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Historical Overview and Future Perspective

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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.

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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

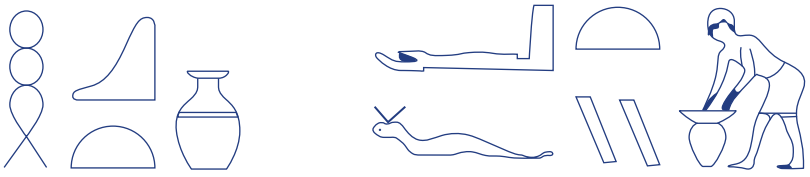

Figure 1.1 More than 3000 year-old Egyptian hieroglyphica, credentials of unconscious use of microorganisms for beer production at ancient times.

(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).

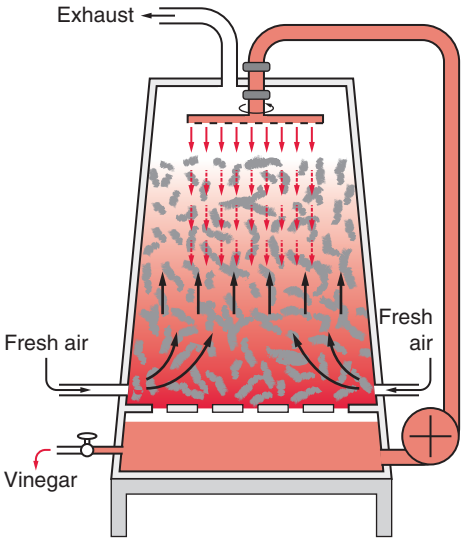

Figure 1.4 Acetic acid production from nineteenth century on. The vessel is filled with beechwood chips, grown over with acetic acid bacteria. 6 – 10% alcohol was trickled from the top of the vessel, at the outlet (bottom) 4 – 10% acetic acid was collected. Aeration was provided by air counterflow to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

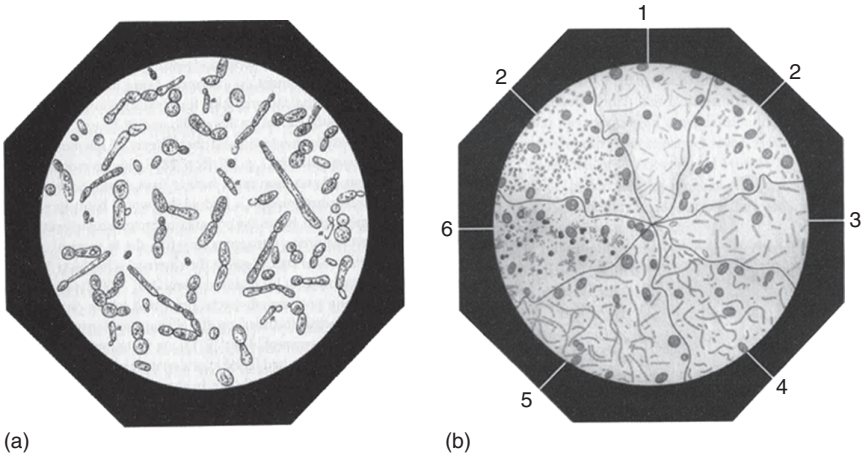

Figure 1.6 Yeast at the beginning of fermentation (a) and from "failed fermentations" (b). Drawing prepared by Pasteur (1876).

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

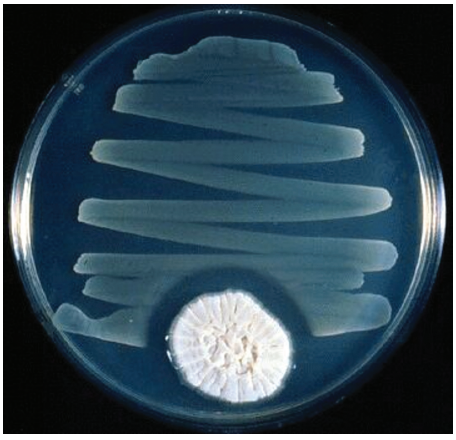

Figure 1.7 Reproduction of an agar plate (solid medium) contaminated with *Penicillium notatum*, randomly observed by Sir Alexander Fleming. *Staphylococcus aureus* do not grow in the neighborhood of the fungus as the penicillin concentration is above the minimal inhibitory concentration (with kind permission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

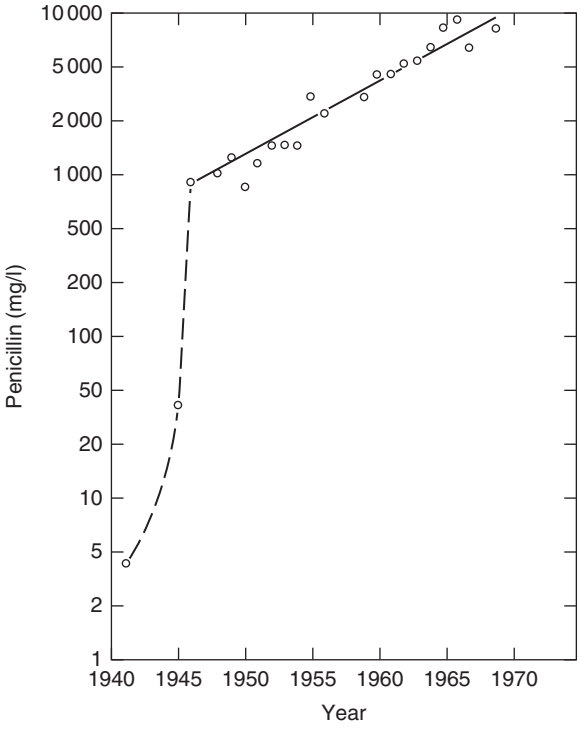

Figure 1.8 Progression of penicillin concentrations in fermentations from 1941 to 1960. Modified from: A.L. Demain (1971). Overproduction of microbial metabolites and enzymes due to alteration of regulation. Advances in Biochemical Engineering/Biotechnology, 1 : 113–142 (with kind permission of Springer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

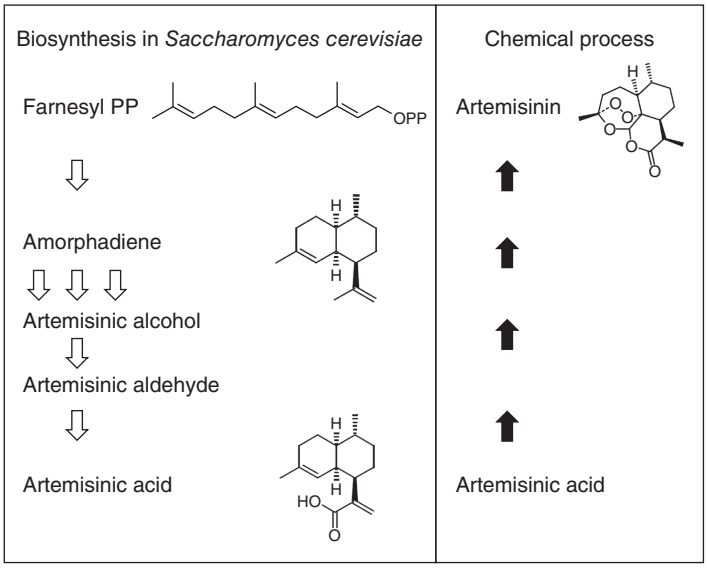

Figure 1.9 Semisynthetic Artemisinin production. Farnesyl-PP is provided as precursor by the *S. cerevisiae* anabolism after overexpression of 10 genes (not shown). Six enzymes (⬆) encoded by genes originating from the plant *Artemisia anna* catalyze the intracellular conversion to Artemisinic acid.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

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