1
History of Industrial Biotechnology

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1.1
Early History

The practise of industrial biotechnology has its roots deep in antiquity. Long before their "discovery," microorganisms were exploited to serve the needs and desires of humans, for example to preserve milk, fruits, and vegetables, and to enhance the quality of life by producing beverages, cheeses, bread, pickled foods, and vinegar. The use of yeasts dates back to ancient days. The oldest fermentation know-how – the conversion of sugar to alcohol by yeasts – was used to make beer in Sumeria and Babylonia as early as 7000 BC. By 4000 BC, the Egyptians had discovered that carbon dioxide generated by the action of brewer's yeast could leaven bread. Ancient peoples are also known to have made cheese with molds and bacteria.

Another ancient product of fermentation, wine, was made in Assyria in 3500 BC and reference to wine can be found in the Book of Genesis, where it is noted that Noah consumed a bit too much of the beverage. According to the Talmud, "a man without salt and vinegar is a lost man." In the field of human health, vinegar has a long history of use: the Assyrians used it to treat chronic middle ear diseases, Hippocrates treated patients with it in 400 BC, and, according to the New Testament, vinegar was offered to Jesus on the cross.

The use of microorganisms in food also has a long history. In 100 BC, Ancient Rome had over 250 bakeries which were making leavened bread. As a method of preservation, milk was fermented to lactic acid to make yogurt and also converted into kefyr and koumiss using *Kluyveromyces* species in Asia. The use of molds to saccharify rice in the koji process dates back at least to AD 700. By the fourteenth century AD, the distillation of alcoholic spirits from fermented grain, a practise thought to have originated in China or the Middle East, was common in many parts of the world. Vinegar manufacture began in Orleans, France, at the end of the fourteenth century and the surface technique used is known as the Orleans method.

In the seventeenth century, Antonie van Leeuwenhoek, a Dutch merchant with no university training but a keen amateur interest in the construction of microscopes, turned his simple lens to the examination of water, decaying matter,

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and scrapings from his teeth. There he reported on the presence of tiny "animalcules," moving organisms less than a thousandth the size of a grain of sand. Leeuwenhoek's lack of university connection might have caused his discoveries to go unrecognized had it not been for the Royal Society in England and its secretary, Henry Oldenburg, who corresponded with European science amateurs. From 1673 to 1723, Leeuwenhoek's observations as a microscopist were communicated to the Society in a series of letters.

Most scientists at the time thought that microbes arose spontaneously from non-living matter. What followed was a 100-year debate over spontaneous generation, aptly called the "War of the Infusions." Proponents had previously claimed that maggots were spontaneously created from decaying meat, but this theory was disputed by Italian physician, Francesco Redi. By this time, the theory of spontaneous generation, originally postulated by Aristotle, among others, had been discredited with respect to higher forms of life, so the proponents concentrated their arguments on bacteria. The theory did seem to explain how a clear broth became cloudy via growth of large numbers of "spontaneously generated microorganisms" as the broth aged. However, others believed that microorganisms only came from previously existing microbes and that their ubiquitous presence in air was the reason that they would develop in organic infusions after gaining access to these rich liquids.

In the early nineteenth century, three independent investigators – Charles Cagniard de la Tour of France, Theodor Schwann, and Friedrich Traugott Kützing of Germany – proposed that the products of fermentation, chiefly ethanol and carbon dioxide, were created by a microscopic form of life. This concept was bitterly opposed by the leading chemists of the period (such as Jöns Jakob Berzelius, Justus von Liebig, and Friedrich Wöhler), who believed fermentation to be strictly a chemical reaction; they maintained that the yeast in the fermentation broth was lifeless, decaying matter.

Organic chemistry was flourishing at the time, and these opponents of the theory of living microbial origins were initially quite successful in putting forth their views. Interest in the mechanisms of these fermentations resulted in the later investigations by Louis Pasteur (Figure 1.1), which not only advanced microbiology as a distinct discipline, but also led to the development of vaccines and concepts of hygiene which revolutionized the practise of medicine.

In 1850, Casimir Davaine detected rod-shaped objects in the blood of anthrax-infected sheep and was able to produce the disease in healthy sheep by inoculation of such blood. In the next 25 years, Pasteur of France and John Tyndall of Britain finally demolished the concept of spontaneous generation and proved that existing microbial life came from preexisting life.

The work of Pasteur originated in his work as a chemist on chirality. In the 1850s, Pasteur had detected two optical types of amyl alcohol, that is, D and L, but he was not able to separate the two. For this reason, he began to study living microbes carrying out fermentation which led to his conclusion, in 1857, that fermentation was a living process of yeast. In 1861, Pasteur proved the presence of microbes in the air, which discredited the theory of spontaneous generation of

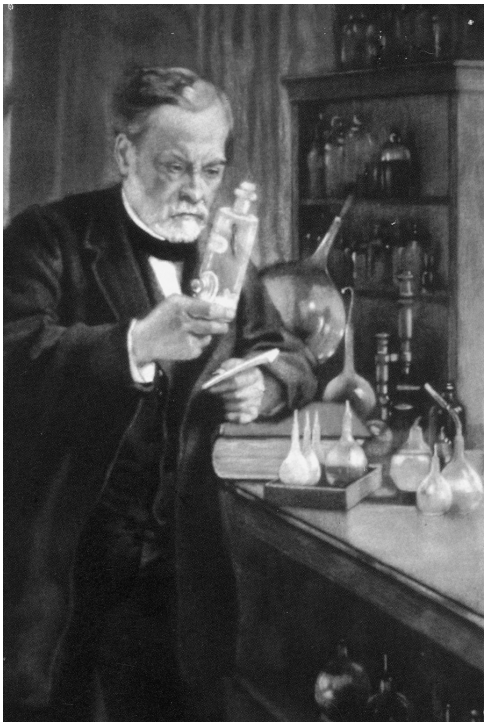

Figure 1.1 Louis Pasteur.

microbes, and it was at this point that fermentation microbiology was born. Nevertheless, it took almost two decades, until 1876, to disprove the chemical hypothesis of Berzelius, Liebig, and Wöhler (i.e., that fermentation was the result of contact with decaying matter).

In 1876, the great German microbiologist Robert Koch (Figure 1.2) proved that bacteria from anthrax infections were capable of causing the disease. His contributions involving the growth of microbes in pure culture led to the decline of the pleomorphism theory, which postulated that one form of bacteria developed into another. It was mainly Koch's work that led to the acceptance of the idea that specific diseases were caused by specific organisms, each of which had a specific form and function. In 1884, his students Gaffky and Loeffler were able to confirm the etiologic role of infectious bacteria in typhoid fever and diphtheria.

Pasteur was called on by the distillers of Lille to find out why the contents of their fermentation vats were turning sour. Using his microscope, he noted that the fermentation broth contained not only yeast cells, but also bacteria, and he already knew that these could produce lactic acid. This observation led to his suggestion that such souring could be prevented by a mild heat treatment, which later became known as "pasteurization."

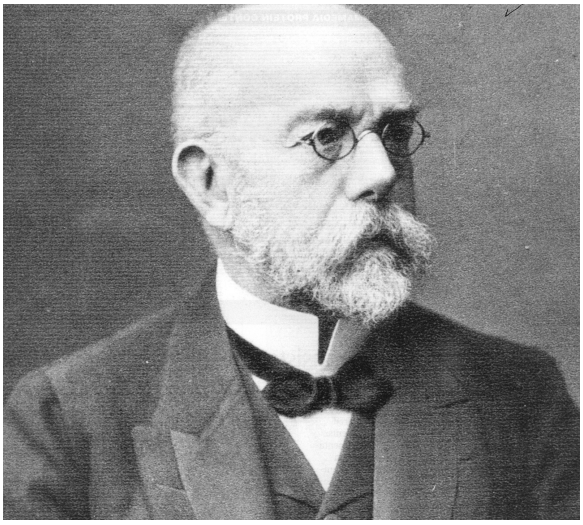

Figure 1.2 Robert Koch.

One of Pasteur's greatest contributions was to establish that each type of fermentation was mediated by a specific microorganism. Furthermore, in a study undertaken to determine why French beer was inferior to German beer, he demonstrated the existence of life forms that were strictly anaerobic. Interest in the mechanisms of these fermentations resulted in the later investigations by Pasteur which not only advanced microbiology as a distinct discipline, but also led to the development of vaccines and concepts of hygiene which revolutionized the practise of medicine.

With the establishment of the germ theory of disease by Pasteur and Koch, the latter half of the nineteenth century was characterized by the fight against disease and the attention of microbiologists was directed towards aspects of microbiology concerned with medicine and sanitation. This resulted in the discovery that the human body has its own defenses in the fight against pathogenic microbes. Pasteur and Koch, among others, found that upon invasion by a bacterium, proteins (i.e., antibodies) are formed in the bloodstream that can specifically neutralize the invading organism. Thus, the science of immunology was founded. By injecting either dead forms or attenuated forms of the disease-producing bacterium, Pasteur could render the individual immune to the disease. The production of these vaccines occupied much of the early research in microbiology.

During Pasteur's life, the application of antiseptics was introduced. It had been shown in 1846 by Ignaz Semmelweis that chlorine could control infection, and in 1865, Joseph Lister showed that the same could be done with carbolic acid. Later, Paul Ehrlich used synthetic dyes and established the concept of the "magic bullet." Towards the end of the nineteenth century, Ehrlich began testing many synthetic compounds. He achieved success in 1909, curing relapsing fever, syphilis, and

trypanosomiasis with an arsenical product called Salvarsan or Compound 606 (because it was his 606th attempt to produce an arsenical compound which killed the syphilis bacterium *in vivo* without harming the host). This was the first chemotherapeutic drug ever discovered and he coined the term "chemotherapy."

This use of drugs selectively toxic to the parasite but not harmful to the host opened an entirely new field for the curing of human diseases. In 1927, this work was continued by Gerhard Domagk in Germany [1] along with his collaborators Mietzsch and Klarer. They were working at the I.G. Farbenindustrie which was the result of a 1924 merger between Bayer and BASF. Their work resulted in the development of the red-colored molecule "prontosil rubrum." This compound was active in mice against streptococci but strangely was not active *in vitro*. Then in 1935, Trefouel and coworkers in France discovered that the red dye was broken down in the animal to the colorless and inhibitory sulfanilamide. This established the important concept that chemicals could kill or inhibit bacteria without toxicity to humans. Although the Nazi government refused to permit Domagk to accept the Nobel Prize in 1939, it was later accepted by him in 1947. Other synthetic chemotherapeutic drugs gained widespread use over the years, including isonicotinic acid hydrazide and para-aminosalicylic acid, both for tuberculosis.

Another discovery in the nineteenth century concerned the way microorganisms interact with one another. For thousands of years, moldy cheese, meat, and bread had been employed in folk medicine to heal wounds. Then in the 1870s, Tyndall, Pasteur, and William Roberts, a British physician, directly observed the antagonistic effects of one microorganism on another. Pasteur, with his characteristic foresight, suggested that the phenomenon might have some therapeutic potential. For the next 50 years, various microbial preparations were tried as medicines, but they were either too toxic or inactive in live animals. This led to the pivotal moment in microbiological history when, in 1927, Alexander Fleming discovered penicillin (see Section 1.2).

In 1877, Moritz Traube proposed that (i) protein-like materials catalyzed fermentation and other chemical reactions and (ii) they were not destroyed by doing such things. This was the beginning of the recognition of what we call enzymes today. He also proposed that fermentation was carried out via multistage reactions in which the transfer of oxygen occurred from one part of a sugar molecule to another, finally forming some oxidized compound (e.g., carbon dioxide) and a reduced compound (e.g., alcohol).

The field of biochemistry became established in 1897 when Eduard Buchner found that cell-free yeast extracts, lacking whole cells, could convert sucrose into ethanol. Thus, the views of Pasteur were modified and it became understood that fermentation could also be carried out in the absence of living cells.

During World War I, the need for glycerol, used to manufacture ammunition, resulted in the application of yeast to convert sugars into glycerol. This development led after the war to an exhaustive study by Neuberg of the mechanisms involved in these reactions and those converting sugars to ethanol. This was followed by the studies of Dutch scientists in Delft looking at oxidation/reduction reactions and the kinetics of enzyme-catalyzed reactions.

Figure 1.3 Chaim Weizmann.

Also during World War I, Chaim Weizmann (Figure 1.3) at the University of Manchester applied the butyric acid bacteria used for centuries for the retting of flax and hemp, for production of acetone and butanol. His use of *Clostridium* to produce acetone and butanol was the first non-food fermentation developed for large-scale production; with it came the problems of viral and microbial contamination that had to be solved. Although use of this fermentation faded because it could not compete with chemical methods of solvent production, it did provide a base of experience for the development of large-scale cultivation of fungi for production of citric acid. Soon after World War I, an aerobic process was devised in which *Aspergillus niger* was used. Not too many years later, the discoveries of penicillin and streptomycin and their commercial development heralded the start of the antibiotic era.

1.2 The Penicillin Story

The golden era of antibiotics began with the accidental discovery of penicillin by Alexander Fleming in 1929 in England [2]. He noted that some of his plates con-

Figure 1.4 Howard W. Florey.

taining *Staphylococcus aureus* were contaminated with a mold, *Penicillium notatum*, and was surprised to see that none of the bacterial colonies could grow in the vicinity of the mold. Fleming concluded that the mold was producing some kind of inhibitory agent. He also observed that filtrates of the mold lyzed the staphylococci and were non-toxic in animals. He named the agent penicillin. Since the activity was very unstable and Fleming could get no encouragement from his fellow scientists concerning the usefulness of such material, the project was abandoned.

The importance of Fleming's discovery was that it led to penicillin, the first successful chemotherapeutic agent produced by a microbe, thus initiating the golden age of the wonder drugs. However, the road to the development of penicillin as a successful drug was not an easy one. For a decade, it remained as a laboratory curiosity, and an unstable curiosity at that. Attempts to isolate penicillin were made in the 1930s by a number of British chemists, but the instability of the substance frustrated their efforts. When World War II arrived and many British soldiers were dying on the battlefield from bacterial infections after being wounded, a study of penicillin began in 1939 at the Sir William Dunn School of Pathology of the University of Oxford by Howard W. Florey (Figure 1.4), Ernst B. Chain (Figure 1.5), Norman Heatley (Figure 1.6), Edward Abraham, and their colleagues.

Figure 1.5 Ernst B. Chain.

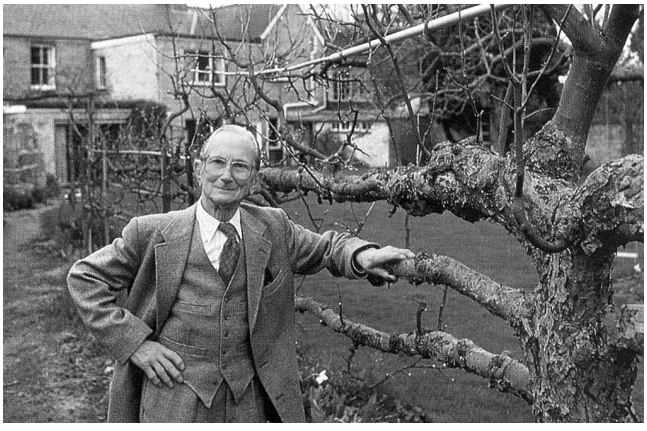

Figure 1.6 Norman Heatley.

This effort led to the successful preparation of a stable form of penicillin and the demonstration of its remarkable antibacterial activity and lack of toxicity in mice.

Unfortunately, production of penicillin by the strain of *P. notatum* in use was so slow that it took over a year to accumulate enough material for a clinical test

on humans [3]. When the clinical tests were found to be successful, however, large-scale production became essential, prompting Florey and his colleague to go to the United States in the summer of 1941 to seek assistance. There they convinced the Northern Regional Research Laboratory (NRRL) of the US Department of Agriculture (USDA) in Peoria, Illinois, and several American pharmaceutical companies (including Merck, Squibb, and Pfizer) to develop the commercial production of penicillin. Heatley remained for a period at the NRRL to work with Moyer and Coghill [4]. Thus began a momentous cooperative effort among university and industrial laboratories in the United States and academic institutions in the United Kingdom which lasted throughout the War.

The result was that thousands of lives were saved, on and off the battlefield. The discovery and development of the beta-lactam antibiotics was among the most powerful achievements of modern science and technology. Since Fleming's accidental discovery of the penicillin-producing mold, years of steady progress followed, and today, the beta-lactam group of compounds is one of the most successful examples of natural product application and chemotherapy.

In the 1940s, a period of intense development in microbial genetics began [5]. Although Fleming's original strain produced only traces of penicillin, "brute force" genetic manipulation made tremendous strides in production ability and led to a whole new technology known as "strain improvement." These early genetic studies concentrated heavily on the production of mutants and the study of their properties. The ease with which "permanent" characteristics of microorganisms could be changed by mutation and the simplicity of the mutation technique had tremendous appeal to microbiologists. A cooperative "strain-selection" program was established between the workers at the USDA in Peoria, the Carnegie Institution, Stanford University, and the University of Wisconsin.

Strain selection began with *Penicillium chrysogenum* NRRL 1951, the well-known isolate from a moldy cantaloupe obtained in a Peoria market. This strain was capable of producing 60 μg/ml. Cultivation of spontaneous sector mutants and single-spore isolations led to more productive cultures from NRRL 1951. One of these, NRRL 1951-1325, produced 150 μg/ml. It was next subjected to X-ray treatment by Demerec of the Carnegie Institution at Cold Spring Harbor, New York, and mutant X-1612 was obtained. This yielded 300 μg/ml. Workers at the University of Wisconsin then obtained ultraviolet-induced mutants of Demerec's strain. One of these, Q-176, which produced 550 μg/ml, became the ancestor of all of the strains used in industry. The "Wisconsin family" of superior strains became well known all over the world, some producing over 1800 μg/ml. The penicillin improvement effort was the start of a long engagement between genetics and industrial microbiology which ultimately demonstrated that mutation was the major factor involved in the hundred- to thousand-fold increases obtained in the production of microbial metabolites.

Originally, penicillin had been produced in surface culture, but it was found that titers were very low. Submerged culture soon became the method of choice. By the use of strain improvement and medium modifications, such as the use of corn steep liquor as additive, the yield of penicillin was increased by 100-fold in

Figure 1.7 Marvin Johnson.

just a few years. Much of the understanding of the physiology of *P. chrysogenum* in relation to penicillin production was achieved by Professor Marvin Johnson (Figure 1.7) and his students at the University of Wisconsin. Further clinical successes were demonstrated in both the United Kingdom and the United States, and finally, in 1943, penicillin was used to treat those wounded in battle.

By the 1950s, it was realized that *P. chrysogenum* could use additional acyl compounds as side-chain precursors (other than phenylacetic acid for penicillin G) and produce new penicillins; one of these, penicillin V (phenoxymethylpenicillin), achieved commercial success. Its commercial application resulted from its stability to acid which permitted oral administration, an advantage it held over the accepted article of commerce, penicillin G (benzylpenicillin). Penicillin G and penicillin V became the main penicillins of commerce. In commercial production, the usual medium had been a complex one, composed of glucose, corn steep liquor, side-chain precursor (phenylacetic acid for penicillin G or phenoxyacetic acid for penicillin V), and mineral salts. The earliest recognition that glucose had a negative effect on penicillin biosynthesis was made by Johnson and his students [6, 7]. Because they found glucose to be excellent for growth but poor for penicillin formation, while lactose showed the opposite pattern, they devised a medium containing both sugars in which growth occurred at the expense of glucose and when it

was exhausted, the mass of cells began to produce the antibiotic at the expense of lactose. Unlike glucose, lactose was utilized slowly and did not exert carbon catabolite repression on the process. Then, Davey and Johnson [8] found that intermittent or continuous feeding of the less expensive glucose could replace batch feeding of lactose. This represented the birth of the fed-batch fermentation which is commonplace in the fermentation industry today.

The biosynthesis of penicillin from its precursors, L-cysteine, L-valine, and phenylacetic acid, was actively studied during the 1950s, 1960s, and 1970s. Of great interest was the relationship between L-lysine and penicillin formation. In 1947, David Bonner had made the observation that 25% of the lysine auxotrophs that he had made from *P. chrysogenum* failed to make penicillin and he predicted that (i) there was some relationship between the antibiotic and the amino acid and (ii) there was a common precursor of the two compounds. That he was absolutely correct was established ten years later when Demain [9] found that lysine was a potent inhibitor of penicillin biosynthesis. That the inhibition could be reversed by L-α-aminoadipic acid led to the postulations [10] that (i) L-α-aminoadipic acid was involved in penicillin biosynthesis although it did not end up in the final penicillin molecule, (ii) penicillin was derived from α-ketoglutarate and acetyl-CoA via the fungal lysine biosynthetic pathway, and (iii) lysine inhibition of penicillin biosynthesis was due to feedback inhibition by lysine of its own biosynthetic pathway, thus limiting L-α-aminoadipic acid formation. Independently, Arnstein and colleagues [11] detected the tripeptide δ-(α-aminoadipyl)-cysteinylvaline (ACV) as an intracellular compound in *P. chrysogenum*. Results in several laboratories established L-α-aminoadipic acid as an important precursor of all penicillins. Soon, δ-(α-L-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV) was established as the crucial intermediate of penicillin biosynthesis. The reaction sensitive to feedback inhibition by lysine was later proven to be the initial step of lysine biosynthesis in fungi, that is, the homocitrate synthase reaction [12, 13].

During the 1950s, the future of penicillins became doubtful as resistant strains of *Staphylococcus aureus* emerged in hospital populations. The staphylococcal population was building up resistance to penicillin via selection of penicillinase-producing strains, and new drugs were clearly needed to combat these resistant forms. The penicillins described up to this point were solvent-soluble, exhibiting a high degree of activity against Gram-positive organisms, but were much less active against Gram-negatives. Fortunately, two developments occurred which led to a rebirth of interest in the penicillins and related antibiotics. One was the 1959 discovery in Japan by Koichi Kato of the accumulation of the "penicillin nucleus" in *P. chrysogenum* broths to which no side-chain precursor had been added [14]. In the United Kingdom, Batchelor *et al.* [15] isolated the compound 6-aminopenicillanic acid (6-APA) which was the "penicillin nucleus" discovered by Kato. 6-APA was used to make "semi-synthetic" (i.e., chemical modification of a natural antibiotic) penicillins with the benefical properties of resistance to penicillinase and to acid, plus broad-spectrum antibacterial activity.

The second development was the discovery of a completely different type of penicillin, a hydrophilic type, showing equivalent activity against both classes of

microorganisms (i.e., penicillin N). This compound was discovered independently by two groups of workers. Brotzu in 1948 [16] published his work in an unknown Sardinian journal on the isolation of an antibiotic-producing culture of the fungus *Cephalosporium acremonium* (later reclassified as *Acremonium chrysogenum*) from sewage. Unable to purify the antibiotic material, he sent the culture to Florey at Oxford where, some ten years earlier, *P. notatum* had been at a similar stage in history. While the British scientists were studying the components of this antibiotic complex, workers at the Michigan Department of Health in the United States [17] announced that a species of *Tilachlidium* produced a new antibiotic which they called "synnematin." After the culture was reclassified as *Cephalosporium salmosynnematum* [18], it was shown that synnematin was a complex of two components, A and B. Almost nothing was ever published on the A component, and we have no knowledge today about its structural relationship to synnematin B. While the above work was going in Michigan, the British announced [19, 20] that Brotzu's strain produced two antibiotics, that is, "cephalosporin P," active only against Gram-positive organisms and "cephalosporin N," which was active against both Gram-positive and Gram-negative bacteria. "Cephalosporin P" was found to be of steroidal nature and not a β-lactam at all. "Cephalosporin N," on the other hand, was found by Abraham and coworkers [21] (Figure 1.8) to be a true penicillin possessing an α-D-aminoadipyl side-chain, and to be identical to synnematin B [22]. It was renamed "penicillin N." In comparison to penicillin G, it was only 1% as active against Gram-positive forms, but had equal to or somewhat greater activity against Gram-negative bacteria. The hydrophilic nature of penicillin N and its roughly equivalent activity against Gram-positive and Gram-negative bacteria were due to the carboxyl group in the side-chain.

Figure 1.8 Guy G.F. Newton and Edward P. Abraham.

The tremendous success attained in the battle against disease with penicillin G not only led to the Nobel Prize being awarded to Fleming, Florey, and Chain, but to a new field of antibiotic research, and a new antibiotic industry. Penicillin opened the way for the development of many other antibiotics, and it still remains the most active and one of the least toxic of these compounds. Today, about 100 antibiotics are used to combat infections in humans, animals, and plants.

1.3 The Coming of the Cephalosporins

A key breakthrough was the finding in Edward Abraham's laboratory at Oxford that a second antibiotic was produced by Brotzu's strain of *A. chrysogenum*. After his important contributions as part of the Florey penicillin team, Abraham had established an independent laboratory at Oxford. Abraham and Newton [23] found the new compound to be related to penicillin N in that it consisted of a β-lactam ring attached to a side-chain which was identical to that of penicillin N, that is, D-α-aminoadipic acid. It differed, however, from the penicillins in containing a six-membered dihydrothiazine ring in place of the five-membered thiazolidine ring of the penicillins. It was called cephalosporin C. Thus, the era of the cephalosporins was launched.

The nucleus of cephalosporin C was named 7-aminocephalosporanic acid (7-ACA). Cephalosporin C strongly absorbed ultraviolet light, was stable to acid and to penicillin β-lactamase, was non-toxic, and had *in vivo* activity in mice. Its mode of action was the same as that of the penicillins; that is, inhibition of bacterial cell wall formation. Although neither penicillin N nor cephalosporin C was ever commercialized, they led to important knowledge on the biosynthesis of these compounds and the development of many powerful semi-synthetic cephalosporins of great use in medicine.

The stability of cephalosporin C to penicillin β-lactamase was a very attractive property. The main disadvantage of the molecule was its weak activity; it had only 0.1% of the activity of penicillin G against sensitive staphylococci, although its activity against Gram-negative bacteria equaled that of penicillin G. However, by chemical removal of its D-α-aminoadipic acid side-chain and replacement with phenylacetic acid, a penicillinase-resistant semi-synthetic compound was obtained which was 100 times as active as cephalosporin C. Many other new cephalosporins with wide antibacterial spectra were developed in the ensuing years, for example, cephalothin, cephaloridine, and cephaloglycin, making the semi-synthetic cephalosporins the most important group of antibiotics at that time. The stability of the cephalosporins to penicillinase was evidently a function of its dihydrothiazine ring since (i) the D-α-aminoadipic acid side-chain did not render penicillin N immune to attack and (ii) removal of the acetoxy group from cephalosporin C did not decrease its stability to penicillinase. Cephalosporin C competitively inhibited the action of penicillinase from *Bacillus cereus* on penicillin G. Although it did not have a similar effect on the *S. aureus* enzyme, certain of its derivatives did. Another

advantage was that cephalosporins could be given to some patients who were allergic to penicillins.

From a biosynthetic sense, the relationship of penicillin N and cephalosporin C was of great interest. An important development that led to rapid progress in this area was the subcellular work done by Abraham and his Oxford colleagues in the 1970s [24]. They used protoplast lysates of *A. chrysogenum* to convert labeled valine into a penicillin. This led to the discovery at Massachusetts Institute of Technology in 1976 of the ring expansion reaction [25], catalyzed by the "expandase" (deacetoxycephalosporin C synthase; DAOCS) enzyme. For many years, it had been thought that penicillin N and cephalosporin C were products of different biosynthetic branches in *A. chrysogenum*. However, the discovery of the ring expansion enzyme showed that cephalosporins were produced from a penicillin. This was confirmed by Yoshida *et al.* in 1978 [26], who showed that the ring expansion enzyme converted penicillin N to deacetoxycephalosporin C. Extracts of mutants which fermentatively produced penicillin N but not cephalosporins failed to carry out this reaction, whereas early blocked mutants (negative for production of both penicillin N and cephalosporins) did expand the ring.

From the 1970s through the 1980s, knowledge accumulated concerning fermentation and biosynthesis of cephalosporin C. Of major importance was the (i) stimulation by DL-methionine via a regulatory mechanism unrelated to its ability to contribute the sulfur atom to the antibiotic [27], (ii) use of acetate as precursor to the acetoxy group [28], (iii) L-cysteine and L-valine [29] as precursors of the nucleus and (iv) L-α-aminoadipic acid as precursor of the D-α-aminoadipyl side-chain of cephalosporin C [30]. An important step forward was provided by Banko *et al.* [31] when they demonstrated that the cell-free activity of the enzyme from *A. chrysogenum* formed the important tripeptide precursor of all penicillins and cephalosporins, that is, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV). The enzyme ACV synthetase was proven to be a single multifunctional enzyme acting on L-α-aminoadipic acid, L-cysteine, and L-valine to produce LLD-ACV. Also of importance was the isolation by Hollander *et al.* [32] of pure isopenicillin N synthetase ("cyclase") which converted the LLD-ACV to isopenicillin N.

The long-held notion that β-lactams were produced only by fungi was shattered by a report from Merck & Co. that a streptomycete produced penicillin N [33]. This provocative finding was presented at the 1962 American Society for Microbiology Annual Meeting and was only published as an abstract. Although much doubt was cast on this report, two reports from Eli Lilly & Co. and Merck published some 9–10 years later [34, 35] reported that various species of *Streptomyces* and *Nocardia* produced cephalosporins modified at C7 (= cephamycins) and/or at the side-chain attached to C3. The discovery of cephamycin C led to much research on and development of prokaryotic cephalosporins since the presence of the methoxy group on the β-lactam ring made the molecule more active against Gram-negative and anaerobic pathogens and more resistant to Gram-negative β-lactamases. For the first time in the history of the β-lactams, molecules were available which showed a high degree of stability to these troublesome enzymes. Like fungal cephalosporin C, cephamycin C was never used clinically but was employed

for semi-synthesis of many medically useful compounds. A more potent semi-synthetic cephamycin, cefoxitin, was rapidly commercialized by Merck, to be followed later by cefmetazole, temocillin, cefotetan, and other semi-synthetic cephalosporins.

In the 1970s to the 1980s, the pathways to the penicillins and the cephalosporins including cephamycin C were worked out, especially after cell-free systems became available [24, 36]. Late in the 1970s came reports on the production of β-lactam antibiotics which were neither penicillins nor cephalosporins. The most important was clavulanic acid from streptomycetes, which possessed only weak antibiotic activity but was an excellent inhibitor of β-lactamase (see Section 1.8.1). It became a blockbuster compound by being co-formulated with broad-spectrum semi-synthetic penicillins that are susceptible to β-lactamase, for example, with amoxycillin, the combination is known as Augmentin.

Another important development in the history of the β-lactam antibiotics was the discovery at Merck of the the carbapenems. The first, called thienamycin, was discovered by Kahan *et al.* [37] with a screening protocol based on inhibition of peptidoglycan synthesis. The antibiotic was produced by *Streptomyces cattleya*, which also made cephamycin C. Carbapenems resembled the penicillins in having a β-lactam ring fused to a five-membered ring. They differed in that the five-membered ring was unsaturated and contained a carbon atom instead of the sulfur. Sulfur was, however, present in another location in all the carbapenems produced by streptomycetes. A large number of carbapenems were reported, but thienamycin was the most important. Indeed, it was the most potent, most broad-spectrum and non-toxic natural antibacterial agent ever found. It inhibited cell wall synthesis, as did the penicillins and cephalosporins, and was relatively resistant to microbial β-lactamases.

The development of commercial process technology for the penicillin nucleus (6-APA) and the cephalosporin nucleus (7-ACA) opened the way for chemical acylation with various side-chains yielding many improved semi-synthetic penicillins and cephalosporins with broader antibacterial activity and improved pharmacokinetic properties. Broad-spectrum penicillins and cephalosporins became the best-selling antibacterial agents in the pharmaceutical arena.

1.4
The Waksman Era

The advent of penicillin, which signaled the beginning of the antibiotic era, was closely followed in the 1940s by the discoveries of Selman A. Waksman (Figure 1.9), a soil microbiologist at Rutgers University. He and his students, especially H. Boyd Woodruff, Albert Schatz, and Hubert Lechevalier, succeeded in discovering many new antibiotics from the filamentous bacteria, the actinomycetes, such as actinomycin D, neomycin, and the best-known of these new "wonder drugs," streptomycin. These discoveries on the antibiotic-producing abilities of the actinomycetes occurred long before the developments, described above, of

Figure 1.9 Selman A. Waksman and H. Boyd Woodruff.

β-lactams produced by these filamentous bacteria. Waksman and Woodruff published in 1940 on the discovery of the actinomycins, which were chromooligopeptides [38]. One such compound, actinomycin D, was used for years to combat Wilms tumor in children and became a very important tool in the development of molecular biology as an inhibitor of RNA polymerase.

After its monumentous discovery in 1944 by Waksman, Schatz, and Bugie [39] as a product of *Streptomyces griseus*, streptomycin was used against tuberculosis caused by *Mycobacterium tuberculosis* and also against Gram-negative bacteria; bacterial meningitis was also treated with streptomycin. Its major impact on medicine was recognized by the award of the Nobel Prize to Waksman in 1952. As the first commercially successful antibiotic produced by an actinomycete, this aminoglycoside led the way to the recognition of these organisms as the most prolific producers of antibiotics. Streptomycin also provided a valuable tool for studying cell function. After a period of time during which it was thought to act by altering permeability, its interference with protein synthesis was recognized as its primary effect. Its interaction with ribosomes provided much information on their structure and function; it not only inhibited their action but also caused misreading of the genetic code and was required for the function of ribosomes in streptomycin-dependent mutants.

With Lechevalier, Waksman reported on the discovery of neomycin in 1948 [40] and candicidin in 1953 [41]. Neomycin, an aminoglycoside produced by *Streptomyces fradiae*, served as a topical antibacterial, and the polyene candicidin, made by *S. griseus*, found use as a topical antifungal antibiotic.

Cooperation on the development of industrial processes between Rutgers University, Princeton University, Columbia University, and Merck & Co., Inc. led to the birth of the field of biochemical engineering. With royalties on streptomycin turned over to Rutgers University by the manufacturer, Merck, Waksman was able to build the world-famed Institute of Microbiology.

The discoveries of the aminoglycosides at Rutgers ushered in the antibiotic era and resulted in the discovery of many more "wonder drugs" such as chloramphenicol in 1947 [42], the tetracyclines in 1948 [43], macrolides such as erythromycin in 1952 [44], glycopeptides such as vancomycin in 1956 [45], additional aminoglycosides such as gentamicin in 1963 [46], β-lactams such as cephamycins in 1970 [34, 35] and carbapenems in 1979 [37], ansamycins such as rifamycin in 1957 [47], and polyene macrolides such as nystatin in 1950 [48]. Approximately 15 000 microbial secondary metabolites have been discovered; of these, about 12 000 are antibiotics. Their unusual chemical structures included β-lactam rings, cyclic peptides containing "unnatural" and non-protein amino acids, unusual sugars and nucleosides, polyenes, and large macrolide rings. Although most were useless for humans, being either too toxic or inactive in higher organisms, others were life savers. The antibiotics were virtually the only drugs utilized for chemotherapy against pathogenic microorganisms and were crucial in the increase in average life expectancy in the United States from 47 years in 1900 to 74 for men and 80 for women in 2000.

For some reason, the actinomycetes were amazingly prolific in the number of antibiotics they could produce. Roughly 70% of all antibiotics were obtained from these filamentous prokaryotes, and 75% of those were in turn made by a single genus, *Streptomyces*. It is quite amazing that strains of *Streptomyces hygroscopicus* produced over 180 different secondary metabolites. About 10% of the antibiotics were made by unicellular bacteria and about 20% by fungi [49]. New bioactive products from microbes were discovered at an amazing pace: 200–300 per year in the late 1970s increasing to 500 per year by the 1990s. Accompanying the natural product antibiotics in the pharmaceutical arena were synthetic antimicrobials such as the quinolones and fluoroquinolones. Even these synthetics traced their discovery back to a natural product, that is, quinine. The first quinolone, nalidixic acid, was modeled after quinine. Commercialization of antibiotics, however, slowed down in the 1980s and only three, daptomycin, caspofungin acetate, and the synthetic oxazolidinone, were commercialized in the ensuing decades.

1.5 Strain Improvement

The experiences described above in which penicillin-producing cultures were mutagenized and screened for improved producers led the way to the extensive use of genetics to improve production ability [50]. From the 1950s on, genetic manipulation such as mutagenesis/screening was replaced by mutagenesis/selection/screening in which various selective means were used to decrease the number

of strains that had to be screened for improved production. Then, it was found that new derivatives, some better than the parent molecule, could be produced by mutants. This was first discovered by Kelner in 1949 [51] but the more active derivatives were not isolated and identified. However, the medically useful metabolites demethyltetracycline [52] and doxorubicin [53] were discovered later by mutation of the cultures producing tetracycline and daunomycin, respectively. In 1969, the technique of "mutational biosynthesis" (= mutasynthesis) was devised by University of Illinois Professors Kenneth Rinehart and David Gottlieb and student W.T. Shier [54]. In this process, a mutant blocked in secondary metabolism was fed analogs of the moiety whose biosynthesis was blocked. If successful, the mutant (called an "idiotroph") produced a new secondary metabolite. Mutational biosynthesis was used for the discovery of many new secondary metabolites. The most well known was the commercial antihelmintic agent doramectin, production of which employed a mutant of the avermectin producer, *Streptomyces avermitilis* [55] (see Section 1.8.5).

For strain improvement, genetic recombination was virtually ignored in industry before 1975, mainly due to the low frequency of recombination, as low as 10^{-6}. However, use of polyethyleneglycol-mediated protoplast fusion in actinomycetes by Okanishi *et al*. [56] changed the situation markedly. Okanishi's work on protoplast formation, fusion, and regeneration accelerated the use of genetic recombination. From then on, there was a heightened interest in the application of genetic recombination to the production of important microbial products. Frequencies of recombination increased to even greater than 10^{-1} in some cases. After 1985, many strain improvement programs routinely included (i) transposition mutagenesis, (ii) targeted deletions and duplications by genetic engineering, and (iii) genetic recombination by protoplast fusion and plasmid transformation. Much was known about genetics and regulation in the actinomycetes due to the elegant research on *Streptomyces coelicolor* by David Hopwood, Keith Chater, Mervyn Bibb, and their colleagues at the John Innes Institute in Norwich, England (sometimes referred to as the "temple of *Streptomyces* genetics") [57]. Their favorite organism made at least five secondary metabolites (an A-factor-like molecule, and the antibiotics actinorhodin, undecylprodigiosin, methylenomycin A, and "calcium-dependent antibiotic" or CDA).

These efforts and others by geneticists in academia and industry throughout the world in the 1970s and 1980s revealed that the genes encoding most antibiotic biosynthetic pathways were clustered into operons, thus facilitating transfer of entire pathways from one organism to another. Such clusters were found to also include regulatory and resistance genes. In 1985, "combinatorial biosynthesis" was born [58]. An international effort from the United Kingdom, Japan, and the United States resulted in the cloning of a pathway from one streptomycete producing the isochromanequinone antibiotic actinorhodin into strains producing granaticin, dihydrogranaticin, and mederomycin (which are also isochromanequinones). This resulted in the discovery of two new hybrid antibiotics, mederrhodin A and dihydrogranatirhodin. Combinational biosynthesis became a widely used technique used for discovery of new hybrid drugs [59] by recombinant DNA (rDNA) technol-

ogy. New antibiotics were also created by changing the order of the genes of an individual pathway in its native host.

Progress in strain development has recently involved extensive use of new genetic techniques such as (i) metabolic engineering, accomplishing quantification, and control of metabolic fluxes, and including inverse metabolic engineering and transcript expression analyses, such as association analysis and massive parallel signature sequencing; (ii) directed evolution (see Section 1.11); (iii) molecular breeding including DNA shuffling and whole genome shuffling; and (iv) combinatorial biosynthesis. These efforts are facilitating not only the isolation of improved strains but also the elucidation and identification of new genetic targets to be used in product discovery.

1.6
Semi-Synthetic Antibiotics to Combat Resistant Microbes

Although there was a feeling expressed by many in the late 1970s that the era of product discovery for bacterial disease was ending, the battle against resistant microbes continued and featured some surprising developments. These included: (i) semi-synthetic variations of old antibiotics (ketolides, clarithromycin, azithromycin, glycylglycines); (ii) older underutilized antibiotics (teicoplanin); (iii) new derivatives of previously undeveloped narrow-spectrum antibiotics (streptogrammins); and (iv) a few newly developed antimicrobial agents (caspofungin, daptomycin, and the synthetic epothilones).

Of great success was the development of semi-synthetic erythromycins [60]. These included clarithromycin, roxithromycin, azithromycin, and the ketolide telithromycin. Whereas the first two showed improved acid stability and bioavailability over erythromycin A, they showed no improvement against resistant strains. On the other hand, azithromycin and telithromycin acted against macrolide-resistant bacteria. All the above semi-synthetic erythromycins were effective agents for upper respiratory tract infections and could be administered parentally or orally. Telithromycin was bacteriostatic, active orally, and of great importance for community-acquired respiratory infections. Of particular interest was its low ability to select for resistance mutations as well as to induce cross-resistance. It also did not induce MLS_B resistance, a problem with other macrolides.

For more than 35 years, the glycopeptides vancomycin and teicoplanin were virtually the only natural antibiotics active against multidrug-resistant Gram-positive bacteria. Their use was cut down by the increase in multidrug resistance. To the rescue came a number of new semi-synthetic antibiotics, one called Synercid. Synercid was composed of a synergistic (by 100-fold) pair of narrow-spectrum streptogramins, that is, quinupristin and dalfopristin, both being semi-synthesized from natural compounds made by a single strain of *Streptomyces pristinaespiralis* [61]. The pair was constituted by a (Group A) polyunsaturated macrolactone containing an unusual oxazole ring and a dienylamide fragment and a (Group B) cyclic hexadepsipeptide possessing a 3-hydroxypicolinoyl exocyclic

fragment. Although the natural streptogramins were poorly water-soluble and could not be used intravenously, the Synercid components were both water-soluble. They inhibited protein synthesis, and were active against vancomycin-resistant *Enterococcus faecium* (VREF), methicillin-resistant *S. aureus* (MRSA), glycopeptide-resistant *S. aureus*, and β-lactam-resistant *Streptococcus pneumoniae*. Synergistic action of the streptogramins was due to the fact that the B component blocked binding of aminoacyl-tRNA complexes to the ribosome while the A component inhibited peptide bond formation and distorted the ribosome, promoting the binding of the B component. Synercid was approved by the US Food and Drug Administration (FDA) in 1999.

A semi-synthetic tetracycline, that is, a glycylcycline, was successfully developed for use against tetracycline-resistant bacteria [62]. The 9-*t*-butylglycylamido derivative of minocycline called tigecycline, was active against resistant Gram-positive, Gram-negative, and anaerobic bacteria possessing the ribosomal protection resistance mechanism or the active efflux mechanism.

1.7 The Primary Metabolites

The development of penicillin fermentation in the 1940s marked the true beginning of what might be called the golden age of industrial microbiology. The work of Louis Pasteur pointed to the importance of the activity of non-pathogenic microbes in wine and beer in producing alcohol. This realization resulted in a large number of microbial primary metabolites of commercial importance being produced by fermentation. Primary metabolism involves an interrelated series of enzyme-mediated catabolic, amphibolic, and anabolic reactions which provide biosynthetic intermediates and energy, and convert biosynthetic precursors into essential macromolecules such as DNA, RNA, proteins, lipids, and polysaccharides. It is finely balanced and intermediates are rarely accumulated. By deregulating primary metabolism, overproduction of many primary metabolites was achieved in the fermentation industry. Commercially, the most important primary metabolites were amino acids, vitamins, flavor nucleotides, organic acids, and alcohols.

1.7.1 Amino Acids

Production of amino acids amounted to 2.3 million tons in 2002. The most important acids made at least partly by biological methods were glutamate (1.6 million tons per year), lysine-HCl (700 000 tons), threonine (70 000 tons), phenylalanine (13 000 tons, including that by chemical synthesis), aspartic acid (10 000 tons made enzymatically), and tryptophan (3000 tons including that made enzymatically). High titers were achieved in fermentation (e.g., 170 g/l L-lysine-HCl).

Monosodium glutamate (MSG) is used as a potent flavor enhancer. The glutamic acid fermentation was discovered in Japan in 1957 by Kinoshita, Udaka, and

Shimono [63] of the Kyowa Hakko Kogyo Company. It is of interest that the founder of the company (Dr, Benzaburo Kato), worrying about the diet of the Japanese population and trying to reduce dependence on rice, had charged Kinoshita with the task of producing edible proteins by fermentation. Instead, the researchers came up with a fermentation process yielding an extracellular L-amino acid from non-proteinaceous material. This major discovery was crucial for the development of the amino acid fermentation industry. MSG was manufactured using various species of the genera *Corynebacterium* and *Brevibacterium*.

Normally, glutamic acid overproduction would not be expected to occur because of feedback regulation. Glutamate feedback controls include repression of PEP carboxylase, citrate synthase, and NADP-glutamate dehydrogenase; the last-named enzyme is also inhibited by glutamate. However, by decreasing the effectiveness of the barrier to outward passage, glutamate was pumped out of the cell, thus allowing its biosynthesis to proceed unabated. The excretion of glutamate freed the glutamate pathway from feedback control until excessive levels accumulated.

Glutamate excretion was intentionally effected by various manipulations, such as limitation of biotin in *Corynebacterium glutamicum*; all glutamate overproducers were natural biotin auxotrophs. Biotin is a cofactor of acetyl-CoA carboxylase which is essential for biosynthesis of fatty acids. The surprising report [64] that the addition of penicillin to cells grown in high biotin resulted in excretion of glutamic acid led Shiio and coworkers [65] to postulate (i) that growth of the glutamate-overproducing bacterium in the presence of non-limiting levels of biotin resulted in a cell membrane permeability barrier restricting the outward passage of intracellular amino acids out of the cell and (ii) that inhibition of cell wall biosynthesis by penicillin altered the permeability properties of the cell membrane and allowed glutamate to pass out of the cell. The commonality in the various manipulations that were found to bring about high-level production of L-glutamic acid, that is, (i) limitation of biotin, (ii) addition of penicillin, or (iii) fatty acid surfactants (e.g., tween 60) to exponentially growing cells, was recognized and the permeability mechanism was strongly supported [66]. Apparently, all of these manipulations result in a phospholipid-deficient cytoplasmic membrane, which favors active exit of glutamate from the cell. This view was further supported by the discoveries that oleate limitation of an oleate auxotroph [67] and glycerol limitation of a glycerol auxotroph [68] brought about glutamate excretion. Both oleate and glycerol were precursors of phospholipids. Glutamate-excreting cells were later found to have a major decrease in cell lipids, especially phospholipids [69]. It thus became clear that high level glutamate excretion required (i) growth inhibition in the presence of unlimited carbon and energy sources and (ii) a change in strain on the membrane caused by deficiency of biotin, oleate or glycerol, or addition of certain agents.

Despite the above evidence, the leaky plasma membrane hypothesis was discounted by certain investigators in favor of an efflux system specific for glutamate and regulated by the energy state of the cell. The action of biotin was attributed to effects on intermediary metabolism, correlating with the activity of fatty acid syn-

thetases. Additional opinions discounting the permeability hypothesis attributed glutamate overproduction to a decrease in the activity of α-ketoglutarate dehydrogenase caused by biotin limitation or by addition of penicillin or surfactants. In 2001, however, the permeability modification hypothesis was supported. The various manipulations leading to glutamate overproduction were shown to cause increased permeability of the mycolic acid layer of the cell wall [70]. The glutamate-overproducing bacteria are characterized by a special cell envelope containing mycolic acids which surrounds the entire cell as a structured layer and is thought to be involved in permeation of solutes. The mycolic acids esterified with arabinogalactan and the non-covalently bound mycolic acid derivatives formed a second lipid layer, the cytoplasmic membrane being the first. As stated by these authors, "The concepts of 'permeability of the cell wall' as originally used in the very first work on L-glutamate production more than forty years ago now takes on a new meaning." Nampoothiri *et al.* [71] provided evidence that overexpression or inactivity of genes involved in lipid synthesis changed glutamate efflux dramatically, altered the chemical and physical properties of the cytoplasmic membrane, and that this was necessary to achieve efflux of L-glutamate. They state "that altering the phosphoplipid content alone is sufficient to enable L-glutamate efflux." Burkovski and Kraemer [72] further stated that "There is no doubt that stimulation of glutamate excretion in *C. glutamicum* is directly or indirectly related to membrane and/or cell wall integrity."

Since the bulk of the cereals consumed in the world were deficient in L-lysine, this essential amino acid became an important industrial product. The lysine biosynthetic pathway is controlled very tightly in an organism like *Escherichia coli* which contains three aspartate kinases, each of which is regulated by a different end-product (lysine, threonine, and methionine). In addition, after each branch point, the initial enzymes were inhibited by their respective end-products. However, in lysine fermentation organisms (e.g., mutants of *C. glutamicum* and its relatives), there is only a single aspartate kinase, which is regulated via concerted feedback inhibition by threonine plus lysine. By genetic removal of homoserine dehydrogenase, a glutamate-producing wild-type *Corynebacterium* was converted into a lysine-overproducing mutant that could not grow unless methionine and threonine were added to the medium [73].

E. coli strains were constructed with plasmids bearing amino acid biosynthetic operons. Plasmid transformation was also accomplished in *Corynebacterium*, *Brevibacterium*, and *Serratia* so that rDNA technology could be used to improve these commercial amino acid-producing strains [74]. Especially useful was the concept of metabolic engineering, that is, the directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or introduction of new ones with the use of rDNA technology [75, 76]. Its essence was the combination of analytical methods to quantify fluxes and the control of fluxes with molecular biological techniques to implement suggested genetic modification. Metabolic control analysis revealed that the overall flux through a metabolic pathway depended on several steps, not just a single rate-limiting reaction [77]. Metabolic flux studies of wild-type *C. glutamicum* and four improved lysine-

producing mutants showed that yield increased in the series from 1.2 to 24.9% relative to the glucose flux [78]. It was found that (i) the pentose phosphate pathway had increased, (ii) anaplerotic net flux increased almost twofold by concerted control of C3 carboxylation and C4 decarboxylation, (iii) the relative flux through isocitrate dehydrogenase decreased from 83 to 60%, and (iv) NADPH demand increased from 109 to 172%.

The value of genetic engineering can be appreciated in the following example on overproduction of threonine. By introduction of feedback-resistant threonine dehydratases and additional copies of genes encoding branched amino acid biosynthetic enzymes, various groups converted their lysine- or threonine-producing strains into L-isoleucine-producing strains. An engineered strain of *E. coli* (made by mutating to isoleucine auxotrophy, cloning in extra copies of the *thrABC* operon, inactivating the threonine-degrading gene *tdh*, mutating to resistance to high concentrations of L-threonine and L-homoserine) produced 80 g/l L-threonine in 1.5 days at a yield of 50% [79].

1.7.2 Nucleotides

Commercial interest in nucleotide fermentations developed due to the discovery of A. Kuninaka in Japan that certain purine ribonucleoside 5′-monophosphates, namely guanylic acid (GMP), inosinic acid (IMP), and xanthylic acid (XMP) were enhancers of flavor for foods, beverages, and seasonings [80, 81]. Interestingly, AMP had no such activity. The intensity of flavor enhancement by these compounds surpassed that of MSG by several orders of magnitude. Combination of a flavor nucleotide with MSG had a synergistic effect on flavor enhancement. Originally, the nucleotides were made by enzymatic hydrolysis of yeast RNA but this was an expensive process. Auxotrophic mutants of the glutamic acid producer, *C. glutamicum*, were then found to produce IMP and XMP [82, 83]. By further mutation of the IMP producer, a strain was obtained which could produce a mixture of the two most potent nucleotides, GMP and IMP [84]. Direct fermentation became the method of choice for the industry [85], although some companies produced the nucleosides (inosine and guanosine) by fermentation and then enzymatically (via phosphokinase catalysis) converted these to the 5′ nucleotides. Production of 5′-flavor nucleotides amounted to about 40 g/l.

1.7.3 Vitamins

Vitamins are made at a rate of 70 000 tons per year by synthesis and fermentation. Of these, riboflavin was produced by these two methods at an annual rate of 4000 tons. Most fungi produce enough riboflavin (vitamin B_2) to satisfy their growth requirements, but a few were natural overproducers of this vitamin. This tendency of uncontrolled synthesis of riboflavin was found primarily in two species of fungi, *Eremothecium ashbyi* and *Ashbya gossypii*. Overproduction in natural

strains of *E. ashbyi* was discovered in 1935 by A. Guilliermond and coworkers [86]. They noted the yellow color of the colonies of both cultures, *E. ashbyi* being the more intense. During the stationary phase of growth, the vacuoles became yellowish and, in some vacuoles, rosettes of needle-shaped crystals of riboflavin were observed. This led to the use of *E. ashbyi* for the industrial production of riboflavin in animal feed formulae [87].

L.J. Wickerham and coworkers at the Peoria NRRL/USDA lab received, in 1943, a culture of *A. gossypii* from W.J. Robbins, Director of the New York Botanical Garden and designated it as NRRL Y-1056. This culture produced pale yellow colonies, but in 1944, Wickerham and his colleagues noted the emergence of a variant with bright orange-yellow colonies [88]. *E. ashbyi* was replaced in industry by the more stable *A. gossypii* [89] which produced, after genetic manipulation, over 20 g/l of the vitamin.

An rDNA process was developed for riboflavin in *Corynebacterium ammoniagenes* by cloning and overexpressing the organism's own riboflavin biosynthesis genes and its own promoter sequences [90]. The resulting culture produced 15 g/l riboflavin in 3 days. Genetic engineering of a *Bacillus subtilis* strain already containing purine analog-resistance mutations led to production of 15 g/l riboflavin [91]. This strain of *B. subtilis* was produced by inserting multiple copies of the *rib* operon at two different sites in the chromosome, expressing these constitutively from strong phage promoters located at the 5′-end and in an internal site in the operon, making purine analog-resistance mutations to increase guanosine triphosphate (GTP; a precursor) production and a riboflavin analog (roseflavin)-resistance mutation in *ribC* that deregulated the entire pathway [92]. The limiting gene in this strain was found to be *ribA*, encoding both 3,4-dihydroxy-2-butanone 4-phosphate synthase (N-terminal half of RibA) and GTP cyclohydrolase (C-terminal domain) [93]. One additional copy of *ribA* increased riboflavin titer much beyond 15 g/l and also raised the yield from glucose by 25%. Due to these efforts, *B. subtilis* became important for the industrial production of riboflavin.

Bacterial formation of vitamin B_{12} by bacteria is a very old phenomenon. The anaerobic pathway is about 4 billion years old whereas the aerobic pathway evolved when our atmosphere became enriched with oxygen about 2 billion years ago [94]. In the late 1940s, a Merck scientist [95] discovered that *Streptomyces griseus* and *Pseudomonas denitrificans* could form vitamin B_{12} [96]. Other studies showed that the vitamin could also be produced by *Propionibacterium shermanii* as well as other bacteria. *P. denitrificans* and *P. shermanii* became the industrial producing organisms. Production of vitamin B_{12} reached levels of over 200 mg/l using *P. denitrificans*. Fermentation was used exclusively at a rate of 12 tons/year in the early 2000s. This may seem like a small number but no more is needed since vitamin B_{12} is such a potent molecule.

At one time, ascorbic acid (vitamin C) was produced by isolation from lemons. This was replaced by the seven-step Reichstein synthesis in 1933 [97], which included a biosynthetic step. After chemical conversion of D-glucose to D-sorbitol, *Gluconobacter oxydans* was used to convert the latter to L-sorbose. L-Sorbose was

then converted in several chemical steps to 2-keto-L-gulonic acid (2-KLGA). 2-KLGA was then chemically converted by acid or base to ascorbic acid. The yield from glucose to 2-KLGA was 50%. The Reichstein process was used for 70 years but newly developed fermentation processes which converted D-glucose, D-sorbitol, or L-sorbose to 2-KLGA became competitive [98].

Some of the microbiological vitamin C processes used single organisms such as species of *Gluconobacter*, *Acetobacter*, or *Pseudomonas*. Mixed culture processes included one developed in China as far back as 1969 and used widely there to convert D-sorbitol to 2-KLGA. In another process, D-glucose was converted to 2,5-diketo-D-gluconic acid by *Erwinia* or *Acetobacter* which was then transformed into 2-KLGA by *Corynebacterium* [99]. Another similar process involved mutants of *G. oxydans* and *Bacillus megaterium* [100]. A genetically engineered strain of *Erwinia herbicola* produced 120 g/l of 2-KLGA [101], whereas recombinant *G. oxydans* made 130 g/l [102]. Ascorbic acid is used in the pharmaceutical, food, beverage, and feed industries. Annual production is 110 000 tons by synthesis and fermentation.

1.7.4 Organic Acids

Organic acids have been an important product of biotechnology. Much information on the history of the organic acid fermentations can be found in the reviews of Miall [103], Mattey [104], Roehr [105], and Magnuson and Lasure [106]. The most important commercial organic acids are citric, acetic, and lactic acids. Fermentation processes are also available for production of succinic, gluconic, oxogluconic, pyruvic, itaconic, shikimic, malic, propionic, butyric, oxalic, kojic, fumaric, erythorbic, *trans*-epoxysuccinic, tartaric, itatartaric, and long-chain α, ω-dicarboxylic acids.

Citric acid production has historic significance since it was the first industrial fermentation to be developed. It had been exclusively produced via isolation from lemons. In 1916, citric acid production by black aspergilli was described by Charles Thom and J.N. Currie [107]. Currie joined Chas. Pfizer and Co. in Brooklyn, NY and developed a commercial production process in 1923. The 1927 patents of Fernbach *et al.* [108, 109] were the basis of citric acid production in England by John & E. Sturge, Ltd. The process was improved in the 1930s by Raistrick's group [110] and by Doelger and Prescott [111]. The crucial metal requirements of the *Aspergillus niger* culture were studied by a number of groups including Tomlinson and coworkers [112] and Adiga *et al.* [113].

During the early years, the acid was produced solely by surface culture in flasks for laboratory studies and in trays for commercial production. However, Amelung [114] and Kluyver and Perquin [115] found that submerged culture was better. Further development of the citric acid fermentation depended greatly on the work of Professor Marvin Johnson with colleagues David Perlman [116] and Ping Shu [117] at the University of Wisconsin during the same years that Johnson was contributing so greatly to the development of the penicillin fermentation. The use

of invert molasses (high-test molasses), treated to reduce its iron content, was pioneered by Miles Laboratories. Mutants producing higher concentrations were obtained by Miles Laboratories [118], James *et al.* [119] and Hannan *et al.* [120].

About 1.5 million tons of citric acid are produced by *A. niger* per year. The commercial process employs *A. niger* in media deficient in iron and manganese. A high level of citric acid production was also associated with an increased intracellular concentration of fructose 2,6-biphosphate, an activator of glycolysis. Other factors contributing to high citric acid production were the inhibition of isocitrate dehydrogenase by citric acid, and the low pH optimum (1.7–2.0). In approximately 4–5 days, the major portion (80%) of the sugar provided was converted to citric acid, titers reaching about 100 g/l. Alternative processes were developed for the production of citric acid by *Candida* yeasts, especially from hydrocarbons. Such yeasts were able to convert *n*-paraffins to citric and isocitric acids in extremely high yields (150–170% on a weight basis). Titers as high as 230 g/l were reached.

The acetic acid bacteria are Gram-negative, obligately aerobic bacteria composed of species of *Acetobacter*, *Gluconoacetobacter*, and *Frateuria*. Their activity as the agents of wine spoilage has been a problem since at least 10 000 BC. *Acetobacter suboxydans* was used to produce vinegar as far back as 4000 BC. Indeed, the Latin word "acetum" means sour wine or sharp wine. Production of vinegar, an aqueous solution of acetic acid, is best carried out with species of *Gluconacetobacter* and *Acetobacter* [121]. A solution of ethanol was converted to acetic acid in which 90–98% of the ethanol was attacked, yielding a solution of vinegar containing 12–17% acetic acid. Industrial production of acetic acid was carried out solely by the conversion of sugar until the latter part of the 1800s when the distillation of wood became competitive with the fermentation process. Petroleum then became a major source of synthetic acetic acid. The first chemical plant for acetic acid production was established in 1916. Acetic acid made by fermentation from corn was mainly used by the food industry. Production of acetic acid amounts to 7.5 million tons per year by synthesis and fermentation.

Today, acetic acid fermentation is a two-step process in which the yeast *Saccharomyces cerevisiae* converts glucose to ethanol and *Acetobacter aceti* produces acetic acid from the ethanol. Cloning of aldehyde dehydrogenase from *Acetobacter polyoxogenes* on a plasmid vector into *A. aceti* subsp. *xylinum* increased the rate of acetic acid production by over 100% (1.8 g/l per h to 4 g/l per h) and titer by 40% (68–97 g/l) [122].

Another group of organisms considered for acetic acid production has been the anaerobic, thermophilic anaerobes of the genus *Clostridium*. In 1940, Wieringa isolated *Clostridium aceticum* [123] which was subsequently lost. However in 1942, Fontaine *et al.* [124] isolated *Clostridium thermoaceticum* which converted sugar quantitatively to acetic acid via the Embden–Meyerhof pathway to pyruvate which was then converted to acetate. As a result, 1 mole of glucose was converted into 3 moles of acetate. Experience with this fermentation showed that 0.85 g of acetic acid could be produced from 1 g of glucose [125] and titers of 83–102 g/l were reached with improved mutants [126]. Production by *C. thermoaceticum* was reviewed by Cheryan *et al.* [127].

Lactic acid production has been used to retard food spoilage for centuries, but the way in which it does this was not known until the Pasteur discovery of 1857 that this favorable activity was caused by microorganisms. In 1878, Joseph Lister [128] isolated the first pure culture of any bacterium which he called *Bacterium lactis*, later to be renamed *Lactobacillus lactis* ssp. *lactis*. The lactic acid bacteria are Gram-positive anaerobes that produce and excrete lactic acid into the medium. They were among the first microbes to be used in the manufacture of foods. Today, lactic acid is used in the food industry as a preservative and as flavor enhancer and in the chemical and pharmaceutical industries. Important applications of L-lactic acid are in the manufacture of polylactide (see Section 1.7.6) and that of the environmentally benign solvent ethyl lactate. About 250 000 tons of lactic acid are produced annually.

In addition to the lactic acid bacteria, the fungus *Rhizopus* is also a producer of the acid. It is sometimes preferred because it does not require supplements such as yeast extract, corn steep liquor, or whey which make product recovery expensive. Also, *Rhizopus oryzae* synthesizes solely the L-(+) isomer of lactic acid, whereas most lactobacilli produce mixed isomers of the acid. Some recombinant lactobacilli can produce individual isomers but yields are low. A six-step strain improvement program resulted in a *R. oryzae* strain producing over 130 g/l of L-(+)-lactic acid and a yield from glucose of near 90% [129].

Although most lactobacilli produce mixed isomers, a strain of *L. lactis* has been isolated which makes 195 g/l of L-lactic acid from 200 g/l glucose [130]. A recombinant *E. coli* strain produced optically pure D-lactic acid from glucose with a yield almost at the theoretical maximum yield (i.e., two molecules from one molecule of glucose) [131]. The organism was engineered by eliminating genes of competing pathways encoding fumarate reductase, alcohol/aldehyde dehydrogenase, and pyruvate formate lyase, and by a mutation in the acetate kinase gene.

1.7.5
Alcohols

Ethanol production is probably the oldest fermentation process known. For 6000 years, since the Sumerian and Egyptian days, the conversion of sugar in fruits and grains to ethanol has been an important process. Up until the 1980s, it was mainly used for to make alcoholic beverages, but in recent years, it has become an important clean and neat feedstock and fuel, especially for automobiles. Ethanol also has applications as: (i) a solvent in the laboratory, in pharmaceuticals, and in cosmetics, (ii) a cosurfactant in oil–water emulsions, and (iii) a sterilizing agent and antiseptic.

It was J.L. Gay-Lussac who, in 1810, first reported ethanol and CO_2 as the principal products of sugar breakdown by yeast (see the fascinating review by F. Schlenk [132]). In 1837, the physicist Charles Cagniard-Latour described the features of yeast cells found in fermented beverages including their shape, reproduction, their need for both fermentable carbohydrate and a nitrogen source for multiplication, and differences between yeasts of wine production and those of

the beer-making process. At about the same time, Theodor Schwann, trained in medicine, found that yeasts were required for alcoholic fermentation. The work of Cagniard-Latour and Schwann was confirmed by Friedrich Traugott Kuetzing, a pharmacist and college teacher. However, the work of these pioneers was rejected by the chemists J.J. Berzelius, J. Liebig, and F. Woehler, who believed the process to be strictly chemical in nature. Interestingly, one of the severest critics of the chemists was Moritz Traube, who had been a student of Liebig. He proposed that fermentation was carried out by oxidizing and reducing cellular components which he called "ferments." It was Pasteur in 1860 who proved that the yeast cells were absolutely required to carry out the series of (bio)chemical reactions. Finally, Eduard Buchner in 1897, with the help of his brother Hans, was able to carry out cell-free synthesis of ethanol with extracts of yeast. For this, he won the Nobel Prize in chemistry in 1907.

Ethyl alcohol is produced via fermentation of sugars (or a polysaccharide that can be depolymerized to a fermentable sugar) by *S. cerevisiae* in the case of hexoses, and *Kluyveromyces fragilis* or *Candida* species with lactose or a pentose, respectively. *S. cerevisiae* and other yeasts were chosen and adapted for specific ethanol fermentations. They included baker's yeast (different strains for compressed and active dry yeast), wine yeasts (including special flocculent strains for the production of champagne and film-forming strains for the production of flor sherry), sake yeast, top and bottom fermenting brewing strains (varying in the degree of flocculation occurring during fermentation), and distiller's strains used for alcohol production from cereal starch. About 2 million tons of yeast are produced annually for the distilling, brewing, and baking industries each year. The production of beverage alcohol was restricted to the use of microorganisms (e.g., yeast) but that of industrial and fuel alcohol was usually carried out by chemical synthesis from petroleum; this eventually changed in favor of yeasts. Under optimum conditions, approximately 10–12% ethanol by volume was obtained in yeast fermenations within 5 days. Such a high concentration slowed down growth and the fermentation ceased. With special (saki) yeasts, the fermentation could be continued to alcohol concentrations of 20% by volume but these concentrations were attained only after months of fermentation.

In 1977, yeast production of beverage, fuel, and fuel alcohol was 20% less than by chemical synthesis. However, by 1984, yeasts provided 87% more ethanol than did chemical synthesis. The percentage of total alcohol made by yeasts continued to increase over the years. By 2006, 13.2 million tons of ethanol were produced from corn annually in the United States by fermentation compared with 0.65 million tons by synthesis. Due to the elimination of lead from gasoline, ethanol was substituted as a blend to raise gasoline's octane rating. Later, it was added to gasoline as an oxygenate to reduce CO_2 emissions by improving the overall oxidation and performance of gasoline. This was due to the phasing out of the use of methyl tert-butyl ether (MTBE) as oxygenate, as ruled by many state legislatures in the United States. Ethyl alcohol was produced in Brazil from cane sugar at a rate of over 4 billion gallons per year and was used either as a 25% blend or as a pure fuel.

Fuel ethanol produced from biomass is being considered as a means to provide relief from air pollution caused by use of gasoline without contributing to the greenhouse effect [133] and of eliminating the dependence of the United States on foreign sources of petroleum. The available feedstock in the United States could supply 20 billion gallons of fuel ethanol. New processes have been developed to convert biomass to ethanol and rDNA technology has been used to convert *E. coli* and its close relatives into efficient producers of ethanol (43% yield, v/v) [134]. Alcohol dehydrogenase II and pyruvate decarboxylase genes from *Zymomonas mobilis* were inserted in *E. coli* and became the dominant system for NAD regeneration. Ethanol represented over 95% of the fermentation products in the genetically engineered strain. Some genetically engineered *E. coli* strains made as much as 60 g/l of ethanol. By cloning and expressing the same two genes into *Klebsiella oxytoca*, the recombinant strain was able to convert crystalline cellulose to ethanol in high yield when fungal cellulase was added [135]. Maximum theoretical yield was 81–86% and titers as high as 47 g/l of ethanol were produced from 100 g/l cellulose.

Bacteria such as clostridia and *Zymomonas* have also been reexamined for their utility in ethanol production after years of neglect. *Clostridium thermocellum*, an anaerobic thermophile, converts waste cellulose (i.e., biomass) and crystalline cellulose directly to ethanol, without the need for fungal cellulase [136–139].

Butanol is another alcohol which could help solve the problem of overdependence on petroleum as a motor fuel [140, 141]. Cloning of its *ace* operon genes *adc* (encoding acetoacetate decarboxylase), *ctfA* and *ctfB* (two genes encoding coenzyme A transferase) on a plasmid containing the *adc* promoter into *Clostridium acetobutylicum* resulted in a 95% increase in production of acetone, a 37% increase in butanol, a 90% increase in ethanol, a 50% increase in solvent yield from glucose, and a 22-fold lower production of acids [142].

An *E. coli* culture has been developed that grows on glucose and produces 1,3-propanediol (PDO; trimethylene glycol; 3G) at 135 g/l, with a yield of 51% and a rate of 3.5 g/l per h [143]. This effort was jointly carried out by scientists from Genencor International and DuPont and was achieved by introducing eight new genes to convert dihydroxyacetone phosphate (DHAP) into PDO. These included yeast genes converting dihydroxyacetone to glycerol and *Klebsiella pneumoniae* genes converting glycerol to PDO. The researchers improved production in the recombinant by modifying 18 *E. coli* genes, including regulatory genes. PDO was the monomer used to chemically synthesize polyurethanes and the polyester fiber Sorono™ by DuPont (see Section 1.7.6). PDO is also used as a polyglycol-like lubricant and as a solvent.

Other alcohols that can be made by fermentation are glycerol, erythritol, mannitol, sorbitol, and xylitol.

1.7.6 Polymers

Thirty thousand tons of the polysaccharide xanthan are produced annually for use in the oil, pharmaceutical, cosmetic, paper, paint, and textile industries. Genetic

manipulation increased titers of xanthan by twofold and increased pyruvate content by 45% [144]. Cloning genes, which complemented xanthan-negative mutants, into wild-type *Xanthomonas campestris* increased xanthan production by 15% [145].

A solution to the polluting effects of chemically produced plastics was provided by a group of microbially produced biodegradable plastics, known as polyhydroxyalkanoates (PHAs). One PHA, polyhydroxybutyrate, became available from microorganisms in the 1980s [146]. PHAs accumulate intracellularly to levels of 30–80% of cell dry weight and under certain conditions, in *Alcaligenes eutrophus,* reach 96% of the cell material [147].

Another new bioplastic is polytrimethylene terephthalate (3GT polyester; 3G+), made by reacting terephthalic acid with PDO produced by fermentation [148]. 3G+ is a new, environmentally friendly polyester. Another environmentally friendly plastic is polylactide, made chemically from fermentation-derived L-(+)-lactic acid [149].

1.7.7
Specialty Sugars, Sugar Alcohols, L-Sugars, Oligosugars, Novel Extracellular Polysaccharides, Biopigments, Cosmetics Including Fragrants, and Microbial Enzymes for Chiral Synthesis and Other Applications

An important specialty sugar is L-ribulose [150] which can be made by bioconversion, that is, dehydrogenation of ribitol. This compound is then converted to L-ribose which is used for the synthesis of nucleoside analogs as antiviral agents. A useful homopolysaccharide of glucose is dextran, which is secreted by strains of *Leuconostoc, Streptococcus,* and *Lactobacillus.* Dextran can be produced by use of dextransucrase [151]. Cosmetics (personal care products) were used by ancient Egyptians, Greeks, Romans, Incas and Aztecs centuries ago and are extensively used today [152]. They include hyaluronic acid, chitosan, xanthan, ceramides, amino acids, ectoines, provitamins, dihydroxyacetone, *Clostridium botulinum* toxin, organic acids, cyclodextrins, biosurfactants, indigo, biopigments, fatty acids, and microbial enzymes. Important biopigments are riboflavin, β-carotene, astaxanthin, zeaxanthin, and monaascin. Biofragrants include a peach aroma 4-decalactone, a butter aroma *R*-δ-dodecanolide, and cheese aromas butyric acid and its ethyl ester [153]. Biocatalysis (see Sections 1.12 and 1.13) has been extensively used in the bulk and fine chemicals areas and also in the environmental sector [154,155].

1.8
The Shift from Antibiotics to Pharmacological Agents

During the 3 billion years in which bacteria have inhabited the earth, Nature has developed a unique chemistry in the form of hundreds of thousands of identified and isolated novel secondary metabolites. These natural products, with structures much more spatially complex than those of synthetic chemicals, have been an

overwhelming success in their usage by humans. Secondary metabolites of microorganisms have reduced pain and suffering, and revolutionized medicine. Natural products have been the most important anti-infective and anticancer agents. The inhibitors of HIV reverse transcriptase and protease used in combination against AIDS were all modeled after leads obtained from natural products at the National Cancer Institute of the United States. The anti-herpes agent acyclovir and the non-Hodgkin's lymphoma drug cytarabine derive from sponges. At the turn of the century, over half of the approved drugs available were either natural products or were related to them, and that did not even include biologicals such as vaccines and monoclonal antibodies.

Of all the traditional products made by fermentation, the most important to human health are the secondary metabolites (idiolites). These (i) are often produced in a developmental phase of batch culture (idiophase) subsequent to growth, (ii) have no function in growth, (iii) are produced by narrow taxonomic groups of organisms, (iv) have unusual and varied chemical structures, and (v) are often formed as mixtures of closely related members of a chemical family. In nature, their functions serve the survival of the strain, but when the producing microorganisms are grown in pure culture, the secondary metabolites have no such role. Thus, production ability in industry is easily lost by mutation ("strain degeneration"). In general, both the primary and the secondary metabolites of commercial interest have fairly low molecular weights, that is, less than 1500 Da.

Whereas primary metabolism is basically the same for all living systems, secondary metabolism is mainly carried out by plants and microorganisms and is usually strain-specific. The best-known secondary metabolites are the antibiotics, as discussed above.

In the latter half of the twentieth century, more attention was placed on the use of microbial secondary metabolites as pharmacological agents. No longer were microbial sources looked upon solely as potential solutions for microbial diseases. With great vision, Hamao Umezawa (Figure 1.10) began, in the 1960s, his pioneering efforts to broaden the scope of industrial microbiology to low molecular weight secondary metabolites which had activities other than, or in addition to, antibacterial, antifungal, and antitumor action. He and his colleagues at the Institute of Microbial Chemistry in Tokyo focused on enzyme inhibitors [156, 157] and over the years, discovered, isolated, purified, and studied the *in vitro* and *in vivo* activity of many of these novel compounds. Similar efforts were conducted at the Kitasato Institute in Tokyo led by Satoshi Omura [158]. This change in screening philosophy was followed by ingenious applications of molecular biology to detect activities of compounds from microbes and plants for non-antibiotic applications.

1.8.1 Enzyme Inhibitors

The most important group of enzyme inhibitors are the statins, used for cholesterol-lowering in humans. These extremely successful agents also have antifungal

Figure 1.10 Hamao Umezawa.

activities, especially against yeasts. Independently, Brown *et al.* [159] in the United Kingdom and Endo in Japan [160] discovered the first member of this group, compactin (ML-236B; mevastatin) as an antibiotic product of *Penicillium brevicompactum* and *Penicillium citrinum*. Endo *et al.* discovered compactin in broths as an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the regulatory and rate-limiting enzyme of cholesterol biosynthesis. Later, Endo [161] and Alberts *et al.* [162] (at Merck, USA) independently discovered the more active methylated form of compactin known as lovastatin (monacolin K; mevinolin) in broths of *Monascus ruber* and *Aspergillus terreus*, respectively.

Lovastatin (mevinolin) was produced by *Aspergillus terreus* and in its hydroxyacid form (mevinolinic acid), was a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase from liver. Lovastatin led the way to the development of pravastatin, Zocor (simvastatin), and Lipitor (atorvastatin). Pravastatin was produced by bioconversion, that is, hydroxylation of compactin using actinomycetes such as *Streptomyces carbophilus* [163] and *Actinomadura* sp. [164]. Interestingly, the enzymatic mechanism in the two actinomycetes was found to be quite different [165]. Zocor was prepared by semi-synthesis from lovastatin, whereas Lipitor was made by total synthesis, modeled after the structure of the other statins.

The statins were found to inhibit *de novo* production of cholesterol in the liver, the major source of blood cholesterol. High blood cholesterol leads to atheroscle-

rosis which is a causal factor in many types of coronary heart disease, a leading cause of human death. Statins were a success because they reduced total plasma cholesterol by 20–40%, whereas the previously used fibrates only lowered it by 10–15%. Statins were not only useful for reduction in the risk of cardiovascular disease, they also prevented stroke, reduced development of peripheral vascular disease, and had antithrombotic and anti-inflammatory activities. It is obvious why this group of compounds became the leading drugs of the pharmaceutical industry.

Anti-enzyme screens also led to the discovery of the pseudotetrasaccharide acarbose, a natural inhibitor of intestinal α-glucosidase and sucrase [166]. Acarbose was produced by an actinomycete of the genus *Actinoplanes*. It decreased hyperglycemia and triacylglycerol synthesis in adipose tissue, liver, and the intestinal wall of patients with diabetes, obesity and type IV hyperlipidemia. Additional valuable enzyme inhibitors included lipstatin, used to combat obesity and diabetes by inhibiting gastrointestinal absorption of fat. It was a pancreatic lipase inhibitor produced by *Streptomyces toxytricini*. The commercial product was called Orlistat (tetrahydrolipstatin) [167].

Also discovered at this time was a very important enzyme inhibitor for medicine called clavulanic acid [168] (see Section 1.3) and several for agriculture such as the polyoxins [169] and the phosphinothricins (see Section 1.8.5). Polyoxins are antifungal agents which inhibit cell wall formation via inhibition of chitin synthetase.

Inhibitors of metal uptake also became important. One, called desferal, was a siderophore produced by *Streptomyces pilosus* which was used in iron-overload disease (hemochromatosis) and for aluminum overload in kidney dialysis patients [170].

1.8.2 Immunosuppressants

Umezawa's concepts also led to development of the hugely important immunosuppressants, such as ciclosporin, tacrolimus, sirolimus, and mycophenolic acid, which revolutionized the field of organ transplantation. Ciclosporin (initially known as cyclosporine A) was originally discovered as a narrow-spectrum antifungal peptide produced by the mold *Tolypocladium niveum* (previously *Tolypocladium inflatum*) [171]. Discovery of its immunosuppressive activity led to its use in heart, liver, and kidney transplants and to the overwhelming success of the organ transplant field.

Although ciclosporin had been the only product on the market for many years, two other products from actinomycetes provided further opportunities. These were sirolimus (rapamycin) and tacrolimus (FK-506). Both were narrow-spectrum polyketide antifungal agents, which were 100-fold more potent than ciclosporin as immunosuppressants and less toxic.

Sirolimus was discovered by a small group of scientists at Ayerst Laboratories in Montreal, Canada, under the leadership of Claude Vezina [172]. In 1964, a

Canadian scientific expedition had traveled to Easter Island (Rapa Nui) in the South Pacific to gather samples of soils and plants. Fortunately, they shared the soil samples with the Ayerst team and in 1972, the latter isolated sirolimus (called rapamycin at that time) from *Streptomyces hygroscopicus*. This remarkable molecule exhibited potent activity against the pathogenic yeast *Candida albicans* and other yeasts. Due to the efforts of Suren Sehgal, it was later found to have both immunosuppressive and anticancer activities [173]. Rapamycin was patented in 1975, but because of corporate lack of interest, it was not commercialized as an agent to facilitate organ transplantation until 1999. Sirolimus did not exhibit the nephrotoxicity of ciclosporin and tacrolimus, and was synergistic with both compounds in immunosuppressive action. The drug found a new use in cardiology when it was used for impregnation of stents because sirolimus-impregnated stents were less prone to proliferation and restenosis, which usually occur after treatment of coronary artery disease.

Developments on the fermentative production of sirolimus and the production of new analogs of this multipotent molecule were aided by the discoveries in 1991–1993 by Paiva and coworkers [174–176] of its biosynthetic precursors (i.e., acetate, propionate, methionine, pipecolic acid, and shikimic acid).

Tacrolimus was discovered by the Fujisawa Pharmaceutical Co. (now Astellas) [177] but almost abandoned after initial animal studies showed dose-associated toxicity. However, Dr. Thomas Starzl of the University of Pittsburgh, realizing that the immunosupressant was 30- to 100- fold more active than ciclosporin, tried lower doses which were very effective and non-toxic, thus saving the drug and many patients after that, especially those that were not responding to ciclosporin. Since its introduction in 1993 in Japan and 1994 in the United States, tacrolimus has been used for transplants of liver, kidney, heart, pancreas, lung, intestines, and for prevention of graft-versus-host disease. A topical preparation was shown to be very active against atopic dermatitis, a widespread skin disease.

A very old broad-spectrum antibiotic compound, mycophenolic acid, has an amazing history. The unsung hero of the story is Bartolomeo Gosio, the Italian physician who discovered the compound in 1893 [178]. Gosio isolated a fungus from spoiled corn which he named *Penicillium glaucum*, and which was later reclassified as *P. brevicompactum*. He isolated crystals of the compound from culture filtrates in 1896 and found it to inhibit growth of *Bacillus anthracis*. This was the first time an antibiotic had been crystallized and the first time that a pure compound had ever been shown to have antibiotic activity. The work was forgotten but fortunately the compound was rediscovered by Alsberg and Black in 1913 [179] and given the name mycophenolic acid. They used a strain originally isolated from spoiled corn in Italy called *Penicillium stoloniferum*, a synonym of *P. brevicompactum*. The chemical structure was elucidated many years later by Raistrick and coworkers in England. Mycophenolic acid has antifungal, antiviral, antitumor, antipsoriasis, and immunosuppressive activities. It was never commercialized as an antibiotic because of its toxicity, but its 2-morpholinoethylester was approved as a new immunosuppressant for kidney transplantation in 1995 and for heart transplants in 1998. The ester is called mycophenolate mofetil (CellCept) and is a prodrug which is hydrolyzed to mycophenolic acid in the body.

1.8.3 Antitumor Agents

Natural products became the leading chemotherapeutic drugs against cancer. Cytarabine (Cytostar®) for non-Hodgkin's lymphoma was isolated from a sponge [180]. Most of the other important antitumor compounds were microbially produced secondary metabolites. These included actinomycin D, mitomycin, bleomycins, and the anthracyclines daunorubicin and doxorubicin. The bark of the Pacific Yew tree (*Taxus brevifolia*) yielded taxol (paclitaxel), the microtubule stabilizer discovered by Wall and Wani [181] with excellent activity against breast and ovarian cancer. Of particular interest is that it is also produced by endophytic fungi isolated from the same source [182]. Taxol was the first antitumor drug known to act by blocking depolymerization of microtubules. Another plant compound used for cancer and discovered by Wall and Wani is camptothecin [181].

For a number of years, scientists worked on the "magic bullet" approach which would utilize monoclonal antibodies specific for tumor cells to bring very toxic chemotherapeutic agents into intimate contact with tumor cells and thus provide a specific, and hopefully a safe, way to kill these cells. Problems included less than required specificity, immunological complications, and poor penetration of antibody–toxin conjugates into the cancer cells. However, the combination of monoclonal antibodies with chemotherapeutic agents had promise. In the 1990s, the microbially produced toxic enediyne antitumor drug calicheamicin was attached to a humanized monoclonal antibody and was approved for use against acute myeloid leukemia (AML) [183]. The monoclonal antibody was designed to direct it to the CD33 antigen which is a protein expressed by myeloid leukemic cells. The conjugate was called Mylotarg (or gemtuzumab ozogamicin). It became a marketed product in 2001.

1.8.4 Ergot Alkaloids

Broad screening led to the development of ergot alkaloids for various medical uses such as for uterocontraction, hypertension, serotonin-related disturbances, and migraine headaches, among others. These plant alkaloids had been traditionally produced by extraction from sclerotia from parasitic species of the fungal genus *Claviceps*. Later, these alkaloids became products of fungi in submerged commercial fermentations [184].

1.8.5 Agricultural Compounds

The new efforts in pharmacology also paid dividends in agriculture. New products included bioherbicides, antiparasitic agents, bioinsecticides, agricultural antifungal agents, plant growth stimulators, and animal estrogenic agents.

The agricultural use of synthetic chemicals as herbicides had worried many environmentalists as several widely used herbicides were reported to cause cancer

in long-term animal tests. To fill the void, antibiotics were considered for use as agricultural herbicides. One such herbicide, bialaphos (*N*-{4[hydroxyl[methyl] phosphinoyl] homoalanyl}alanylalanine), which was active against broad-leaved weeds, was developed in Japan in 1973 [185]. This *Streptomyces viridochromogenes* product had been discovered one year earlier by Zahner's group in Germany as a broad-spectrum antibiotic active against bacteria and *Botrytis cinerea* [186]. Its hydrolysis product, DL-homoalanin-4-yl (methyl) phosphinic acid (DL-phosphinothricin) is a glutamine synthetase inhibitor which is manufactured by Hoechst as glufosinate (Basta). Of great interest to environmentalists was that bialaphos was easily degraded in the environment, having a half-life only 2 h.

The gibberellins, plant growth stimulators, are produced commercially by *Gibberella fujikuroi*, the conidial state of the fungus *Fusarium moniliforme*, and are used as plant regulators and in the malting of barley in the brewing process [187]. They increase the yield of vegetables and accelerate the development of biennials.

One of the major economic diseases of poultry is coccidiosis caused by species of the parasitic protozoan *Eimeria*. For years, this disease was treated solely by synthetic chemicals and indeed only synthetic compounds were screened for coccidiostat activity. Although they were generally effective, resistance developed rapidly in the coccidia and new chemical modifications of the existing coccidiostats were pursued. Surprisingly, a parenterally toxic and narrow-spectrum antibiotic, monensin, was found to have extreme potency against coccidia [188]. The polyether antibiotics, especially monensin (produced by *Streptomyces cinnamonensis*), lasalocid (produced by *Streptomyces lasaliensis*), and salinomycin (produced by *Streptomyces albus*), have dominated the commercial coccidiostat field ever since.

An interesting sideline in the monensin story is the discovery of its further use as a growth promotant in ruminants. For years, synthetic chemicals had been screened for activity in cattle and sheep diets to eliminate wasteful methane production and increase volatile fatty acid formation (especially propionate) in the rumen, which would improve feed efficiency. Although the concept was sound, no useful products resulted. Experimentation with monensin showed that polyethers had this activity and these compounds are now widely used [189].

Another antiparasitic agent is avermectin which replaced the synthetic products previously used as antihelmintics/endectocides. Direct *in vivo* screening of fermentation broths against nematodes in mice led to the discovery of the potent activity of the avermectins against disease-causing helminths in animals. Avermectin's antihelmintic activity was an order of magnitude greater than that of previously developed synthetic compounds. The *Streptomyces avermitilis* culture, which was isolated by Yoko Takahashi and Satoshi Omura and coworkers at the Kitasato Institute in Japan (described in [190]), produced a family of secondary metabolites having both antihelmintic and insecticidal activities which were named "avermectins" and developed by Merck in the United States [191]. They are disaccharide derivatives of macrocyclic lactones with exceptional activity against parasites, that is, at least 10 times higher than that of any synthetic antihelmintic agent known. Despite their macrolide structure, avermectins lack antibiotic activity

against bacteria and fungi, do not inhibit protein synthesis nor are they ionophores; instead they interfere with neurotransmission in many invertebrates. The activity of avermectins against both nematode and arthropod parasites in sheep, cattle, dogs, horses, was 1000 times more active than that of the previously used synthetic compound, thiobenzole. A semi-synthetic derivative, ivermectin, was soon established as a commercial veterinary product.

A fortunate fallout from the work with avermectin was the finding that ivermectin had activity against the black fly vector of human onchocerciasis ("river blindness") [192]. It interferes with transmission of the filarial nematode, *Onchocerca volvulus*, to the human population. Since 40 million people were affected by this disease, the decision by Merck in the 1980s to supply ivermectin free of charge to the World Health Organization for use in humans in the tropics was met with great enthusiasm and hope for conquering this parasitic disease. Ivermectin is also effective in the Asian human disease strongyloidiasis.

A family of bioinsecticides called the spinosyns were discovered at Eli Lilly and commercialized by Dow AgroSciences [193]. These were non-toxic, non-antibiotic, environmentally friendly tetracyclic macrolides produced by *Saccharopolyspora spinosa* with activity against insects of the orders Coleoptera, Diptera, Hymenoptera, Isoptera, Lepidoptera, Siphonoptera, and Thysanoptera.

Another important bioinsecticide was a non-antibiotic large molecule, the protein crystal of *Bacillus thuringiensis*. The selective toxicity of this protein (delta-endotoxin; BT toxin) against insects of the order Lepidoptera has been exploited successfully in agriculture. The strong selectivity of its toxicity against these insects meant that it did not harm the environment and only little resistance has developed over the years. Other commercialized agricultural products include the bioinsecticide nikkomycin, the agricultural antifungal group known as polyoxins, and the animal estrogenic agent zearelanone.

1.9
The Biopharmaceutical Revolution

Major milestones in genetics include the findings of Gregor Mendel in the mid-nineteenth century on the inheritance of characters in peas. In 1944, a major discovery, that DNA was the genetic material, was made by Avery, McCloud, and McCarty at the Rockefeller Institute. Two years later, Lederberg and Tatum discovered sex in bacteria. The revolution in biotechnology was sparked by the 1953 discovery of the double-stranded structure of DNA by Watson and Crick (Figure 1.11). The use of microorganisms and their antibiotics as tools of basic research is mainly responsible for the remarkable advances in the fields of molecular biology and molecular genetics. The biotechnology revolution did not come immediately but required some 15 years for additional basic discoveries to be made by others in molecular biology/genetics. In 1956, Alexander Rich (Figure 1.12) and David Davies reported that two single-standed RNA molecules could spontaneously organize themselves to form a double helix. Although this was doubted by

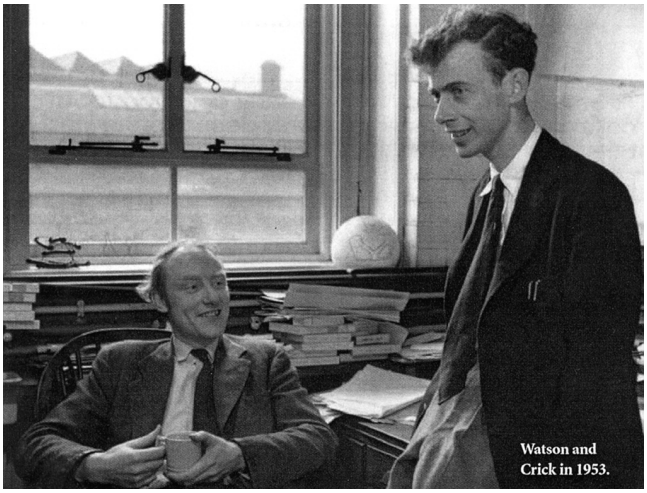

Figure 1.11 James D. Watson and Francis Crick.

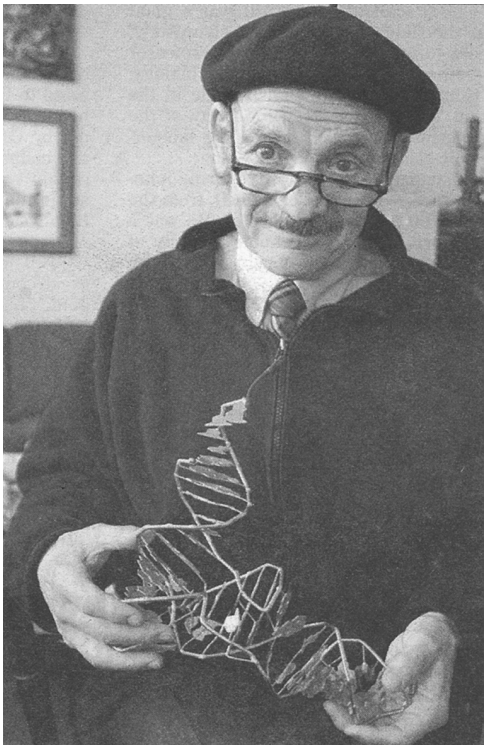

Figure 1.12 Alexander Rich.

many, the RNA double helix led about 40 years later to the discovery of micro RNAs and RNA interference. Beadle and Tatum received the Nobel Prize in 1958 for demonstrating the relationship between genes and enzymes. Three years later, Monod and Jacob discovered the regulation of gene expression and, in 1962, Smith and Arbor described restriction endonucleases.

The year 1966 was a very important one, during which the genetic code was deciphered by Nirenberg, Matthei, Leder, Khorana, and Ochoa. Shapiro and Beckwith isolated a gene in 1969 and Khorana chemically synthesized a gene in 1970. Until this point, genetic recombination was recognized to occur only between organisms of the same species or of closely related species. Even in the laboratory, protoplast fusion was restricted to genetically related species. All organisms had restriction endonucleases that recognized foreign DNA and destroyed it so that "illegitimate recombination" would not occur.

Then in 1972–1973, the development of recombinant DNA by Berg, Cohen, and Boyer at Stanford University and the University of California, San Francisco, triggered the birth of modern biotechnology [194]. These workers discovered how to use restriction enzymes to cut DNA molecules, how to use another enzyme, DNA ligase, to join DNA molecules from different organisms, and how to introduce the rDNA via a vehicle (e.g., plasmid, phage) into *E. coli*. They thus defied nature and carried out recombination across species barriers. This propelled biotechnology to new heights and led to the establishment of a new biopharmaceutical industry in the United States and around the world.

The revolutionary exploitation of basic biological discoveries, which began in 1971, did not take place in a vacuum but heavily depended upon the solid structure of the fermentation industry. At that time, a physician (Peter Farley), a biochemist (Ronald Cape, Figure 1.13), and a Nobel Laureate physicist (Donald Glaser), with several others, conceived of the commercialization of rDNA technology and established the Cetus Corporation in Berkeley, California, in 1971. Thus began one of the most exciting adventures in the history of industrial biotechnology. The vision of these Cetus founders led to the establishment of a major biotechnology industry, serving the needs of patients throughout the world and revolutionizing the practise of industrial microbiology.

The second biotechnology company was established in 1976, across the bay from Cetus in South San Francisco, by Herbert Boyer and Robert Swanson. In that same year, a human gene was expressed in bacteria and yeast DNA was replicated and expressed. By 1978, Genentech had developed human insulin and tissue plasminogen activator (tPA). Also in 1978, bacterial DNA was successfully inserted into yeast chromosomes and Biogen was founded in Cambridge, Massachusetts. In 1979, yeast protoplasts were transformed by a hybrid *E. coli*/yeast plasmid. Amgen was founded in southern California in 1980, the same year that a monumental ruling was made by the US Supreme Court stating that living organisms could be patented. This was based on the work of Ananda Chakrabarty.

In 1981, Genetics Institute, Chiron, and Genzyme were formed and the first recombinant diagnostic kit was approved by the FDA. In 1982, recombinant human insulin was ready for the marketplace as a Genentech/Eli Lilly endeavor.

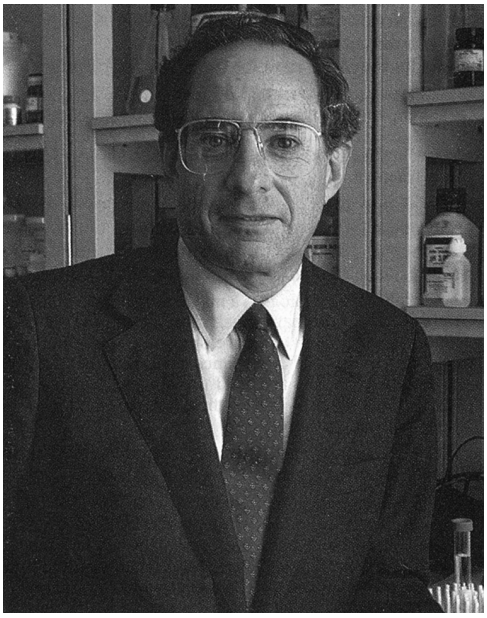

Figure 1.13 Ronald Cape.

Other products soon followed: human growth hormone in 1983; α-interferon, and recombinant hepatitis B vaccine in 1986; tPA in 1987; erythropoietin (EPO) in 1989; granulocyte colony-stimulating factor (G-CSF) in 1991; Factor VIII in 1992; and β-interferon in 1993.

Although Cetus is no longer in existence as an independent corporate entity, having been incorporated into Chiron Corporation in 1991, it should long be remembered as the founder of modern biotechnology and the developer of the polymerase chain reaction (PCR) in 1985, a technique of enormous importance today. Indeed, the PCR principal investigator, Cary Mullis, holds the only Nobel Prize (awarded in 1993) ever given to a scientist for their work in the biotechnology industry.

Since then, thousands of companies have been established, including Immunex, Centocor, MedImmune, etc. Many of them invested in modern biotechnology with no clear idea of the future but with the faith that genetics would lead to products that could not even be conceived of at the time; indeed this dream came true in a major way. This led to an explosion of investment activity in new companies, mainly dedicated to innovation via genetic approaches. Newer companies entered the scene in various niches such as microbiological engineering and downstream processing.

By 1988, there were about 440 biotechnology companies and 70 large pharmaceutical, chemical, and energy corporations in the United States devoting all or part of their resources to biotechnology. In 1993, the field served the following

areas: 41% therapeutics, 27% diagnostics, 15% supplies, 9% agricultural, and 8% chemical, environmental and services. The number of US companies increased to about 1500 by 2003. The number of US employees was 191 000 in 2002. R&D spending on biotechnology in the United States in 2000 amounted to US$21 billion and revenues were US$36 billion.

A significant number of biotechnology companies and departments of large companies were also established in Europe and Asia. The 2005 world market for rDNA products amounted to US$43 billion. Today, large pharmaceutical companies have major holdings in some of these companies and biopharmaceutical revenues have reached over US$60 billion.

The rDNA pharmaceutical market dealt with four principal areas: (i) blood products (thrombolytics, dismutases, septic shock drugs, clotting agents, erythropoietin); (ii) immunotherapy products (α-, β-, and γ-interferons, interleukins, colony-stimulating factors; (iii) infectious disease combattants (hepatitis B vaccine, AIDS vaccine); (iv) growth factors for mammalian cells (epidermal growth factor, insulin-like growth factors, fibroblast growth factors, transforming growth factors, platelet-derived growth factor, growth hormone releasing factor, lung surfactants, and tumor necrosis factor).

Many benefits to society have come from biotechnology [195]. (i) Diabetics no longer have to fear producing antibodies to animal insulin. (ii) Children deficient in growth hormone no longer suffer from dwarfism or fear the risk of contracting Creutzfeldt–Jakob syndrome. (iii) Children who have chronic granulomatous disease are able to have a normal life by taking γ-interferon therapy. (iv) Patients undergoing cancer chemotherapy or radiation therapy recover more quickly with fewer infections when they use granulocyte colony-stimulating factor (G-CSF).

The success of the biopharmaceutical revolution can be seen in the following data. Between 1997 and 2002, 40% of the drugs introduced into medical practise came from biotechnology companies. The five largest pharmaceutical companies in-licensed from six to ten products from biotechnology or specialty pharmaceutical companies yielding 28–80% of their revenue. The biotechnology industry had two drug/vaccine approvals in 1982, none in 1983/1984, one in 1985, and 32 in 2000! The number of patents granted to biotechnology companies rose from 1500 in 1985 to 9000 in 1999. The major products of the biopharmaceutical industry are discussed below.

1.9.1 Human Insulin (Novolin, Humulin)

Human insulin will always be remembered as the product that launched the biopharmaceutical industry. The first recombinant protein produced and then approved by FDA, human insulin was developed by Genentech in 1979 and produced industrially in 1982 by Eli Lilly. Previously, the process required extraction from the pancreas of dead cattle and pigs and the product was not identical to human insulin. Furthermore, the animal products contained impurities that

caused allergic reactions. These problems were all solved by recombinant human insulin.

1.9.2 Erythropoietin (Epogen, Procrit)

Erythropoietin (EPO) is a bone marrow factor for kidney disfunction and for chemotherapy patients used for treatment of chronic renal failure in patients using kidney dialysis. It acts to ameliorate certain anemias by stimulating production and differentiation of red blood cells. The product entered clinical trials in 1985 and was approved in 1989. It is also useful for anemia caused by azidothymidine (AZT) for AIDS and by chemotherapeutics for cancer. EPO is also given to patients who want to use their own banked blood instead of the blood of others.

1.9.3 Interferons

α-Interferon (Intron-A, Roferon) was cleared by the FDA for use against Kaposi's sarcoma, chronic myeloid leukemia, genital warts, and hairy cell leukemia. It became useful in antiviral therapy. By 1992, it had been approved for hepatitis B and C. β-Interferon ("Betaseron," recombinant interferon β-1a, Avonex) was approved by the FDA for multiple sclerosis in 1993 and γ-interferon in 1990 for treatment of chronic granulomatous disease.

1.9.4 Human Growth Hormone (Somatotropin, Somatropin; Humatrope, Nutropin, Protropin, Somatren, Serostim)

An early effort of the Genentech organization was the production of human growth hormone (hGH) which had immediate application in the treatment of abnormally small children. Up to 1984, hGH had been produced from pituitary glands of human cadavers. It was very expensive and, in some cases, the material was contaminated with Creutzfeldt–Jakob virus, resulting in death. Since then, recombinant hGH has replaced the pituitary material and this has eliminated the problem. Although hGH was originally approved only for treating dwarfism, it was later approved for 11 indications; much of it is sold for burns and as an anti-aging product.

1.9.5 Tissue Plasminogen Activator (Activase, Alteplase)

Tissue plasminogen activator (tPA) dissolves blood clots in human coronary arteries and is prescribed for rapid cessation of heart attacks (acute myocardial infarction), deep vein thrombosis, pulmonary embolism, and stroke. It was

introduced on the market in late 1987. Use of tPA was extended to stroke patients in 1996.

1.9.6 Interleukins

Interleukin 2 (IL-2, proleukin) showed activity against renal cell cancer and was approved in 1992. In early 1998, FDA approved recombinant IL-11 (Neumega) for treatment of cancer chemotheraphy-related thrombocytopenia (i.e., low platelet count), due to its ability to stimulate platelet formation.

1.9.7 Factor VIII

Patients with hemophelia traditionally received blood coagulant products derived from human plasma to correct their deficiency of the blood-clotting protein Factor VIII. Unfortunately, 60% of these patients became infected with HIV, hepatitis, or other diseases whose viruses contaminate such products. Clinical trials began in 1989 on recombinant Factor VIII for use in hemophelia. The FDA approved the product in 1993.

1.9.8 Colony-Stimulating Factors

Colony-stimulating factors are bone marrow factors for kidney disfunction and for chemotherapy patients. Granulocyte colony-stimulating factor (G-CSF; Neupogen, Filgrastim, Leukine) was approved in 1991 for chemotherapy-induced white blood cell deficiency (neutropenia). Granulocyte macrophage colony-stimulating factor (GM-CSF) was also approved in 1991 for stimulation of white cell growth in autologous bone marrow transplants.

1.9.9 Human DNase (Pulmozyme)

Human DNase was approved by the FDA in 1994 for cystic fibrosis (CF). It was the first new drug in 30 years for CF, a disease that affects 30 000 people in the United States. In clinical trials, DNase has also shown efficacy in chronic bronchitis, a disease that affects 2 million people in the United States.

1.9.10 Glucocerebrosidase (Cerezyme)

Glucocerebrosidase was approved in 1994 for the genetic disorder Gaucher's disease. Patients lacking this enzyme cannot prevent lipid accumulation in vital organs and bones.

1.9.11 Monoclonal Antibodies

Monoclonal antiobodies (mAbs) were discovered by Georges Kolter and Cesar Milstein in the United Kingdom in 1975 [196]. They fused a mouse skin cancer cell ("myeloma") with an antibody-producing white cell. The result was a hybrid cell ("hybridoma") which produced a pure specific antibody. The two immunologists were awarded the Nobel Prize in 1984. Previously, polyclonal antibodies (pAbs) had been used but they contained varying specificities and affinities and were very variable. They had been produced by the entire immune system of the animal, whereas mAbs were produced by single cells of the immune system.

For mAb production, mice were immunized with a single antigen, allowed to show an immune response, their spleens were removed, cells extracted, fused with cells of mouse lymphoma cell line (immortal cancerous). Then, the fused "hybridoma" cells were cloned and screened to isolate those hybridoma cells that excreted the specific desired antibody. Since the human body could react in an undesirable way to mouse sequences, the mAbs were "humanized" by genetic engineering techniques. The mice were genetically engineered so that human genes encoding human heavy chains and human kappa light chain replaced the relevant mouse genes which were eliminated. Monoclonals could also be made in human immune cells. They were used to bind or block the binding of a target protein as a "magic bullet" in which a drug or a radioisotope is brought to a designated target.

After 2000, mAbs became the fastest growing therapeutic protein class, reaching a market of US$6.8 billion in 2006. ReoPro was the first successful therapeutic mAb, being approved in 1994 for inhibition of platelet aggregation (blood clotting). It successfully prevented complications of angioplasty such as death, heart attack, and need for repeat angioplasty. It was followed by four more monoclonals in 1998: (i) infliximab (Remicade) inhibited tumor necrosis factor (TNF) and was approved for Crohn's disease and also for rheumatoid arthritis; (ii) basiliximab (Simulect) was used prophylactically against acute organ rejection in patients receiving renal transplantation, along with ciclosporin and corticosteroids; (iii) trastuzumab (Herceptin) targeted the epidermal HER2 growth factor receptor protein oncogene and was used for late-stage metastatic breast cancer in 25–30% of the women with this disease and whose tumors overexpressed HER2; (iv) palivizumab (Synagis, MEDI-493) for prevention of lower respiratory tract disease caused by the respiratory syncytial virus. This was the first mAb against an infectious disease and was used to stop respiratory syncytial virus (RSV) leading to serious lower respiratory tract disease in pediatric patients. Adalimumab (Humira) was approved in 2003 for rheumatoid arthritis. Another very important monoclonal is retuximab (Rituxan), used for non-Hodgkin's lymphoma.

1.9.12 Additional Biopharmaceuticals

Other important products include: (i) etanercept (Enbrel), approved in 1998 for rheumatoid arthritis via its binding and inhibition of TNF, a protein involved in

inflammation; and (ii) imatinib (Gleevec, Glivec), which is active against chronic myelogenous leukemia (CML). CML is a disease caused by genetic translocation between chromosomes 9 and 22, generating an abnormal protein, Brc-Ab1, which causes uncontrolled proliferation of white blood cells resulting in leukemia. Imatinib blocks the action of Brc-Ab1 and is also active against gastrointestinal stromal tumor (GIST).

1.10 Recombinant Hosts

High cell density fermentation of microorganisms reaches levels of 233 g dry cell weight/l for bacteria and 268 g dry cell weight/l for yeasts [197]. Mammalian polypeptides are produced in these microbes at levels up to 70% of cell protein and concentrations as high as 15 g/l.

1.10.1 *E. coli*

The first most popular bacterial system for production of recombinant proteins was *E. coli*. Early in the era of biopharmaceuticals, it was realized that the same milligram quantities of mammalian polypeptides that were being produced in a few liters of recombinant *E. coli* broth previously had to be extracted from the brain tissue of half a million sheep. The benefits of *E. coli* as a recombinant host, in addition to high cell densities and elevated product yields, included the following: (i) it was easy to quickly and precisely modify the genome; (ii) growth was rapid; (iii) culture conditions were simple; (iv) protease activity was easily reduced; (v) avoidance of incorporation of amino acid analogs was possible; (vi) promoter control was simple; (vii) plasmid copy number could be altered easily; (viii) alteration of metabolic carbon flow was not a problem; (ix) formation of intracellular disulfide bonds was easy; (x) accumulation of heterologous proteins amounted to as much as 50% of dry cell weight; (xi) survival was possible under a wide variety of environmental conditions; (xii) expensive medium ingredients were not required; and (xiii) performance was reproducible especially with computer control [195].

One problem with *E. coli* was the formation of the heterologous proteins in the form of inclusion bodies. In this form, the recombinant proteins were inactive, aggregated, and insoluble, usually possessing non-native intra- and intermolecular disulfide bonds and unusual free cysteines. To produce active protein, these bodies had to be removed from the cell by homogenization, washing, and centrifugation, solubilized by denaturants (guanidine HCl, urea, sodium dodecyl sulfate) which unfolded the protein, and treated with reducing agents which broke the disulfide bonds. Then, refolding was carried out by removal of denaturant and reducing agent. The renaturation processes used were (i) air oxidation, (ii) the glutathione reoxidation system, and (iii) the mixed disulfides of protein-*S*-sulfonate and protein-*S*-glutathione systems. Heterologous recombinant proteins were also made

in biologically active soluble form at high levels by fusing their genes to the *E. coli* thioredoxin gene. Many human proteins were produced at levels of 5–20% of total proteins as fusions in *E. coli* cytoplasm. Some fusions retained the thioredoxin properties of (i) being released by osmotic shock or freeze/thaw methods and (ii) high thermal stability.

Another useful method of reducing the formation of inclusion bodies containing heterologous proteins in *E. coli* is to lower the temperature of growth from 37 to 30 °C. Products made in *E. coli* include human insulin, human growth hormone, α β γ-interferons, and G-CSF [195].

Almost all polypeptides excreted by eukaryotes are glycosylated. Glycosylation is species-, tissue-, and cell-type specific. Unfortunately, *E. coli* does not glycosylate proteins. In some cases, a normally glycosylated protein is active without the carbohydrate moiety and can be made in bacteria. This was found to be the case with γ-interferon. In cases where glycosylation is necessary for stability or proper folding (e.g., erythropoeitin), proteins can often be provided by recombinant yeast, mold, insect, or mammalian cells.

1.10.2 Yeasts

Yeasts offer certain advantages over bacteria as a cloning host. (i) They can secrete heterologous proteins into the extracellular broth when proper signal sequences are attached to the structural genes. (ii) They carry out glycosylation of proteins. However, glycosylation by *S. cerevisiae* is often unacceptable for mammalian proteins because the *O*-linked oligosaccharides contain only mannose whereas higher eukaryotic proteins have sialylated *O*-linked chains. Furthermore, *S. cerevisiae* overglycosylates *N*-linked sites which led to reduction in both activity and receptor-binding, causing immunological problems.

The methylotrophic yeast *Pichia pastoris* was found to possess advantages over *S. cerevisiae* as a host for heterologous genes. (i) This yeast could be grown at extremely high cell densities in protein-free media. (ii) It had a higher level of protein productivity. (iii) It did not overglycosylate. (iv) Foreign genes were incorporated in multiple copies into the chromosome. (v) The levels of protein production by these yeasts were relatively high. For example, *P. pastoris* can produce extracellularly 4 g/l of intracellular IL-2, 4 g/l of human serum albumin, and 10 g/l of TNF. (vi) The expression cassette was stably integrated into the host genome at specific locations. (vii) *P. pastoris* was haploid and amenable to traditional mutagenesis.

1.10.3 Molds

When foreign genes are introduced via plasmids into filamentous fungi, they integrate stably into the chromosome as tandem repeats. As many as 100 copies of a gene are observed. Production of bovine chymosin by recombinant *A. niger*

var. *awamori* amounts to 1 g/l and that of human lactoferrin by *A. awamori* is 2 g/l of extracellular protein.

1.10.4 Insect Cells

Insect cells in culture are good hosts for recombinant protein production [198]. Recombinant insect cell cultures have yielded over 200 proteins encoded by genes from viruses, bacteria, fungi, plants, and animals. Expression vectors have been prepared from the baculovirus which attacked invertebrates but not vertebrates or plants, thus insuring safety. The most widely used baculovirus is the nuclear polyhedrosis virus (*Autographa californica*) which contains circular double-stranded DNA, is naturally pathogenic for lepidopteran cells, and can be grown easily *in vitro*. The virus contains a gene encoding the protein polyhedrin which is normally made at very high levels and is not necessary for virus replication. The gene to be cloned was placed under the strong control of the viral polyhedrin promoter and suitable levels of proteins were produced with many of the posttranslational modifications of higher eukaryotes, including phosphorylation, glycosylation, correct signal peptide cleavage, proteolytic processing, palmitylation, and myristylation.

The usual host is the fall armyworm (*Spodoptera frugiperda*) in suspension culture. Alternatively, a larval culture is used which is much cheaper than cell culture. Larval systems have produced 600 mg/l of recombinant protein.

1.10.5 Mammalian Cells

The use of mammalian cell culture, chiefly immortalized Chinese hamster ovary (CHO) cells, was mandated by the need for EPO and tPA production in the early 1980s [195]. The development of mammalian cell culture was facilitated by prior developments in microbial fermentation technology. Mammalian cell cultures were useful in that the proteins were made in a properly folded and glycosylated form, thus eliminating the need to renature them. The production of recombinant proteins by mammalian cells was also done in N50 murine myeloma cells, baby hamster kidney cells, green monkey kidney cells, and human embryonic kidney cells. Mammalian cell culture became the leading source of recombinant biopharmaceuticals and was used for production of human growth hormone, GM-CSF, G-CSF, EPO, and Pulmozyme, among others. CHO cell processes were developed that yielded 3–5 g/l of recombinant protein.

1.10.6 Transgenic Animals

Transgenic animals were developed as production systems for recombinant peptides. tPA was made in milk of transgenic goats at a level of 3 g/l. Cows produce 30l of milk per day, of which protein amounts to 35 g/l; thus the total protein

produced per day is 1 kg. Production titers are 2 g/l of antithrombin III and 4 g/l of human growth hormone in the milk of mice, 5 g/l of recombinant fibrinogen in sheep milk, 8 g/l of α-glucosidase in rabbit milk, 14 g/l of antithrombin III in goat milk, 35 g/l of α-1-antitrypsin in sheep milk, and 40 g/l of hemoglobin in pigs; all genes were human in origin.

In most cases, the protein is as active as the native protein. Transgenic goats produce a tPA with glycosylation different from that produced in cell culture and with a longer half-life than native tPA. One of the negative points in production of proteins by transgenic animals is the length of time needed to assess production levels. This takes 3.5 months in mice, 15 months in pigs, 28 months in sheep, and 32 months in cows.

1.10.7
Transgenic Plants

Transgenic plants could also be used to produce valuable products, including β-glucuronidase (GUS), avidin, laccase, and trypsin. Oilseed rape plants have been used to produce enkephalin and a neuropeptide. Recombinant proteins can be produced in transgenic plants at levels as high as 14% of total tobacco-soluble protein (in the case of phytase from *A. niger*) and 1% of canola seed weight (hirudin from *Hirudo medicinalis*). Potential advantages include satisfactory glycosylation, targeting, compartmentalization, and natural storage stability.

1.11
Enzymes

The term "enzyme" was first coined by Kuhne in 1877 meaning "in yeast." When the field of biochemistry was born in 1897 via Buchner's discovery that cell-free extracts of yeast could carry out the production of ethanol from sugar, Buchner referred to the glycolytic enzyme complex as "zymase," meaning "the enzyme of yeast itself." Enzymes became valuable in manufacturing because of their rapid and efficient action at low concentrations under mild pH values and temperatures, their high degree of substrate specificity (which reduced side-product formation), their low toxicity, and the ease of terminating their action by mild treatments. Some microbial strains produced very high concentrations of extracellular enzymes. Wild strains of *Bacillus licheniformis* produced 5 g/l of protease and commercial strains made 20 g/l. High-yielding strains of *Aspergillus* produced 20 g/l of glucoamylase.

Additional reasons for using microbial cells as sources of enzymes were as follows: (i) enzyme fermentations were quite economical on a large scale due to short fermentation cycles and inexpensive media; (ii) screening procedures were simple and thousands of cultures could be examined in a reasonably short time; and (iii) different species produced somewhat different enzymes catalyzing the same reaction, allowing one flexibility with respect to operating conditions in the

reactor. This versatility is illustrated by the fact that α-amylase from *Bacillus amyloliquefaciens*, a commercial enyzme used for years for hydrolysis of starch at a temperature as high as 90 °C, was forced to compete in 1972 with a similar enzyme from *B. licheniformis* which could operate at 110 °C The optimal temperatures for the *B. amyloliquefaciens* and the *B. licheniformis* α-amylases were 70 °C and 92 °C respectively.

In the 1980s and 1990s, microbial enzymes were increasingly used for applications which traditionally employed plant and animal enzymes. These shifts included the partial replacement of (i) amylases of malted barley and wheat in the beer, baking, and textile industries by amylases from *Bacillus* and *Aspergillus*; (ii) plant and animal proteases by *Aspergillus* protease for chill-proofing beer and tenderizing meat; (iii) pancreatic proteases by *Aspergillus* and *Bacillus* proteases for leather bating and in detergent preparations; and (iv) calf stomach rennet (chymosin) by *Mucor* rennins for cheese manufacture. Later, cloning of mammalian chymosin became of interest to cheese manufacturers and tests on cheese made with the recombinant enzyme showed commercial success. Recombinant chymosin was approved in the United States and its price was half that of natural calf chymosin. Important industrial enzymes included the following: (i) glucose isomerase for production of high-fructose corn sirup; (ii) penicillin acylase for production of semi-synthetic penicillins; (iii) peroxidase for manufacture of phenolic resins (which could replace synthetic phenol-formaldehydes); and (iv) nitrile hydrolase for hydration of acrylonitrile to acrylamide. Glucose isomerase was used in conjunction with α-amylase and glucoamylase to convert starch to mixtures of glucose and fructose known as "high fructose corn syrup." The development of glucose isomerase permitted the corn wet milling industry to capture 30% of the sweetener business from the sugar industry in the 1970s. In the United States alone, high fructose corn syrup is produced at 30 billion pounds per year.

The industrial enzyme market reached US$2 billion in 2000 divided into the following application areas: food, 45% (of which starch processing represents 11%); detergents, 34%; textiles, 11%; leather, 3%; pulp and paper, 1.2%. This does not include diagnostic and therapeutic enzymes. The world market for products of enzyme reactions were as follows: high fructose corn syrup, US$1 billion; aspartame, US$800 million; acrylamide, US$300 million; 6-aminopenicillanic acid (6-APA) and 7-aminodeacetoxycephalosporanic acid (7ADCA), US$200 million.

Certain microorganisms ("extremophiles") can grow in extreme environments such as 100 °C, 4 °C, 250 atm, pH 10, pH 2, or 5% NaCl. "Extremozymes," that is, enzymes from these diverse organisms, have industrial significance. A commercial example is Cellulase 103 from an alkaliphile. The enzyme broke down the microscopic fuzz of cellulose fibers which trapped dirt on the surface of cotton textiles. The enzyme was commercialized by Genencor International in 1997 for use in detergents to return the "newness" of cotton clothes even after many washings.

With the development of rDNA methodology it became possible to clone genes encoding microbial enzymes and express them at levels hundreds of times higher than those naturally produced. The industrial enzyme business adopted rDNA

methods eagerly to increase production levels and to produce enzymes from non-industrial microorganisms in industrial organisms, such as species of *Aspergillus* and *Trichoderma*, as well as *Kluyveromyces lactis*, *S. cerevisiae*, *Yarrowia lipolytica*, and *B. licheniformis*. Over 50% of the market is provided by recombinant processes. Sixty per cent of the calf rennin (chymosin) used for cheese making in the United States is supplied by recombinant *E. coli* and the two lipases used industrially (i.e., *Humicola* lipase produced in *Aspergillus* and *Pseudomonas* lipase) are both recombinant. Heat-stable amylase from *B. licheniformis* has been made in a gene-amplified strain of the same species. Plant phytase (produced in recombinant *A. niger*) is used as a feed for 50% of all pigs in Holland. A 1000-fold increase in phytase production was achieved in *A. niger* by use of recombinant technology. Scientists at Novo Nordisk have isolated a very desirable lipase for use in detergents from a species of *Humicola*. For production purposes, the gene was cloned into *A. oryzae* where it produced 1000-fold more enzyme [199]. It became a commercial product for laundry cleaning, for interesterification of lipids and for esterification of glucosides producing glycolipids which have applications as biodegradable non-ionic surfactants for detergents, skincare products, contact lenses, and as food emulsifiers.

Virtually all laundry detergents now contain genetically engineered enzymes and much cheese is made with genetically engineered microbes. Over 60% of the enzymes used in the detergent, food, and starch processing industries are recombinant products [200].

The properties of many enzymes have been altered by genetic means. "Brute force" mutagenesis and random screening of microorganisms over the years have led to changes in pH optimum, thermostability, feedback inhibition, carbon source inhibition, substrate specificity, V_{max}, K_m, and K_i. This information has been exploited by the more rational techniques of protein engineering. Single changes in amino acid sequences have yielded similar types of changes in a large variety of enzymes. For example, a protease from *Bacillus stearothermophilus* was increased in heat tolerance from 86 °C to 100 °C, that is, it was made resistant to boiling! The enzyme was developed by site-directed mutagenesis [201]. Only eight amino acids had to be modified. Temperature stability at 100 °C was increased 340-fold and activity at lower temperature was not decreased. All eight mutations were far from the enzyme's active site.

An excellent method for improving enzymes is directed evolution (also known as applied molecular evolution or directed molecular evolution [202]. DNA shuffling, one type of directed evolution, has achieved significant improvement of catalytic activity, modified specificity, and improved stability of enzymes. This method of pooling and recombining parts of similar genes from different species or strains has yielded remarkable improvements in enzymes in a very short period of time. The procedure actually mimicks nature in that mutation, selection, and recombination were used to evolve highly adapted proteins, but it was much faster than nature. Enzyme activity has been improved up to 32 000-fold (TEM-1 β-lactamase), substrate specificity by 1000-fold (β-galactosidase), protein folding by 48-fold (green fluorescent protein), antibody activity by over 400-fold, expression by 100-fold, arsenate resistance by 40-fold, atrazine degradation by 80-fold, etc. [203].

Proteins from directed evolution work first went on the market in 2000. These were green fluorescent protein of Clonetech and Novo Nordisk's LipoPrime® lipase. Directed evolution provided β-glucosidase activity to a β-galactosidase, converted a β-glucuronidase into a β-galactosidase, gave phospholipase activity to a lipase, and converted an indole-3-glycerol-phosphate synthase into a phosphoribosylanthranilate isomerase [204].

1.12 Bioconversions

The first example of the use of a biological process to compete with a chemical process in the petrochemical industry was the production of acrylamide, made at 200 000 tons per year as a flocculant, a component of synthetic fibers, a soil conditioner, and a recovery agent in the petroleum industry [205]. The chemical process involving copper salt catalysis of the hydration of acrylonitrile had problems associated with it. A bioconversion using *Pseudomonas chlororaphis* B23 or *Rhodococcus rhodochrous* J1, in which nitrile hydratase was induced by methacrylamide and catalyzed the hydration, competed with the chemical reaction. The conversion yield was over 99.99%, was carried out at 10 °C, and the cells were used many times. The titer was 656 g/l after 10 h. Today, bioconversions are used widely in chemical manufacture. They have also become essential to the fine chemical industry because of the demand for single-isomer intermediates.

1.13 Vaccines

Protein antigens for vaccines have been made by cloning and expressing genes coding for surface antigens of viruses, bacteria and parasites. The first subunit vaccine on the market was that of hepatitis B virus surface antigen which was produced in yeast. In 1994, the first recombinant live veterinary vaccine was approved by the USDA. The VectorVax FP-N vaccine produced by Syntro Corp. used a fowlpox virus vector whose two disease-causing genes were deleted to produce a vaccine against both fowlpox virus and Newcastle disease.

1.14 Systems Microbiology

"Systems microbiology" emerged as a term and a scientific field to describe an approach that considers genome-scale and cell-wide measurements in elucidating processes and mechanisms carried out by microbial cells [206]. An expanded view of the microbial cell became possible due to the impressive advances in (i) genomics and in other "omic" techniques (e.g., proteomics, metabolomics) and

(ii) high-throughput technologies for measuring different classes of key intracellular molecules. For discovery of novel active secondary metabolites of commercial importance, genomics have provided a huge group of new targets against which natural products are being screened. The human genome has 30 000–35 000 genes, less than 50% having a putative function. These genes have the potential to produce over 100 000 proteins. Estimates of the number of proteins acting as useful targets range from 600 to 10 000.

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