, up to 75% of the BOD_5 is respired to CO_2 and water, and only 25% of intermediary metabolites are channeled into anabolism (Table 14.4). The much higher specific amount of ATP per substrate unit in low-load activated sludge systems as compared to high-load activation systems is required for cell maintenance during starvation periods and only about 25% are dissipated for bacterial growth and surplus sludge formation. Anaerobic digestion systems for (rather dilute) sewage and digestion under ambient temperatures are not yet successfully implemented at STPs. Thus, anaerobic digesters are standard treatment systems for many highly concentrated industrial effluents. In municipal STPs anaerobic digesters are used for stabilization of more concentrated primary and secondary sewage sludge.

Biogas, formed during methanogenesis of primary and secondary sewage sludge in anaerobic digesters by a complex microbial population is the final product of anaerobic degradation of dissolved, colloidal, or particulate organic matter. As anaerobic bacteria cannot use an oxygen-dependent respiratory chain like aerobic bacteria, the ATP gain per unit of substrate is much less and thus little energy is available for growth and surplus sludge formation. Less than 10% of degraded COD of wastewater is channeled into bacterial growth versus about 25–50% in low-load or high-load activated sludge systems, respectively (Table 14.4). Consequently, less N and P is required for growth of anaerobic bacteria so that the ideal COD:N:P ratio in the raw wastewater or sludge is 800 : 5 : 1. A large part of the energy of the degraded compounds is released with the methane in the biogas. Little energy is transformed into heat, not enough to maintain the optimal temperature for mesophilic digestion at $35 \pm 2\,^{\circ}C$ in non-insulated digesters and far from enough for thermophilic digestion. Thermophilic digestion of sewage at a temperature of $\leq 55\,^{\circ}C$ is still a stable process that can be technically managed and leads to a sanitized effluent but requires energy for maintaining the high reactor temperature.

Aerobic degradation of biopolymers to CO_2 and H_2O (plus ammonia and sulfide when the wastewater contains protein in addition to carbohydrates and fat/oil; Figure 14.1) can be catalyzed by only one species of bacteria, which hydrolyze biopolymers with exoenzymes and respire the monomers to CO_2 and water. Anaerobic degradation is much more complex and requires either hydrolytic/acidogenic (fermentative) + methanogenic *Bacteria* and *Archaea* (Figure 14.2a, left panel) or hydrolytic/acidogenic + acetogenic + methanogenic *Bacteria* and *Archaea* (Figure 14.2b, right panel) that must cooperate not only in a food chain but also by syntrophic coupling of metabolic reactions of the different microorganisms (Table 14.3). Hydrolysis of biopolymers and glycolytic degradation to pyruvate or acetate is similar during aerobic and anaerobic degradation and does not require oxygen. Aerobic bacteria decarboxylate pyruvate and oxidize the acetate (acetyl-CoA) without oxygen in the TCA to carbon dioxide. The electrons of the reducing equivalents from glycolysis,

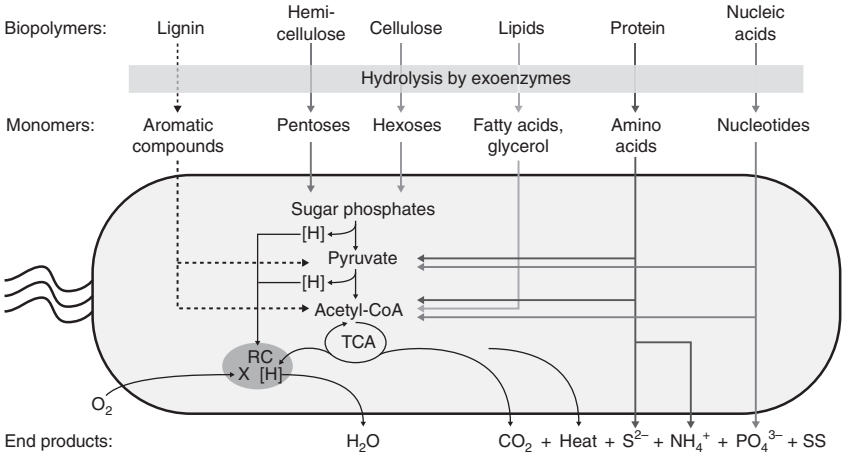


Figure 14.1 Scheme of aerobic degradation of biopolymers (RC = respiratory chain with electron carriers X, TCA = tricarboxylic acid cycle, SS = surplus sludge).

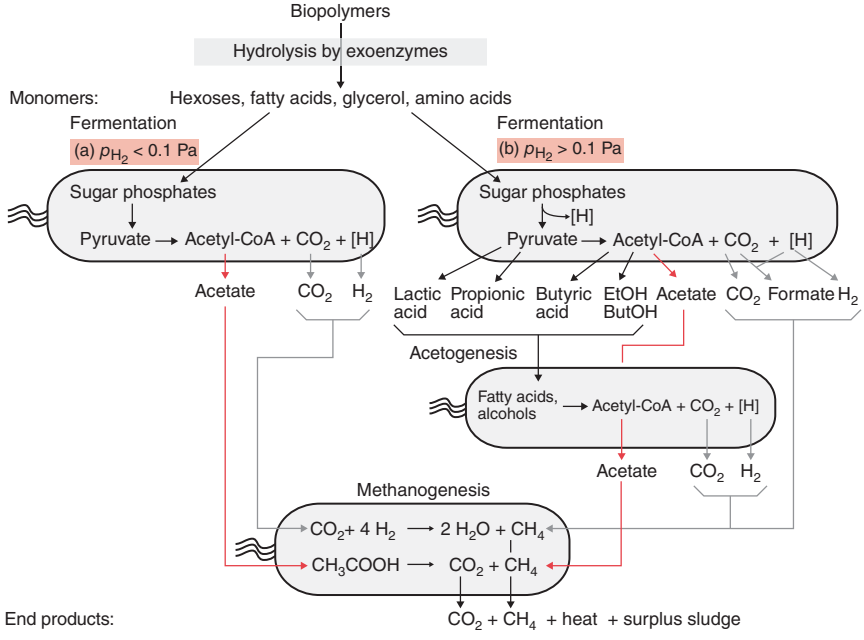


Figure 14.2 Scheme of anaerobic degradation of biopolymers (a) for limiting substrate supply and low hydrogen partial pressure p_{H_2} (left panel) and (b) for surplus substrate supply and a high hydrogen partial pressure p_{H_2} (right panel).

pyruvate decarboxylation, and acetate oxidation drive the respiratory chain and generate ATP while forming water from oxygen and protons. Anaerobic bacteria degrade biopolymers to acetate, CO_2, H_2, heat and surplus sludge (low load). when methanogenic *Archaea* retain the hydrogen partial pressure $p_{H_2} < 0.1$ Pa by methane formation from CO_2 and H_2 (Figure 14.2a, left panel). With surplus substrate or in the presence of toxic substances, hydrogenotrophic methanogenesis ($CO_2 + 4H_2 \rightarrow CH_4 + 2\ H_2O$) as well as acetoclastic methanogenesis ($CH_3{-}COOH \rightarrow CH_4 + CO_2$) is overloaded or inhibited and the hydrogen partial pressure p_{H_2} increases. The fermentative bacteria regenerate their hydrogen-transferring coenzymes by formation of "highly reduced" metabolites such as the volatile fatty acids formate, *n*-butyrate, propionate, and lactic acid or alcohols (ethanol, propanol, and butanol) from more oxidized intermediates such as pyruvate or acetate (Figure 14.2b, right panel). The increased formation of volatile fatty acids decreases the pH and finally leads to failure of methanogenesis. As soon as propionate, *n*-butyrate, and alcohols are formed and excreted, acetogenic bacteria are required for their syntrophic degradation. Fatty acids and alcohols must be degraded to acetate, CO_2, and H_2 (and heat and surplus sludge, high load) which for thermodynamic reasons is only possible in the presence of hydrogen-utilizing bacteria such as methanogens or acetogens (reverse acetogenesis) in biogas digesters or alternatively sulfate reducers, lowering the p_{H_2} to <0.1 Pa. Anaerobic propionate oxidation at standard conditions (Eq. (14.1)) is an endergonic process and proceeds only if hydrogen-utilizing syntrophic partners are present, which lower the hydrogen partial pressure to less than 0.1 Pa. When the p_{H_2} is less than 0.1 Pa, $\Delta G^{0\prime}$ becomes slightly negative, shifting the reaction equilibrium to the right and anaerobic propionate degradation can proceed Eq. (14.2):

$$CH_3{-}CH_2{-}COO^- + 2H_2O \xrightarrow{p_{H_2} > 0.1\ \text{Pa}} CH_3{-}COO^- + CO_2 + 3H_2$$
$$\Delta G^{\circ\prime} = +71.6\ \text{kJ/mol} \tag{14.1}$$

$$CH_3{-}CH_2{-}COO^- + 0.5H_2O \xrightarrow{p_{H_2} < 0.1\ \text{Pa}} CH_3{-}COO^- + 0.25CO_2 + 0.75CH_4 \quad \Delta G^{\circ\prime} = -25.2\ \text{kJ/mol} \tag{14.2}$$

This syntrophic interaction of hydrogen producers and hydrogen consumers (Table 14.3) is called **interspecies hydrogen transfer** and allows complete anaerobic degradation of fatty acids and alcohols in anaerobic digesters. The range of possible p_{H_2}-values for syntrophic propionate oxidation is called the "thermodynamic window."

In sulfate-poor environment, methanogenic *Archaea* dominate as hydrogen scavengers, whereas in the presence of sulfate, sulfate-reducing bacteria are dominating. Hydrogen or the p_{H_2}, respectively, exert a central control function during anaerobic degradation of organic substances to biogas.

14.2.1.1 Mass and Energy Balance

Carbon sources in wastewater and sludge serve the bacteria for catabolism and anabolism. Under optimal growth conditions aerobic bacteria invest up to 50% by

weight of metabolites into cell proliferation (surplus sludge formation), whereas anaerobic bacteria take up maximally 10% by weight for cell growth.

Stoichiometry:	$C_6H_{12}O_6 + 6O_2$	$\rightarrow$	$6CO_2 + 6H_2O$ + heat
Energy balance:	2870 kJ	$\rightarrow$	0 kJ + 0 kJ + 2870 kJ
Mass balance:	180 g + 192 g	$\rightarrow$	264 g + 108 g

Scheme 14.1 Aerobic, growth-uncoupled degradation of glucose.

For a total oxidation of 1 mol glucose (MW = 180 g/mol = 1.07 mol BOD_5), the most often used "model substance" for carbohydrates, 6 mol of oxygen (=192 g) are required (Scheme 14.1). However, carbohydrates or other carbon sources in wastewater are only respired completely if energy conservation during degradation and thus growth is prevented. In **high load activated sludge tanks** up to 50% of the intermediates of glycolysis and the TCA are used for cell proliferation, thus only 3 mol oxygen are necessary for respiration of the residual degradation intermediates to CO_2 and H_2O (Scheme 14.2). In **low-load activated sludge tanks**, when substrate is restricting growth, much more energy must be invested for cell maintenance processes (e.g. for osmoregulation or for pumping of ions through the cytoplasmic membrane). In this situation, energy for growth is restricted and significantly less than 50% by weight of the initial substrate can be incorporated into cells for biomass formation (Scheme 14.3).

Stoichiometry:	$C_6H_{12}O_6 + 3O_2$	$\rightarrow$	$3CO_2 + 3H_2O$ + biomass + heat
Energy balance:	2870 kJ + 0 kJ	$\rightarrow$	0 kJ + 0 kJ + 1980 kJ + 890 kJ
	100% + 0%	$\rightarrow$	0% + 0% + 69% +31%
Mass balance:	180 g + 96 g O_2	$\rightarrow$	132 g + 54 g + 90 g

Scheme 14.2 Aerobic degradation of glucose in a high-load activated sludge tank with surplus substrate supply and maximal growth.

The final oxidation products of aerobic respiration are CO_2 and H_2O. These do not contain energy, whereas methane in the biogas from anaerobic degradation contains most of the energy content of the substrates. Thus during anaerobic degradation little energy is available for cell growth and surplus sludge formation (Scheme 14.4).

Most of the energy that is released as heat during aerobic degradation of organic substances in activated sludge processes is stripped out with the off-gas from aeration, so that unlike composting, where the heat cannot escape from the center of a compost heap or windrow, no self-heating is observed. The implementation of microbiological degradation processes for organic pollutants in wastewater treatment systems will be described in Section 14.3.

In comparison to aerobic wastewater treatment during anaerobic wastewater treatment or sludge digestion, little energy is available for cell growth, leading only to 5–10% surplus sludge. Approximately 90% of the energy of the substrate is conserved into methane and a very small portion of the energy is lost as heat. According to Scheme 14.4, from 1 mol glucose, 5.7 mol biogas, theoretically composed of 50% CO_2 and 50% CH_4, and 9 g of surplus sludge are generated during

Stoichiometry:	$C_6H_{12}O_6 + 4.5O_2$	$\rightarrow$	$4.5CO_2 + 4.5H_2O$ + biomass + heat
Energy balance:	2870 kJ + 0 kJ	$\rightarrow$	0 kJ + 0 kJ + 990 kJ + 1880 kJ
	100 %+ 0 %	$\rightarrow$	0% + 0% + 34.5% + 65.5%
Mass balance:	180 g + 144 g O_2	$\rightarrow$	198 g + 81 g + 45 g

Scheme 14.3 Aerobic degradation of glucose in a low-load activated sludge tank with growth-limiting substrate supply and highly restricted growth.

Stoichiometry:	$C_6H_{12}O_6$	$\rightarrow$	$2.85CO_2 + 2.85CH_4$ + biomass + heat
Energy balance:	2870 kJ	$\rightarrow$	0 kJ + 2541 kJ + 198 kJ + 131 kJ
	100 %	$\rightarrow$	0 % + 88.5 % + 6.9 % + 4.6 %
Mass balance:	180 g	$\rightarrow$	125.4 g + 45.6 g + 9 g

(Energy content of biomass: 22 kJ/g TS, Jördening & Winter 2005)

Scheme 14.4 Anaerobic degradation of glucose to biogas in low-load systems.

$$C_cH_hO_nN_nS_s + \tfrac{1}{4}\,(4c - h - 2o + 3n + 2s)\,H_2O \rightarrow \tfrac{1}{8}\,(4c - h + 2o + 3s + 2s)\,CO_2 + \tfrac{1}{8}\,(4c + h - 2o - 3n - 2s)\,CH_4 + nNH_3 + sH_2S$$

Valid for 100% degradation without consideration of biomass formation (sludge growth)

Scheme 14.5 Buswell equation.

methanogenesis. Using the molar gas volume of 22.4 l/mol from 180 g of glucose, 127.7 l_N (l_N means volume at STP) biogas or 63.8 l_N methane, respectively, is produced, and about 5% of the substrate (9 g) is used for cell growth. In anaerobic digesters, less gas is released as some CO_2 is absorbed during "alcaline digestion": The higher the pH during digestion (e.g. by ammonia formation during protein degradation), the more CO_2 is absorbed forming ammonia bicarbonate and resulting in a higher than theoretically expected methane content in the biogas.

As wastewater from different origins is very different and contains a mixture of complex, dissolved, colloidal, and small-particulate solid substances at highly differing concentrations, deduction of biogas production from the degradation stoichiometry of single compounds is not applicable. To quantify the theoretical amount of biogas and its composition that can be expected from complex wastewater after anaerobic digestion, the input material must be analyzed for its elemental composition. Then, the theoretical amount and the biogas quality can be calculated with the Buswell equation (Scheme 14.5; Buswell and Mueller 1952). For an estimation of the true gas amount, in addition, the degree of degradation, pH, and proportion of the substrate that is diverted for cell growth must be known.

14.2.2 Fundamentals of Nitrification

Municipal wastewater contains many nitrogenous compounds such as urea, amino-N in protein, and heterocyclic N-compounds. Elimination of nitrogen from wastewater requires urea hydrolysis, ammonification of protein and heterocyclic N-compounds, nitrification of ammonia, and denitrification of

nitrate during wastewater treatment. Most of the urea is rapidly hydrolyzed by urease-expressing bacteria from feces Eq. (14.3). Since the travel time of sewage in the sewage systems from distant locations may be several hours, some of the generated ammonia-N ($NH_3 \leftrightarrow NH_4^+$) may also stem from protein hydrolysis and deamination of amino acids Eq. (14.4):

$$NH_2\text{–}CO\text{–}NH_2 + H_2O \xrightarrow{\text{fast}} 2NH_3 + CO_2 \tag{14.3}$$

$$\text{Protein} \xrightarrow{\text{slow}} \text{Oligopeptides/Dipeptides} \xrightarrow{\text{slow}} \text{Amino acids} \xrightarrow{\text{slow}} \text{Fatty acids} + NH_3 \tag{14.4}$$

Ammonia is highly toxic to the water fauna, especially fish, and requires oxygen for nitrification in surface water. Thus, ammonia must be eliminated from wastewater by nitrification/denitrification during treatment in an STP.

Two groups of nitrifying bacteria catalyze nitrification: **Ammonia-oxidizing bacteria (AOB**) that oxidize ammonia to nitrite and **nitrite-oxidizing bacteria (NOB)** that oxidize nitrite to nitrate (for reaction stoichiometries see Table 14.5). Nitrifying bacteria are slow growing microorganisms and must be enriched in activated sludge. The energy gain of AOB from nitritation is $\Delta G^{0'} = -274.7$ kJ/mol, but for nitratation by NOB, only $\Delta G^{0'} = -74.1$ kJ/mol (Table 14.5). As oxidation rates of NOB for nitrite are higher than those of AOB for ammonia under most conditions in STPs, toxic nitrite is not accumulating. AOB and NOB grow autotrophic with CO_2 as the only carbon source and thus generation times and growth rates are much longer/lower and cell yields much smaller than those of heterotrophic sludge bacteria. Average generation times of nitrifiers, for instance, are in the order of six days, whereas those of heterotrophic bacteria are in the order of hours or even less. Nitrification proceeds fastest at a pH of 7.2. At a higher pH ($pH \gg 7.2$), nondissociated ammonia (NH_3) inhibits nitratation, whereas at a lower pH ($pH \ll 7.2$), nondissociated nitric acid (HNO_2) inhibits nitratation (Table 14.5). The higher nitrite oxidation rates of NOB result in significantly higher growth rates than those of AOB at typical sewage temperatures of 16–22 °C. At a sewage temperature of 30 °C, AOB and NOB grow equally fast, whereas at a wastewater temperature of more than 30 °C, AOB grow faster and thus ammonia and nitrite are available for disproportionation by ANAMMOX bacteria (Section 14.2.4). At normally prevailing conditions in conventional STPs, highly toxic nitrite should not accumulate.

Nitrification of 1 mol ammonia-N requires 2 mol oxygen. For oxidation of 1 g NH_4^+-N to nitrate 3.2 l O_2, or about 16 l air are required, provided that this amount of oxygen can be absorbed by the wastewater volume that contains 1 g of ammonia. As none of the aeration systems in activated sludge treatment tanks can provide oxygen transfer rates even close to 100%, high aeration rates with pure oxygen and even higher aeration rates with compressed air must be maintained to avoid oxygen deficiency for carbon removal and nitrification. Because of the higher C- than N-content of sewage and the much higher growth rates of organic compound degrading heterotrophs than of autotrophic nitrifiying bacteria in activated sludge systems a layering of these bacteria in sludge

Table 14.5 Nitrification: comparison of nitritation and nitratation.

Parameter	Nitritation by AOB	Nitratation by NOB
Process	Oxidation of ammonia to nitrite	Oxidation of nitrite to nitrate
Biochemical reaction	$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O$	$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$
Free energy $\Delta G^{0'}$ (kJ/mol)	−274.7	−74.1
Oxygen requirement g O_2/g N	3.43	1.14
Genera	***Nitroso****monas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosolobus*, *Nitrosovibrio*	***Nitro****bacter*, *Nitrococcus*, *Nitrospina*, *Nitrospira*
Growth rates μ[a)]		
At 10 °C	0.29	0.58
At 20 °C	0.76	1.04
At 30 °C	1.97	1.87
Half saturation concentrations (mg N/l)[b)]	2.8	2.3
Inhibition by NH_3[c)] (mg/l)	10–150	0.1–10
Inhibition by HNO_2[d)] (mg/l)	0.15×10^{-3}	0.6
Optimal pH	7.2–8.8	7.2–9.0

AOB = ammonia-oxidizing bacteria; NOB = nitrite-oxidizing bacteria.
a) Taken from Mudrack and Kunst (1991).
b) Reported for nitrification in trickling filters (Siegrist and Gujer 1987).
c) Reported by Anthonisen et al. (1976).
d) Reported by Bergeron (1978).

flocs or aggregates is observed. In the surface layers, fast-growing heterotrophic bacteria are found almost exclusively, which consume all the dissolved oxygen ($c_{max.}$ = 9.6 mg O_2/l) in the wastewater, as long as biodegradable organic pollutants are available. Only when the heterotrophic bacteria have degraded all organic compounds that are represented by the BOD_5 (e.g. in low load activation tanks), oxygen can diffuse into deeper layers of the sludge flocs, allowing the slow-growing autotrophic nitrifiers in this reagion to oxidize ammonia via nitrite to nitrate. For complete oxidation of 500 mg/l BOD_5 in sewage (a typical average concentration), theoretically slightly more than 500 mg O_2/l must be available, and for nitrification of 92 mg/l TKN (typical concentration in sewage, Table 14.1), 420 mg O_2/l is required. As only 9.6 mg of oxygen can be dissolved per liter sewage under standard conditions and much less under ambient conditions in an STP, a greater than 60-fold resaturation of sewage from 0 to 9.6 mg/l is necessary. This high amount of oxygen can only be supplied at high aeration rates as 100% oxygen transfer in STPs cannot be obtained. Conditions

for implementation of nitrification in addition to carbon removal in an activated sludge treatment system or in trickling filters are described in Sections 14.3.2 and 14.3.3.

14.2.3 Elimination of Nitrate by Denitrification

Denitrifying bacteria are mainly aerobic, oxygen-respiring bacteria that, in the absence of oxygen, can switch their metabolism to respiration of organic compounds with nitrate (facultative anaerobic metabolism). The energy gain from nitrate respiration is a little lower than that of oxygen respiration. For denitrification of nitrified sewage (ideally, all BOD_5 removed and all ammonia oxidized), organic carbon sources such as methanol or acetate from external sources or organic compounds from by-passed clarified sewage are supplemented as electron donors. Technically, pre- and post-denitrification systems are installed in STPs (Section 14.3.4). In both arrangements, the carbon sources (e.g. acetate, Scheme 14.6a) are oxidized completely and the electrons are used for N_2 formation from nitrate (Scheme 14.6b). Five mole of electrons are required for denitrification of 1 mol of nitrate (Scheme 14.6b) while only three mole of electrons are necessary for denitrification of 1 mol of nitrite (Scheme 14.6c). Scheme 14.6d,e presents stoichiometries of denitratation and denitritation with acetate, respectively. Incomplete nitrate removal in denitrification tanks may lead to N_2 formation in the adjacent secondary sedimentation pond during the settling time and thus to insufficient settling with a high SVI of the settling sludge. Also emission of climate relevant N_2O is possible.

If nitrification could effectively be interrupted at the stage of nitrite, only 1.5 instead of 2 mol oxygen would be required, which would significantly reduce oxygen requirement and thus aeration costs. Furthermore, less methanol or acetate would be required for denitrification of nitrite, reducing sewage treatment costs even more. Laboratory results were promising and full-scale operation of such a process (so-called "short cut biological nitrogen removal process") for sewage treatment is performed in a few but increasing number of STPs.

14.2.4 New Nitrogen Elimination Processes

Nitrogen removal by disproportionation of nitrite and ammonia in the absence of organic carbon sources by anaerobic ammonia oxidation (ANAMMOX) is a recently implemented process in some STPs. The ANAMMOX bacteria belong to the evolutionarily deep-branching bacterial superphylum of *Planctomycetes*,

(a) $5CH_3–COOH + 10H_2O \rightarrow 10CO_2 + 40H^+ + 40e^-$
(b) $8HNO_3 + 40H^+ + 40e^- \rightarrow 4N_2 + 24H_2O$
(c) $8HNO_2 + 24H^+ + 24e^- \rightarrow 4N_2 + 16H_2O$
(d) $5CH_3–COOH + 8HNO_3 \rightarrow 10CO_2 + 4N_2 + 14H_2O$
(e) $3CH_3–COOH + 8HNO_2 \rightarrow 6CO_2 + 4N_2 + 10H_2O$

Scheme 14.6 Denitrification with acetate as an electron donor: acetate oxidation, nitrous and nitric acid reduction, and denitrification stoichiometries.

Verrucomicrobia, and *Chlamydiae*. Only a few species were isolated recently in pure culture, e.g. *Kuenenia stuttgartiensis*. Currently, five ANAMMOX bacterial genera have been described. Growth is extremely slow with doubling times of around 11 days. For sewage treatment, in STPs several months of enrichment with sludge recycling and the presence of stoichiometric amounts of nitrite and ammonia seem to be required. CANON (*C*ompletely *A*utotrophic *N*itrogen *R*emoval *O*ver *N*itrite), SHARON (*S*ingle *R*eactor system for *H*igh Activity *A*mmonium *R*emoval *O*ver *N*itrite), and OLAND (*O*xygen-*l*imited *A*mmonia *N*itrification and *D*enitrification) are alternative N-elimination processes. Not enough experience is available from full-scale plants to consider such processes already as state of the art for sewage treatment. The main problem seems to be a long-lasting, permanent interruption of nitrification at the stage of nitrite. Once nitrate is formed, conventional denitrifiers outcompete ANAMMOX bacteria.

14.2.5 Microbial Phosphate Elimination

Despite the replacement of phosphate based laundry detergents by zeolite (aluminum silicate)-based laundry detergents, sewage still contains about 20 mg/l total P, mainly in aqueous solution as orthophosphate, polyphosphate, and organic phosphate (Table 14.1). In Germany as in many European countries, the wastewater ordinance requires a reduction of total P-concentrations in treated sewage to less than 1 mg/l, whereas in most STPs of the United States, phosphorus concentration limits are not established. A phosphorus concentration of less than 3–5 mg/l (values according to Litke 2008) in STP effluents is required in some areas of the United States. An even better elimination of phosphorus in STP effluents, as it is required in European countries, would have a beneficial effect on water quality of surface waters.

Classically, the phosphorus of sewage is precipitated with trivalent ferrous or aluminum salts (sulfates or chlorides) when the sewage arrives at the STP. Most of the precipitated phosphorus is removed from the sewage together with primary sewage sludge or at least later on with secondary sewage sludge. If precipitants are dosed only after activated sludge treatment, less precipitants are required since the bacteria in activated sludge take up 1% by weight of phosphate, but an intensive mixing of the precipitant into the effluent of activated sludge tanks and a long time for the reaction to proceed before sedimentation in the secondary sedimentation tank are required. These requirements are considered counter arguments for phosphorus precipitation after activated sludge treatment.

Biological phosphorus removal, exceeding the amount that is required for carbon-removing cells proliferation in sewage, requires polyphosphate accumulating organisms (PAO, e.g. *Acinetobacter* spp., *Microlunatus phosphovorus*, and *Lampropedia* spp.). PAO must be specifically enriched by alternating anaerobic/aerobic phases during sewage treatment (Figure 14.3). PAO are obligate aerobic bacteria accumulating reserve material in three distinct cell deposits that can be distinguished microscopically after specific staining: polyphosphate (PP_n), poly-β-hydroxybutyrate (PHB), and glycogen (Glyc). In a simplified view, PAO in sludge flocs hydrolyze phosphoanhydride bonds of PP_n under anaerobic conditions and release P_i into the sewage, thereby generating ATP for survival

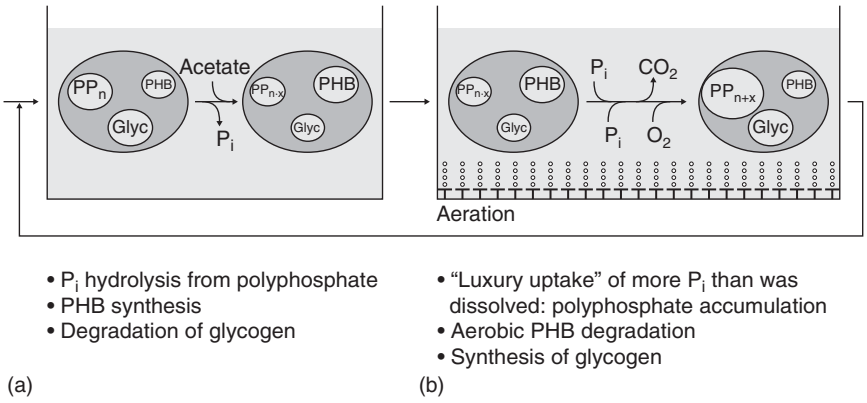


Figure 14.3 Phosphorus elimination by aerobic PAO during alternating anaerobic (a) –aerobic (b) exposition for "luxury uptake of phosphate". PHB = poly-β-hydroxybutyrate; Glyc = glycogen; PP = polyphosphate.

during anaerobiosis. After an anaerobic phase, if oxygen becomes available again, e.g. during activated sludge treatment, PAO take up much more phosphate (P_i) from the sewage than they have liberated under anaerobic conditions, forming PP_{n+x} (Figure 14.3). This phenomenon is called "**luxury uptake**" and is the principle of biological phosphate removal by PAO.

In more detail, aerobic PAO that are exposed to an anaerobic environment, e.g. during controlled anaerobic preincubation, liberate P_i from PP_n ("phosphate redissolution"; Figure 14.3a) and use the energy of the cleaved phosphoanhydride bonds as an energy source for cell maintenance and poly-β-hydroxybutyrate (PHB) synthesis from acetate. Acetate is excessively available in clarified sewage and reducing equivalents for PHB formation (and some ATP) are made available from degradation of part of the cell-internal glycogen reserves. The biochemical reactions for PHB formation do not require much energy. Subsequently in the aerobic environment of an activated sludge tank, PAO have an optimal oxygen supply for respiration of organic substances (BOD_5) that are taken up from raw sewage and/or of metabolites that are released during degradation of internally stored PHB, thereby generating energy for maximal growth, for glycogen synthesis, and for uptake of more phosphate than was released before ("luxury uptake"), respectively (Figure 14.3b). PAO must compete with heterotrophic bacteria for oxygen and for BOD_5. BOD_5 is often the limiting factor for PAO, restricting their energy metabolism. Thus, in addition to BOD_5-compounds from raw sludge PAO respire some β-hydroxybutyrate that is mobilized by hydrolysis of deposited PHB.

In STPs, sludge flocs or aggregates contain PAO together with heterotrophic bacteria and nitrifiers. They are separated with the sludge by gravity sedimentation in the secondary clarifier. The secondary sludge, containing the PAO is mixed with raw sludge in an anaerobic tank arranged upstream of the activated sludge tank. A flow-scheme for one possible option of full-scale treatment with biological phosphorus removal is shown in Section 14.3.5.

As the geological phosphorus deposits are running out worldwide and sewage sludge from phosphorus-removing STPs often contains even more P in the form

of Fe or Al salts and as phosphate in biomass than most primary resources, intensive research on different ways of phosphorus recovery from sewage and sewage sludge is under way.

14.3 Wastewater Treatment Processes

14.3.1 Typical Process Sequence in Municipal Sewage Treatment Plants

Organic and inorganic pollutants of sewage are removed by sequentially arranged mechanical, chemical, and biological purification processes in municipal STPs as shown in Figure 14.4 A, B. The sewage of larger settlements is collected in sewers and flows into the STP inlet structure (1). The first treatment step is mechanical separation of coarse material by gross rakes, fine screens, or sieves (2) and of sand and grease by sedimentation/flotation in sand and grease traps (3), respectively. The suspended material in the sewage is removed in the primary clarifier, and the clarified sewage is treated biologically for carbon and nitrogen removal. The effluent of biological treatment, containing the activated sludge, is again clarified in a secondary clarifier before discharge into surface waters. For removal of soluble phosphorus from sewage, chemical precipitation by Fe or Al salts and separation of precipitates by sedimentation or concentration of sludge is performed, whereas for biological phosphorus removal, PAO must be enriched in the sludge by maintaining anaerobic and subsequently aerobic conditions (Figure 14.3). One process option for biological phosphate removal in a STP is schematically presented in Figure 14.8.

The carbon and nitrogen in sewage is removed by biological treatment. To achieve optimal removal results, in STPs with a pre-denitrification unit (Figure 14.5a), nitrate-containing wastewater from the activated sludge tank and surplus activated sludge from the secondary clarifier is pumped back and mixed with clarified raw sewage for anoxic pre-denitrification (Section 14.3.4). Alternatively, to an anoxic pre-denitrification, a post-denitrification tank for the effluent of the activated sludge treatment tank may be installed (Section 14.3, Figure 14.5b).

Carbonaceous and nitrogenous compounds are converted during aerobic and anoxic wastewater treatment to H_2O, CO_2, and N_2. A significant fraction of intermediary metabolites are used for growth of sludge bacteria. Thus, surplus sludge is formed, which must be separated in the secondary clarifier. As normally high amounts of relatively dilute secondary sewage sludge (TS content most often $\ll$4%) are mixed with a smaller volume of more concentrated primary sewage sludge, the mixture is still very dilute and must be dewatered statically. Dewatered sludge mixtures are then digested in anaerobic reactors to biogas (Figure 14.4c). About 50% of the TS content of the digester effluent is nondigestible suspended residues that are separated from the sludge water by centrifugation and further dewatered in filter presses or with washer disk dryers. The sludge water fraction is highly polluted with COD (including some BOD_5) and ammonia and is recycled into the activated sludge treatment unit. Solidified digester residues from filter

presses are disposed of in sanitary landfills, incinerated, or processed to reusable biosolids (fertilizer), if they are free of toxicants and heavy metals according to boundary values listed in Table 14.2.

Similar processes and technologies for sewage treatment are used in the United States STPs where different levels of treatment must be achieved to ensure protection of public health and the environment (Figure 14.4B). During primary treatment, suspended solids and organic matter are removed. In the secondary treatment, biodegradable organic matter and suspended solids are removed and the wastewater is disinfected by addition of chlorine or by UV

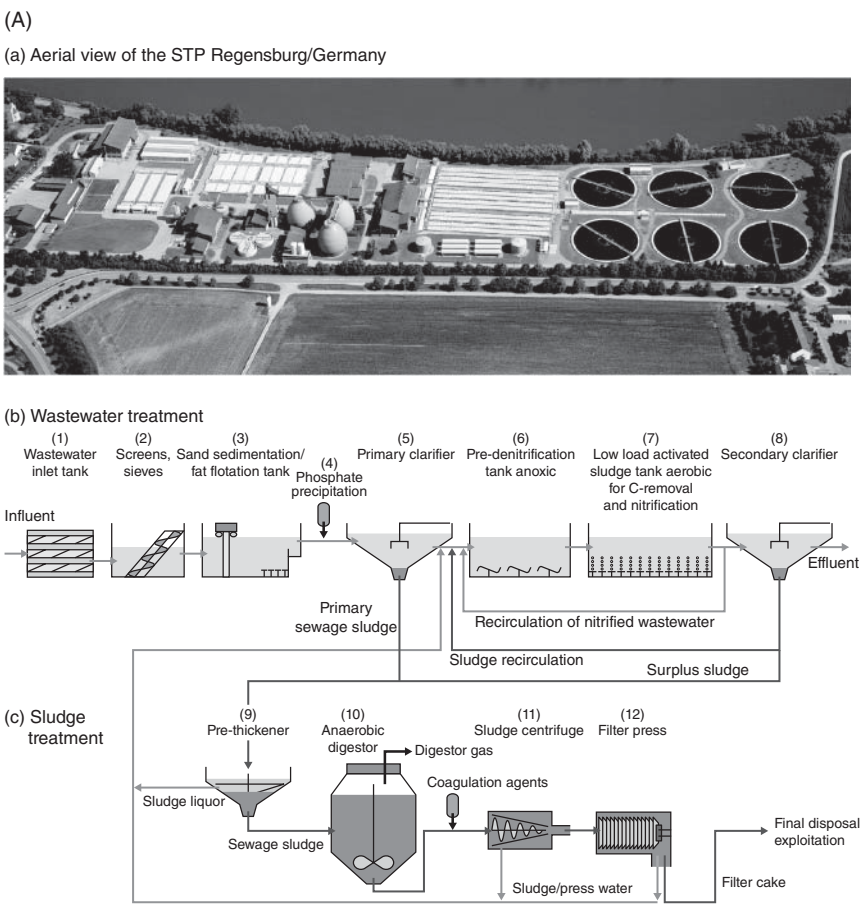


Figure 14.4 Typical sewage treatment plants (STPs) in Germany (A) and in the United States (B). Figure (A) shows an aerial view (a) and the process scheme (b, c) of the municipal STP Regensburg/Bavaria. Source: Photo H. Stolz. Figure (B) shows an aerial view (a) and the process scheme (b, c) of the municipal STP of Boston/Massachusetts. Source: Photos by C. Gallert. In German STPs, the pre-denitrification tank (6) in (b) may be replaced by a post-denitrification tank, which must be fitted in between the low-load activation tank (7) and the secondary clarifier (8). C-sources for denitrification such as methanol, acetate, or glycerol must then be supplied in stoichiometric amounts. Alternatively, adequate amounts of clarified raw sewage from the primary clarifier must be by-passed for post-denitrification.

(B)

(a) Aerial view of the STP Boston/USA

(b) Wastewater treatment

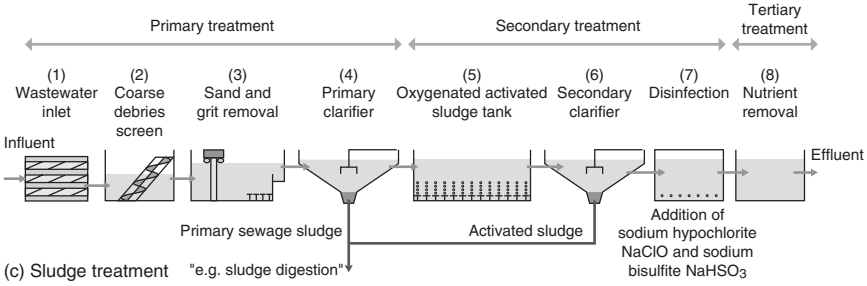


Figure 14.4 (*Continued*)

(ultraviolet) irradiation. If nutrient removal is required secondary or tertiary treatment processes for nitrogen and phosphorus elimination must be installed. The primary and secondary sludges from wastewater treatment must be digested by available technologies (Section 14.3.6).

14.3.2 Activated Sludge Process

Biological purification of mechanically clarified raw sewage in most cases is performed by activated sludge treatment. Dissolved carbonaceous and nitrogenous compounds in the wastewater are respired or nitrified by the aggregated bacteria in sludge flocs. Aeration is required for oxygen supply and for hydropneumatic mixing to prevent gravity sedimentation of the sludge, as well as for carbon dioxide stripping and heat removal. To maintain a high efficiency of BOD_5 removal from sewage in activated sludge tanks at *h*ydraulic *r*etention *t*imes (HRTs) of 12–16 hours (average HRTs in most activated sludge tanks), a suspended sludge concentration of 4.5–5 kg TS/m^3 must be maintained. This requires sludge recirculation from the secondary clarifier. At 16 hours HRT of sewage in activated sludge tanks, carbon removal and nitrification can only proceed close to completion when the sludge age (average sludge retention time (SRT) in the activated sludge tank, including recirculated sludge) exceeds six days and the sludge loading is kept below 0.15 kg $BSB_{5\ added}$/kg sludge TS and day. From a biological standpoint, more than 5 kg of activated sludge per m^3 would be favorable, but optimal process management (mainly oxygen supply) restricts a higher sludge

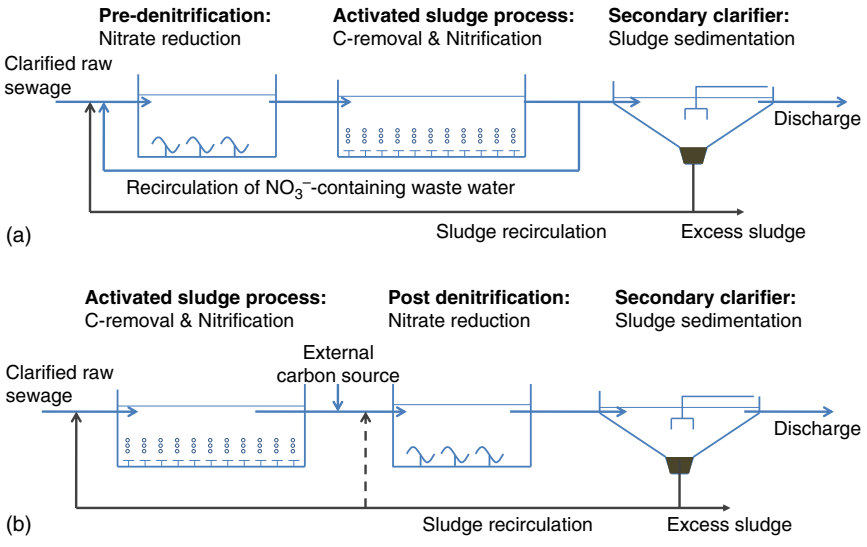


Figure 14.5 Denitrification before (14.5 a, pre-denitrification) and after activated sludge treatment (14.5 b), post-dentrification).

concentration in activated sludge tanks. Increasing viscosity leads to immediate bubble agglomeration after detachment of fine air bubbles from air jet-injection nozzles, causing low oxygen transfer rates into the liquid sewage and finally leads to oxygen deficiency.

In high-load activated sludge tanks (selectors) with a loading of 0.3–2 kg BOD_5/kg TS/d, up to 50% of the BOD_5 is used for surplus sludge formation (Scheme 14.2). BOD_5 may still be degraded almost quantitatively, but there is no nitrification. High-load activated sludge systems can thus only be planned and installed as a first treatment step for removal of as much BOD_5 as possible in a short time at supersaturated carbon supply. To prevent oxygen limitation in selectors, pure oxygen is often used for aeration. The effluent of high-load activated sludge tanks or of selectors never reaches the legally required low discharge boundary values for C and N and thus must be further treated in low-load activated sludge systems to complete degradation of BOD_5 and allow nitrification of ammonia (Figure 14.6a: high-load activated sludge treatment).

If the space loading in low-load activated sludge treatment systems is kept between 0.15 and 0.3 kg $BOD_{5\,added}$/kg TS/d, the BOD_5 is totally degraded (Figure 14.6b: low load activated sludge treatment). Less surplus sludge is formed as compared to high-load activated sludge treatment (minimally 25% at 0.15 kg BOD_5/kg TS/d, Scheme 14.3). The lower the space loading with BOD_5, the more ammonia is nitrified in the activated sludge process. If nitrification in the activated sludge process is not complete, it can be completed in an adjacent trickling filter (Figure 14.7). Growth rates of autotrophic nitrifiers are much lower than those of heterotrophic BOD_5-degrading sludge bacteria and are highly dependent on the temperature of the wastewater (Table 14.5). When the temperature of the wastewater decreases to 10 °C or lower, growth rates of the nitrifiers are very low. Fortunately, NOB still grow about twice as fast as AOB,

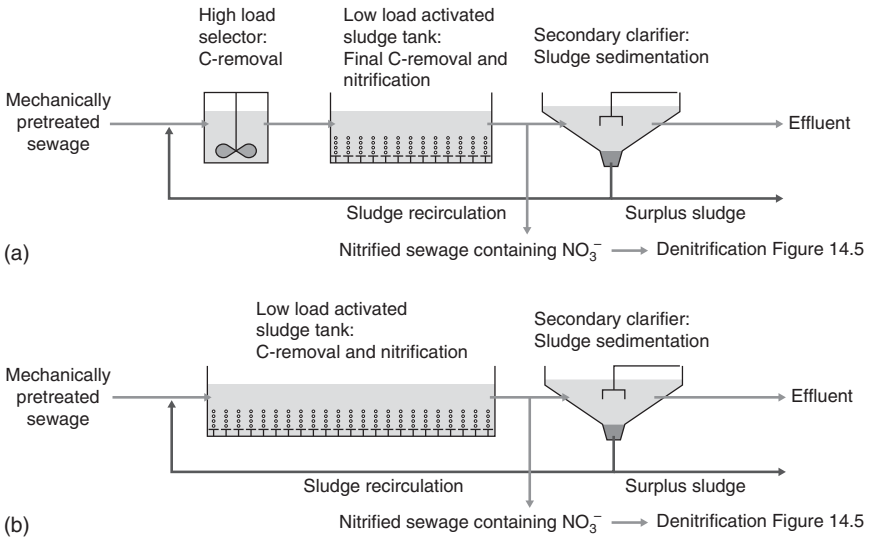


Figure 14.6 Two-stage activated sludge treatment: high-load activated sludge tank (selector) for high-rate BOD_5 removal followed by a low-load activated sludge tank for final C-removal and nitrification (a) and one-stage low-load activated sludge treatment for C-removal and nitrification (b).

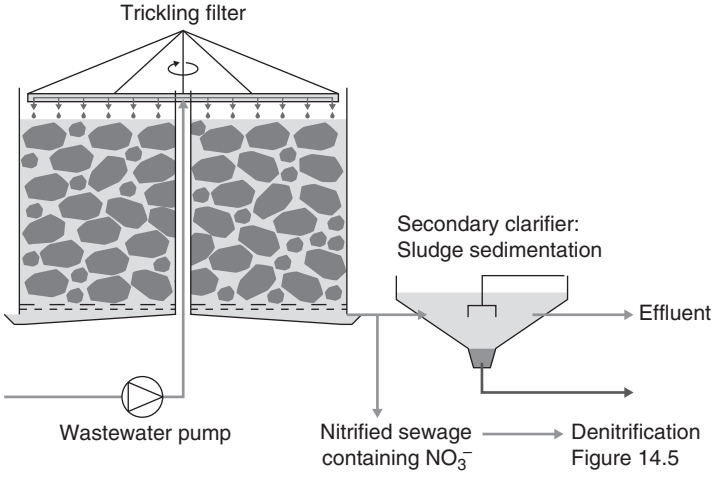


Figure 14.7 Trickling filter for final C-removal and nitrification.

thus preventing accumulation of toxic nitrite concentrations in cold wastewater in the winter season.

14.3.3 Trickling Filters

In most cases, trickling filters in STPs are installed for nitrification or completion of incomplete nitrification of ammonia after activated sludge treatment.

Normally, trickling filters are not used for degradation of carbonaceous compounds in sewage, where much surplus sludge is formed that might lead to irreversible clogging. Wastewater bacteria grow as a biofilm on the surface of irregularly arranged, poured lava stones or on the surface of regularly arranged plastic packings. Mechanically and biologically prepurified wastewater is pumped to the top of trickling filters into rotary distributors and trickles into the filter bed (Figure 14.7). Biological reactions (residual BOD_5 degradation, mainly nitrification of ammonia) are catalyzed by immobilized bacteria in the biofilm on the support material. Surplus biofilm is grazed in a well-balanced manner by protozoa. Sometimes, too much grazing of the biofilm disturbs the biological balance and leads to failure. While oxygen supply in activated sludge tanks is maintained by electricity-consuming surface aerators or by more economic jet injectors from the bottom of the tanks, the oxygen supply for the biofilm on support materials in trickling filters is "passive." Respiration of organic substances or nitrification of ammonia is accompanied by heat release. This leads to an increase of the temperature in the filter material and causes a "chimney effect," sucking fresh air from the bottom to the top of the filter material and thus supplying oxygen for biological reactions.

For sizing of trickling filters, either space loading (kg $BOD_{5\ added}/m^3$ d) or area loading (kg BSB_5/m^2 surface/d) is used. The higher the space loading, the thicker the biofilm grows. Slipping off and wash-out of surplus biofilm is restricted and increases the danger of clogging. To minimize rinse cycles for avoidance of bio-clogging, the loading of trickling filters should be kept low.

Nitrification of all ammonia in mechanically pretreated sewage requires a sequence of two trickling filters, a first one with a larger void volume and higher loading for final carbon removal and a second one with a much smaller void volume and low loading for nitrification. At a space loading of more than 0.75 kg $BOD/m^3/d$, nitrification in a trickling filter is not proceeding, whereas at a space loading of 0.2–0.45 kg $BOD_5/m^3/d$, partial nitrification occurs, and at a space loading below 0.2 kg $BOD_5/m^3/d$, nitrification is complete.

14.3.4 Technical Options for Denitrification

Nitrate that is initially present in wastewater or is generated by nitrification of ammonia during aerobic sewage treatment must be removed by denitrification. The most often used options are pre-denitrification or post-denitrification. In general, pre-denitrification requires energy for pumping to recirculate the nitrified wastewater into the pre-denitrification tank, whereas post-denitrification requires much less energy for pumping but extra costs for external carbon sources and maintaining online nitrate analysis for automated dosage of stoichiometric amounts of the external carbon sources, such as methanol, acetate, or glycerol.

For pre-denitrification of wastewater after activated sludge treatment (Figure 14.5a), electrons are derived from degradation/mineralization of BOD_5 compounds in raw clarified wastewater. All nitrified wastewater must be recirculated into an anoxic tank, arranged in front of the activated sludge treatment unit, and intensively mixed with clarified raw wastewater. To improve denitrification and the following activated sludge treatment efficiency within a

HRT of 12–16 hours, a great part of the activated sludge from the secondary clarifier must also be recirculated to maintain a sludge concentration around 5 kg TS/m^3. Higher sludge concentrations might improve reaction rates for denitrification but would reduce oxygen transfer rates in the subsequent activated sludge tank because of the increase in viscosity. The nitrified wastewater or, in particular sewage from activated sludge treatment, must be recirculated into the pre-denitrification tank at least four times to comply with boundary values for, e.g. N_{total} that must fall below 13 mg/l (Table 14.1) according to legal specification in Germany.

In STPs with post-denitrification, the nitrified wastewater from activated sludge treatment flows into an anoxic denitrification tank, that is following the activated sludge treatment tank (Fig. 14.5b). Electrons for post-denitrification must be supplied by addition of external carbon sources such as methanol, acetate, glycerol, or by adding a respective amount of BOD_5 with clarified raw wastewater. The external carbon sources must be dosed in stoichiometrically required amounts as derived from online measurement of the nitrate concentration in the wastewater. The stoichiometric requirement of acetic acid for denitrification of nitrate or nitrite can be deduced from Scheme 14.6d,e by converting molar amounts into gram amounts or liters. Advantages of a post-denitrification process for nitrogen removal are fast nitrate respiration rates (the bacteria must only metabolize a single, easily degradable carbon source for denitrification of nitrate) and no pumping costs for wastewater recirculation if a natural flow gradient exists in the STP. Disadvantages of post-denitrification are high costs for external carbon sources and online nitrate measurement and installations for dosage of external carbon sources. Concerning the external substrate requirement, slightly over-stoichiometric amounts over the actual nitrate concentration must be added for removal of residual oxygen in the nitrified wastewater that comes from activated sludge treatment.

Alternatives for pre- or post-denitrification of nitrate in wastewater are sequencing batch reactors (SBR), where carbon removal, nitrification, and denitrification proceed sequentially in the same tank by providing alternately aerobic and anoxic phases for the sludge bacteria. During aeration, organic pollutants are respired to CO_2, water, and ammonia (e.g. from protein degradation) and at low loading the ammonia is nitrified to nitrate. In a subsequent anoxic phase without aeration but with stirring/mixing (to avoid sludge sedimentation), nitrate is denitrified with residual BOD_5. In the final phase, stirring must be switched off for sludge sedimentation and withdrawal of the purified and clarified wastewater. Treatment of sewage in SBRs requires at least two tanks: The incoming wastewater must be collected in an empty tank that only contains the sludge sediment after withdrawal of the clarified wastewater, whereas the wastewater in the previously filled tank must undergo the biological and mechanical treatment sequences. The interim "storage volume" of the empty tank must be high enough to collect all incoming wastewater while treatment of the wastewater in the full SBR is running.

Alternatively, a simultaneous C- and N-elimination can be established in round or oval loop basins by slowly circulating wastewater through the loop with the help of mammoth rotating aerators. The wastewater is circulated though the loop several times. BOD_5 respiration and nitrification of ammonia takes place

immediately after the mammoth aerators works until the oxygen in the water is depleted. In the following anoxic flow stretch, denitrification with residual BOD_5 proceeds. Normally, two aerators are installed in a loop.

Alternatively, to pre- or post-denitrification in anoxic tanks or nitrification/denitrification in SBR or loop reactors, denitrification may be carried out in abundant trickling filters (if available) after closing the openings for wastewater outflow and filling the fixed bed volume with wastewater for submersed operation.

14.3.5 Biological Phosphate Elimination

Biological P-elimination can be carried out by installation of "mainstream or sidestream" P-elimination processes (e.g. Pinnekamp et al. 2007; Egle et al. 2016). After enrichment of PAO's they take up more phosphate from wastewater in the activated sludge tank and store it as polyphosphate (PP_n) in granules inside single PAO cells than they redissolved earlier under anaerobic conditions (Section 14.2.5). In so-called "mainstream processes" for biological P-removal (Figure 14.8), the phosphorus is eliminated from the wastewater by removal of the surplus sludge. In an anaerobic tank that is arranged in front of the pre-denitrification tank, recirculated sludge is mixed with raw sewage. Aerobic POA in this sludge sustain anaerobiosis with energy that is generated by phosphate release from PP_n (Figure 14.3). After that, PAO "travel" through the anoxic pre-denitrification tank where denitrifying bacteria reduce nitrate to gaseous nitrogen with electrons mainly from acetate mineralization. The denitrifying bacteria are direct competitors of PAO for acetate or other organic substances (BOD_5). In the activated sludge tank, oxygen is supplied for all bacteria, including the PAO for respiration of BOD_5 ($c_{oxygen} \geq 1.5$ mg/l all time). To prevent redissolution of phosphate during sludge separation in the secondary clarifier, residual oxygen must be available for respiration since redissolution of phosphate would start after oxygen depletion. Gravity-settled sludge must be removed periodically after short sedimentation times. As in all biological processes, Bio-P-removal is temperature dependent and less effective at low water temperatures. For this reason, Bio-P-removal tanks can be equipped with devices for addition of Fe- or Al-salts and rapid mixing to precipitate phosphorus, if it is not incorporated totally into PP_n by PAO, to safely maintain final P-concentrations below the legally required boundary values, as specified in the Wastewater Treatment Ordinance for different size classes of WTPs in Germany (Table 14.1) or according to the EPA data access tool (EPA 2011) for prioritized watersheds with phosphorus loading reductions.

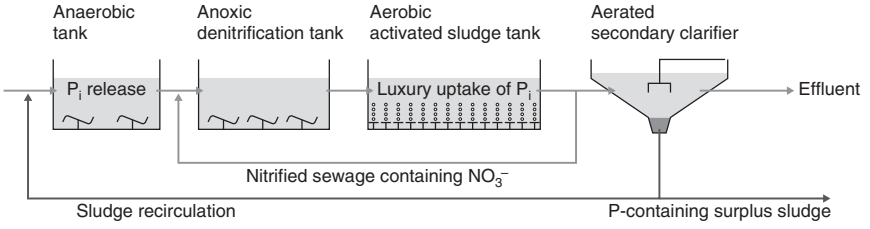


Figure 14.8 Biological phosphorous removal ("mainstream process").

14.3.6 Sewage Sludge Treatment

Sludge from primary and secondary clarifiers as well as flotation sludge contain up to 97% water and must be thickened statically by gravity sedimentation or dynamically by centrifugation or filtration for further aerobic stabilization or anaerobic digestion. A large portion of sludge in STPs is primary sewage sludge, accounting for 40 g of TS/(person d). Aerobic degradation of soluble organic compounds in clarified sewage results in generation of an additional 30–45 g TS/(person d) as surplus sludge, consisting mainly of heterotrophic C-degrading and denitrifying and autotrophic nitrifying sludge bacteria. Chemically precipitated phosphorus contributes about 3–7 g TS/(person d) to the total sludge amount.

14.3.6.1 Aerobic and Anaerobic Sewage Sludge Treatment

Aerobic sewage sludge stabilization in tanks is not widely applied since it requires intensive aeration with pure oxygen or with high flow rates of air, and thus it is not economic. In smaller STPS sometimes pile composting is used, requiring a substantial reduction of the moisture content to about 55 %. Similar or identical respiration reactions take place during composting as during activated sludge treatment (Figure 14.1). Certain biopolymers and a few dissolved organic compounds are carbon sources for the heterotrophic bacteria in the water film around the solids. The biopolymers are hydrolyzed by exoenzymes to water-soluble monomers, which are taken up from the water film by heterotrophs and are respired with oxygen to CO_2 and H_2O (+ ammonia in case of protein degradation). A significant proportion of the metabolites are used for cell proliferation. Aerobic bacteria are living in the water film around the particulate matter where they find plenty of substrates for growth and a good oxygen supply through the loose pore system in a compost heap. For optimal growth conditions, a water activity $a_W \geq 0.97$ must be maintained, if necessary by initial moistening of dry material or remoistening during composting. To prevent self-heating to more than 70 °C in large windrow compost rows, regular turning – at least in the first months – is required to release the heat generated during aerobic degradation. The composted material contains the biologically nondegradable sludge components and bacteria, which are highly "mineralized" (single cells are starved without internal carbon reserve deposits) if sludge stabilization proceeded to completion.

For anaerobic sludge stabilization by sludge digestion, no oxygen may be present and a low redox potential is required. The sequence of reactions that lead to hydrolysis of biopolymers, acidogenesis, acetogenesis, and methanogenesis are catalyzed by different groups of strictly anaerobic bacteria, including methanogenic *Archaea*, that produce biogas (Figure 14.2). Biogas is a climate-relevant gas and may not be released into the atmosphere. The biogas from STPs must thus be collected and either incinerated in gas torches (when energy recovery is not economic because of low amounts) or used as an energy source for heating or for generation of electricity in a CHP (combined heat and power plant). Completely stirred tank reactors (CSTR), either egg-shaped (allow good bottom withdrawal of sediments, Figure 14.9) or cylindrical, are used for anaerobic stabilization of sewage sludge. For optimal degradation rates of the biodegradable portion, the bacteria in anaerobic sludge must be

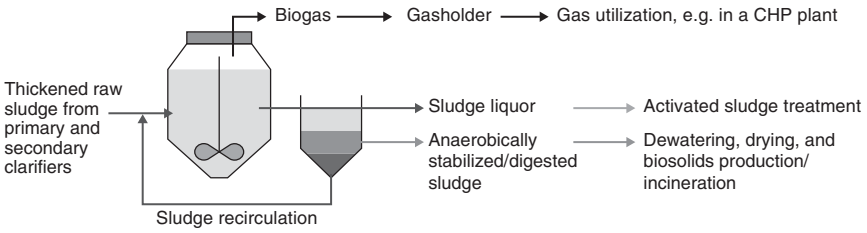


Figure 14.9 Sewage sludge digestion in a completely stirred tank reactor with sludge recirculation (contact process). CHP = combined heat and power plant.

kept in suspension either mechanically by agitators or hydropneumatically by sludge recirculating from the top through bottom inlets of the reactor (allowing re-heating of sludge by passage through externally mounted heat exchangers to maintain the reactor temperature at e.g. 34 °C) or pneumatically by recirculating biogas from the gas head of the reactor through gas nozzels at the bottom of the reactor. For digestion of sewage sludge, mainly CSTRs at a SRT of 10–20 days (or even much longer) are fed with a mixture of primary and secondary (activated) sewage sludge. SRTs longer than 10–20 days were maintained in earlier days for killing of worm eggs to obtain partial sanitation. To improve biogas production rates during anaerobic sludge treatment, sludge from digester effluent may be separated by centrifugation or sedimentation and recirculated into the reactor together with fresh sewage sludge. Reactors with recirculation of digested sludge concentrates are called contact reactors (Figure 14.9). For digestion in the mesophilic temperature range, the sludge in the reactor should be kept at 34 (37) ± 1 °C, and for digestion in the thermophilic range, the digester temperature should be kept at 55 ± 0.5 °C, a temperature that allows sanitation of the effluent after a long enough SRT. Anaerobic sludge treatment is performed either in one-stage systems with parallel reactors if required or in two-stage systems with sequentially arranged identical or different reactor types. The first reactor is fed with fresh material at a high load whereas the second reactor is fed with effluent of the first reactor at a lower load. A combination of mesophilic digestion in the first reactor and thermophilic digestion in the second reactor or vice versa is favorable if sanitation of the effluent is required.

During biological treatment of clarified sewage in a municipal STP, a BOD_5 elimination of ≥97% can be achieved by respiration and incorporation of metabolites during growth of heterotrophic sludge bacteria (Table 14.1), and this high BOD_5 elimination causes almost complete VS elimination. However, the VS elimination of primary and secondary sewage sludge in CSTRs after an SRT of about 10 days does not exceed 50–60%. Even much longer SRTs do not lead to significant further degradation, as the "technical digestion limit" of sewage sludge is reached. The specific biogas production of sewage sludge mixtures is about 740 l_N/kg $VS_{degraded}$ or about 350 l_N/kg VS_{added}.

Degradation rates in mesophilic and thermophilic digesters are not significantly different as judged by biogas production. The reaction temperature has only a small effect on degradation efficiency. Hydrolysis and acidification may proceed a little faster in thermophilic sewage digesters but methanogenesis proceeds at equal rates. Thus, the effluent of thermophilic digesters may contain

more volatile fatty acids than that of mesophilic sewage sludge digesters. Thermophilic digestion at temperatures above 55 °C tends to be unstable and sludge separation from the effluent is less effective. Separated water from dewatering of mesophilically or thermophilically digested sewage sludge is highly polluted with dissolved and colloidal COD and with high concentrations of ammonia and must be recirculated into the STP.

14.3.6.2 Sanitation and Quality Assurance of Sewage Sludge

For quality enforcement in the German sewage ordinance (AbfKlärV 2017), to date only boundary values for heavy metals and organic toxicants are set (Table 14.2). Toxicant-containing sludge should be incinerated. The fertilizer regulation (DüMV 2012) includes a maximum level of the pathogen *Salmonella* spp. and no other specific pathogenic microorganisms (Table 14.2). To avoid direct contact of domestic animals with possibly infectious pathogenic bacteria, the use of treated but nonsanitized sewage sludge as an organic fertilizer on grass land of pastures or on agricultural land for production of forage crops is forbidden. The risk of infection must be minimized by sanitation/decontamination of anaerobically digested sewage sludge. Toxicant containing sludge should be incinerated.

Pathogen reduction in biosolids class A or B in the United States must be carried out by processes with high temperature and time, alkaline treatment, or by other processes to significantly reduce pathogens (PSRP, Table 14.2) such as aerobic digestion, air drying, anaerobic digestion, composting, lime stabilization, or by processes to further reduce pathogens (PFRP) such as composting, heat drying, heat treatment, thermophilic aerobic digestion, β ray irradiation, γ ray irradiation, or pasteurization (EPA 1995, Biosolids Management Handbook).

14.4 Advanced Wastewater Treatment

Carbon removal as well as, microbial nitrogen and phosphorus elimination during sewage treatment are state-of-the-art technologies. Established processes at full-scale achieve required boundary values (Table 14.1). In some regions or in special cases, where surface water is the main source of drinking water, boundary values for phosphorus may be stricter than listed in Table 14.1 (according to AbwV 2004). This is the case for Lake Constance in Southern Germany, which serves as a drinking water reservoir for southern Baden-Württemberg, including its capital Stuttgart. The phosphorus concentration of treated sewage that is discharged into Lake Constance may not exceed 0.3 mg/l, as compared with 1 mg/l in treated sewage of a STP class 5. This requires additional phosphorus removal processes such as flocculation filtration or membrane filtration/ultrafiltration. Phosphorus loading reductions in prioritized watersheds like the Great Lakes or the Mississippi river are examples for stricter regulations in the United States.

In future regulations, removal of xenobiotic micropollutants and of pathogenic bacteria during wastewater treatment will be tightened. Xenobiotic micropollutants ($c < 1$ mg/l) are composed of pharmaceuticals for humans and animals, endocrine disruptors (e.g. hormones), disinfectants, personal care products (e.g.

musks), tensides, pesticides, insecticides, as well as industrial chemicals such as flame retardants. Some of these substances are toxic to the water fauna or cause femalization, are persistent to microbial degradation, and are enriched in the food chain. In health care, more than 60% of medication is used at home ("outpacient-care"). Consequently, residues of pharmaceuticals are reaching STPs dispersed and at a very dilute concentration. Many of the pharmaceuticals are not biodegradable and reach surface waters after sewage treatment. In addition to micropollutants, purified sewage contains significant amounts of, e.g. fecal bacteria, even if total bacterial elimination efficiencies reach 99.99% (4 log decades removal). This means that at e.g. initial concentrations of 10^7 bacteria/ml after treatment, 10^3 bacteria/ml are still present and reach the outfalls of STPs.

14.4.1 Elimination of Micropollutants

Elimination of micropollutants can be achieved by adsorption onto charcoal added as a powder into the activated sludge tank or for more specific adsorption into the effluent of the secondary clarifier, requiring an additional separation device. Alternatively, micropollutants may be adsorbed onto particulate charcoal in filters. Addition of powdered charcoal into the activated sludge treatment tank leads to a better sedimentation of surplus sludge in the secondary clarifier and to less turbidity of the supernatant sewage. A reduction of the amount of bacteria and of micropollutants in effluent of the secondary clarifier may also be achieved by slow sand filtration (biofilm formation and adsorption) or by restraint of bacteria and pollutants during membrane filtration, using ultrafiltration or reversed osmosis. All wastewater must pass the filters, causing high costs for filters, their maintenance, and their safe operation. Alternatively to elimination by filter processes micropollutants and bacteria may be further eliminated from purified wastewater by physicochemical oxidation/inactivation processes. Addition of ozone or $H_2O_2 \pm$ UV irradiation leads to oxidation of micropollutants and inactivates bacteria in the purified wastewater.

14.4.2 Wastewater Disinfection

The inactivation of bacteria in WTP effluents of countries of the EU should follow at least the targets that are defined in the EU Bathing Water Directive (2006/7/EG). Public bathing water is categorized according to the amount of bioburden into "inadequate," "sufficient," "good," and "excellent" quality. In Europe, all bathing waters had to reach the category "sufficient" by the end of the bathing season of 2015. Furthermore, measures had to be demonstrated that will lead to an increase to the categories "good" or "excellent" quality. Microbiological parameters in the German bathing water ordinance are "**intestinal Enterococci** (IE)" and "***Escherichia coli***," determined as "colony forming units" (cfu) per 100 ml. To achieve a bathing water of "excellent quality," IE and *E. coli* may not exceed 200 or 500 cfu /100 ml (95 percentile enumeration). To achieve the goals of World Health Organization (WHO) or EU guidelines, improvements of wastewater purification by advanced (fourth) treatment techniques, such as UV irradiation, photo-Fenton reaction, ozonation, chlorination, filtration, or

combined treatments are useful. Inactivation and decay of bacteria results in the release of genetic material that includes resistance genes for heavy metals or antibiotics. Such resistance genes could be transferred by naturally occurring horizontal gene transfer to the resident bacterial community.

14.5 Future Perspectives

The availability of 100–150 l of sanitized drinking water per person and day and increasing demand for domestic water free of pollutants to ensure safe and sufficient food production for nutrition of the population in industrialized countries requires further improvements of STPs. At the same time, in developing countries, water consumption is increasing and wastewater is often not purified. As the climate changes progressively and costs for drinking water increase sharply, the use of purified wastewater as domestic water or for production processes may become "the rule" in the future. For this reason, "end of pipe" solutions for wastewater treatment can no longer be the only accepted options. Streams of differently polluted wastewater must be separately collected and treated as required, as is done in some industry branches already. To optimize existing or newly detected microbial processes for wastewater treatment, the microbial fundamentals and requirements for growth of respectively required bacteria (e.g. ANAMMOX or N-damo [nitrite-dependent anaerobic methane oxidation] process) need to be studied. Respective microorganisms and their growth requirements are not yet known in detail. It is doubtful if genetically modified bacteria, added as microbial amendments, will be able to compete with the endogenous population during municipal sewage treatment because of the high permanent fecal contamination, the vast gene pool in sewage, and the complexity of sewage. Next-generation sequencing (NGS) technologies will help to understand population community structures and networks in complex ecosystems such as sludge flocs and biofilms. In order to improve sewage treatment or to prevent process failure, a more detailed basic knowledge of biological N- and P-elimination from wastewater is still necessary. Once more basic knowledge is available, it must be transferred by engineers into technical processes for stable operation of STPs.

References

AbfKlärV (2017). Verordnung über die Verwertung von Klärschlamm, Klärschlammgemisch und Klärschlammkompost (Klärschlammverordnung - AbfKlärV). Klärschlammverordnung vom 27. September 2017 (BGBl. I S. 3465), die zuletzt durch Artikel 6 der Verordnung vom 27. September 2017 (BGBl. I S. 3465) geändert worden ist.

AbwV (2004). Verordnung über Anforderungen an das Einleiten von Abwasser in Gewässer in der Fassung der Bekanntmachung vom 17. Juni 2004 (BGBl. I S. 1108, 2625), die durch Artikel 1 der Verordnung vom 1. (BGBl. I S. 1108, 2625), die zuletzt durch Artikel 1 der Verordnung vom 22. August 2018 (BGBl. I S. 1327) geändert worden ist.

Anthonisen, A.C., Loehr, R.C., Prakasam, T.B.S., and Srinath, E.G. (1976). Inhibition of nitrification by ammonia and nitrous acid. *J. Water Pollut. Control Fed.* 48: 835–852.

APHA, American Public Health Association (2003). *Standard Methods for the Examination of Water and Wastewater*. Washington DC: American Public Health Association.

Bergeron P (1978). Untersuchungen zur Kinetik der Nitrifikation. Karlsruher Berichte zur Ingenieurbiologie, 12.

Buswell, A.M. and Mueller, H.F. (1952). Mechanism of methane fermentation. *Ind. Eng. Chem.* 44: 550–552.

Center for Sustainable Systems, University of Michigan (2015). U.S. Wastewater Treatment Fact Sheet. Pub. No. CSS04-14. http://css.snre.umich.edu/css_doc/CSS04-14.pdf. (accessed 7 August 2019)

DEV (Deutsche Einheitsverfahren) (1983). *Deutsche Einheitsverfahren zur Wasser-, Abwasser und Schlammuntersuchung*. Weinheim: Verlag Chemie.

DüMV (2012). Verordnung über das Inverkehrbringen von Düngemitteln, Bodenhilfsstoffen, Kultursubstraten und Pflanzenhilfsmitteln vom 5. Dezember 2012 (BGBl. I S. 2482).

Dunbar, W.P. (1908). *Principles of Sewage Treatment*. London: Charles Griffin & Co.

DWA (2018). 30. Leistungsvergleich kommunaler Kläranlagen. https://de.dwa.de/files/_media/content/06_SERVICE/Zahlen%20|%20Fakten%20|%20Umfragen/leistungsvergleich_2017_kl.pdf. (accessed 7 August 2019).

Egle, L., Rechberger, H., Krampe, J., and Zessner, M. (2016). Phosphorus recovery from municipal wastewater: an integrated comparative technological, environmental and economic assessment of P recovery technologies. *Sci. Total Environ.* 2016 https://doi.org/10.1016/j.scitotenv.2016.07.019.

EPA (1995) Biosolids Management Handbook. https://www.epa.gov/sites/production/files/documents/handbook1.pdf.

EPA (2011). Data access tool to help states develop strategies to address nitrogen and phosphorus pollution. https://www.epa.gov/nutrient-policy-data/nitrogen-and-phosphorus-pollution-data-access-tool. (accessed 7 August 2019).

European Parliament & Council (2006). Directive 2006/7/EC of the European Parliament and of the council of 15 February 2006 concerning the management of bathing water quality and repealing directive 76/160/EEC. Off. J. L 064, 37e51.

Gerardi, M.H. (2006). *Wastewater Bacteria. Wastewater Microbiology Series*. Hoboken, NJ: Wiley.

Imhoff, K. and Imhoff, K.R. (2007). *Taschenbuch der Stadtentwässerung*, 30. Auflage. Oldenbourg Industrieverlag GmbH.

Jördening, H.-J. and Winter, J. (2005). *Environmental Biotechnology – Concepts and Applications*. Weinheim: Wiley-VCH.

Litke, D.W. (2008) Review of Phosphorus Control Measures in the United States and Their Effects on Water Quality. U.S. Geological Survey Water Resources Investigations Report 99-4007. http://pubs.usgs.gov/wri/wri994007/. (accessed 7 August 2019).

Metcalf & Eddy, Inc. (2003). *Wastewater Engineering –Treatment and Reuse*, 4e. New York: Published by McGraw-Hill Comp. Inc.

Mudrack, K. and Kunst, S. (1991). *Biologie der Abwasserreinigung*, 3. Auflage. Stuttgart: Verlag G. Fischer.

Pinnekamp, J., Montag, D., Gethke, K. et al. (2007). Rückgewinnung eines schadstofffreien mineralischen Kombinationsdüngers "Magnesiumphosphat -MAP" aus Abwasser und Klärschlamm. Forschungsbericht 202 33 308 UBA-FB 001009 Texte 25/07. ISSN: 1862-4804. https://www.umweltbundesamt.de. (accessed 7 August 2019).

Siegrist, H. and Gujer, W. (1987). Demonstration of mass transfer and pH effects in a nitrifying biofilm. *Water Res.* 21: 1481–1487.

United States Senate (2002). Clean Water Act: Federal Water Pollution Control Act (As Amended Through P.L. 107–303, November 27, 2002). http://www.epw .senate.gov/water.pdf. (accessed 7 August 2019).

Further Reading

Brisolara, K. F., Qi, Y., Kendrick, Q., and Davis, Y. (2018). Biosolids and Sludge Management. *Water Environment Research*, 90(10), 978–1006.

Sun, J., Dai, X., Wang, Q., van Loosdrecht, M. C., and Ni, B. J. (2019). Microplastics in wastewater treatment plants: Detection, occurrence and removal. Water research.

Agrawal, S., Seuntjens, D., De Cocker, P., Lackner, S., and Vlaeminck, S. E. (2018). Success of mainstream partial nitritation/anammox demands integration of engineering, microbiome and modeling insights. Current opinion in biotechnology, 50, 214–221.

Pei, M., Zhang, B., He, Y., Su, J., Gin, K., Lev, O., … and Hu, S. (2019). State of the art of tertiary treatment technologies for controlling antibiotic resistance in wastewater treatment plants. Environment international, 131, 105026.

Melia, P. M., Cundy, A. B., Sohi, S. P., Hooda, P. S., and Busquets, R. (2017). Trends in the recovery of phosphorus in bioavailable forms from wastewater. Chemosphere, 186, 381–395.

Index

Industrial Microbiology, First Edition.
Edited by David B. Wilson, Hermann Sahm, Klaus-Peter Stahmann, and Mattheos Koffas.

b

WILEY END USER LICENSE AGREEMENT

Go to www.wiley.com/go/eula to access Wiley’s ebook EULA.