Industrial Biotransformations

Second, Completely Revised and Extended Edition

Edited by
Andreas Liese, Karsten Seelbach, Christian Wandrey

WILEY-VCH Verlag GmbH & Co. KGaA

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Preface to the first edition

The main incentive in writing this book was to gather information on one-step biotransformations that are of industrial importance. With this collection, we want to illustrate that more enzyme-catalyzed processes have gained practical significance than their potential users are conscious of. There is still a prejudice that biotransformations are only needed in cases where classical chemical synthesis fails. Even the conviction that the respective biocatalysts are not available and, if so, then too expensive, unstable and only functional in water, still seems to be widespread. We hope that this collection of industrial biotransformations will in future influence decision-making of synthesis development in such a way that it might lead to considering the possible incorporation of a biotransformation step in a scheme of synthesis.

We therefore took great pains in explicitly describing the substrates, the catalyst, the product and as much of the reaction conditions as possible of the processes mentioned. Wherever flow schemes were available for publication or could be generated from the reaction details, this was done. Details of some process parameters are still incomplete, since such information is only sparingly available. We are nevertheless convinced that the details are sufficient to convey a feeling for the process parameters. Finally, the use of the products is described and a few process-relevant references are made.

We would go beyond the scope of this foreword, should we attempt to thank all those who were kind enough to supply us with examples. Of course, we only published openly available results (including the patent literature) or used personally conveyed results with the consent of the respective authors. We are aware of the fact that far more processes exist and that by the time the book is published, many process details will be outdated. Nonetheless, we believe that this compilation with its overview character will serve the above-mentioned purpose. This awareness could be augmented if the reader, using his or her experience, would take the trouble of filling out the printed worksheet at the end of this book with suggestions that could lead to an improvement of a given process or the incorporation of a further industrial process into the collection.

Requesting our industrial partners to make process schemes and parameters more accessible did not please them very much. Even so, we are asking our partners once again to disclose more information than they have done in the past. In

many instances, far more knowledge of industrial processes has been gained than is publicly available. Our objective is to be able to make use of these "well known secrets" as well. We would like to express our gratitude to all those who supplied us with information in a progress-conducive manner. Thanks also go to those who did not reject our requests completely and at least supplied us with a photograph in compensation for the actually requested information.

The book begins with a short historical overview of industrial biotransformations. Since the process order of the compilation is in accordance with the enzyme nomenclature system, the latter is described in more detail. We also include a chapter on reaction engineering to enable an easier evaluation of the processes. The main part of the book, as you would expect, is the compilation of the industrial biotransformations. The comprehensive index will allow a facile search for substrates, enzymes and products.

We sincerely hope that this book will be of assistance in the academic as well as the industrial field, when one wants to get an insight into industrial biotransformations. We would be very thankful to receive any correction suggestions or further comments and contributions. At least we hope to experience a trigger effect that would make it worth while for the readership, the authors and the editors to have a second edition succeeding the first.

We are indebted to several coworkers for screening literature and compiling data, especially to Jürgen Haberland, Doris Hahn, Marianne Hess, Wolfgang Lanters, Monika Lauer, Christian Litterscheid, Nagaraj Rao, Durda Vasic-Racki, Murillo Villela Filho, Philomena Volkmann and Andrea Weckbecker.

We thank especially Uta Seelbach for drawing most of the figures during long nights, as well as Nagaraj Rao and the "enzyme group" (Nils Brinkmann, Lasse Greiner, Jürgen Haberland, Christoph Hoh, David Kihumbu, Stephan Laue, Thomas Stillger and Murillo Villela Filho).

And last but not least we thank our families for their support and tolerance during the time that we invested in our so called 'book project'.

Preface to the second edition

After more than five years since the first edition of "Industrial Biotransformations" many new examples have become industrially relevant, others have lost importance. Therefore we had to enlarge the chapter "Processes" by 20%. If new information about the processes of the first edition was available, this information was incorporated. All processes were checked with respect to the literature (including patent literature). We have included all the valuable corrections suggestions or further comments and contributions of many readers. This might perhaps be of great importance for the reader of the second edition. Expecting that a first edition could not be perfect, we stated in the preface to the first edition: "We would be very thankful to receive any correction suggestions or further comments and contributions. At least we hope to experience a trigger effect that would make it worthwhile for the readership, the authors and the editors to have a second edition succeeding the first." We were astonished how carefully many readers checked the information given. So the reader of the second edition will not only have an enlarged chapter "Processes", but also an updated version with – me must admit – many useful corrections. The best criticism will be given by an experienced reader. We hope very much that the "old" and the "new" readers will realize that the second edition is more than a remake of the first edition.

Since the first edition was sold out earlier than we had expected, the publisher found it scientifically – and economically – more reasonable to have a second edition than to have a reprint of the first edition. Finally after all the additional work was done we agreed with the publisher. Perhaps it is worth to be mentioned that in the meantime also the first Chinese edition appeared.

The focus of the book is still the chapter "Processes". Nevertheless all the other chapters were carefully reevaluated. In the chapter "History of Industrial Biotransformations" we included a new part "History of Biochemical Engineering".

Entirely new is the chapter "Retrosynthetic Biocatalysis". The basic idea comes from classical organic chemistry, where a complex chemical structure is reduced to building blocks, which might even be commercially available. Similarly, one can find out which easily available building blocks can be used for industrial biotransformations. We hope that the reader will find this concept useful. Especially we hope that the classical organic chemistry becomes more part of biotechnology this way.

Entirely new is the chapter "Optimization of Industrial Enzymes by Molecular Engineering". The field of technical evolution of enzymes has become so important that we think it is justified to have a chapter devoted to the interesting and relevant findings in this field. There is no longer an "excuse" that there is no sufficiently stable, selective and active enzyme for a desired reaction. Technical evolution of enzymes has become similarly important as screening of enzymes from the environment. The chapter "Basics of Bioreaction Engineering" has been carefully checked and hopefully improved due to many valuable suggestions of the readers. We hope that bioreaction engineering will be understood as of equal importance for industrial biotransformations as enzyme engineering.

An additional short chapter is entitled "Quantitative Analysis of Industrial Biotransformation". Here the reader can find some quantitative information about the fact that it is a prejudice to believe that only hydrolases in water are useful for industrial biotransformations. Redox reactions and C-C-bond formations even in organic solvents or biphasic systems are also industrially relevant today.

Our original understanding of "Industrial Biotransformations" was a one step reaction of industrial relevance. This definition might become less clear in the future, because also two or three step biotransformations are or might be included. So it will become more and more difficult to distinguish an industrial biotransformation from a fermentation process. This is especially true in the age of "designer bugs", where a microorganism is first grown and than used as a more or less "non-growing catalyst" for industrial biotransformation. But we should not bother too much with definitions. Our aim was and is to show that biotransformations are of great importance in the academic and industrial fields. Since the first edition the field of "White Biotechnology" (formally known as Industrial Microbiology) has developed a lot. A quantative understanding of complex microbial systems by means of the "polyomics" techniques (genomics, proteomics, and metabolomics) has improved so much that we can expect recombinant pathways for industrial biotransformations used in a biocatalysis under non-natural conditions. We can foresee that the technical evolution not only of biocatalyst but also of bioprocesses will lead to many more industrial biotransformations. Thus, sooner or later we expect the burden/pleasure that we will have to prepare a third edition of "Industrial Biotransformations".

We would like to ask the reader again to help us with "correction suggestions or further comments and contributions". The best compensation for all the work the author of a book can get is the feeling that it is read by colleagues who understand the subject.

Last but not least we would like to mention in addition to the many coworkers who have contributed to the first edition, now the additional valuable contributions of many more who helped us to prepare the second edition, especially Karl-Heinz Drauz, Kurt Faber, Katja Goldberg, Udo Kragl, Peter Stahmann, Trevor Laird, John Villadsen, Ulrike Zimmermann. Especially we thank our families for their support during the time that we invested in the second edition of this book.

Dezember 2005

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1
History of Industrial Biotransformations – Dreams and Realities

Durda Vasic-Racki

Throughout the history of mankind, microorganisms have been of enormous social and economic importance. Without even being aware of their existence, very early on in history man was using them in the production of food and beverages. The Sumerians and Babylonians were practising the brewing of beer before 6000 BC, references to wine making can be found in the Book of Genesis and the Egyptians used yeast for baking bread. However, knowledge of the production of chemicals such as alcohols and organic acids through fermentation is relatively recent and the first reports in the literature only appeared in the second half of the 19th century. Lactic acid was probably the first optically active compound to be produced industrially by fermentation. This was accomplished in the USA in 1880 [1]. In 1921, Chapman reviewed a number of early industrial fermentation processes for organic chemicals [2].

In the course of time, it was discovered that microorganisms could modify certain compounds by simple, chemically well defined reactions, which were further catalyzed by enzymes. Nowadays, these processes are called "biotransformations". The essential difference between fermentation and biotransformation is that there are several catalytic steps between the substrate and the product in a fermentation while there are only one or two in a biotransformation. The distinction is also in the fact that the chemical structures of the substrate and the product resemble one another in a biotransformation, but not necessarily in a fermentation.

1.1
From the "Flower of Vinegar" to Recombinant *E. Coli* – The History of Microbial Biotransformations

The story of microbial biotransformations is closely associated with vinegar production which dates back to around 2000 years BC.

Vinegar production is perhaps the oldest and best known example of microbial oxidation, which can illustrate some of the important developments in the field of biotransformations by living cells (Fig. 1.1).

Since ancient times, man has wanted to see things that are far smaller than can be perceived with the naked eye. In the 16th century this led to the construction of a magnifier

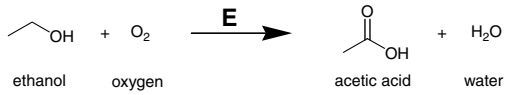


Fig. 1.1 Vinegar production.

consisiting of a single convex lens, and this, in turn, led eventually to the development of the microscope (Fig. 1.2). Antony von Leeuwenhoek (1632–1723) became the first person, or microscopist [3], to make and use a real microscope. He described microorganisms including bacteria, algae and protozoa in fresh water (Fig. 1.3). In fact he constructed a total of 400 microscopes during his lifetime. Subsequently, the compound microscope system was invented in the 17th century. This type of microscope, incorporating more than one lens, has made tremendous contributions to the progress of science. Using this microscope Hooke (1635–1703) discovered the fact that living things are composed of cells, and later on Pasteur, among others, discovered yeast fungus. The microscope has possibly had a greater impact on the development of knowledge than any other scientific instrument in history [4]. The discovery of new microscopic life was the starting point for experimental biology as a basis for the development of the biotransformations.

A prototype bioreactor with immobilized bacteria has been known in France since the 17th century. The oldest bioreactor to use immobilized living microorganisms, a so-called generator, was developed in 1823 [5, 6]. Even today, acetic acid is still known as "vinegar" if it is obtained by oxidative fermentation of ethanol-containing solutions by acetic acid bacteria [7].

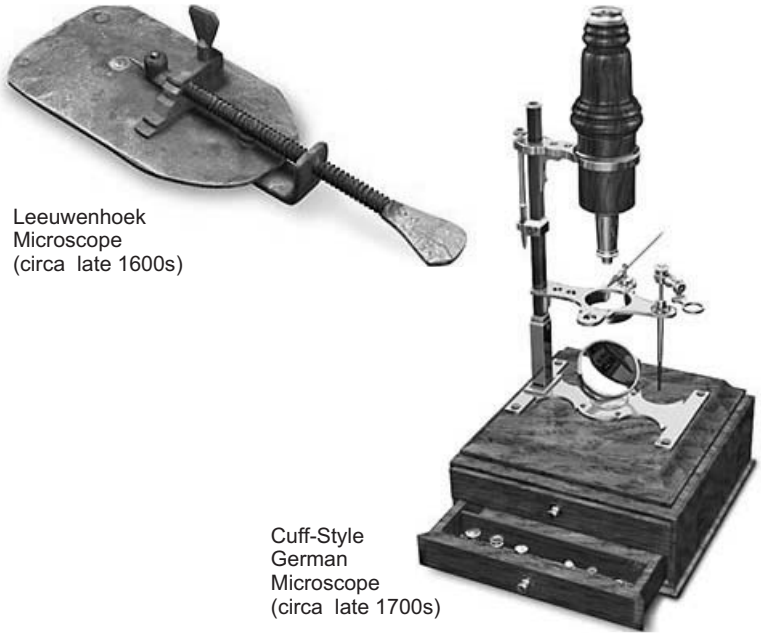


Fig. 1.2 Historical microscopes (photographs courtesy of Michael W. Davidson).

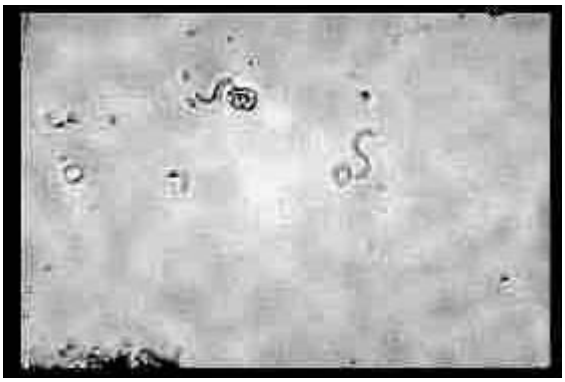


Fig. 1.3 Spiral bacteria (photograph courtesy of Michael W. Davidson).

In 1858, Pasteur [8] was the first to demonstrate the microbial resolution of tartaric acid. He performed fermentation of the ammonium salt of racemic tartaric acid, mediated by the mold *Penicillium glaucum*. The fermentation yielded (–)-tartaric acid (Fig. 1.4).

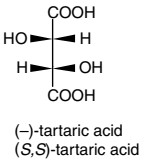


Fig. 1.4 Pasteur's product of the first resolution reaction.

This was also the first time that a method was used where the microorganisms degraded one enantiomer of the racemate while leaving the other untouched.

In 1862, Pasteur [9] investigated the conversion of alcohol into vinegar and concluded that pellicle, which he called "the flower of vinegar", "served as a method of transport for the oxygen in air to a multitude of organic substances".

In 1886 Brown confirmed Pasteur's findings and gave the causative agent in vinegar production the name *Bacterium xylinum*. He also found that it could oxidize propanol to propionic acid and mannitol to fructose (Fig. 1.5) [10].

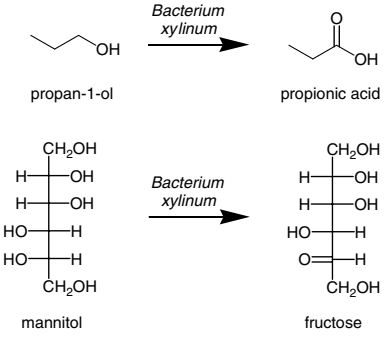
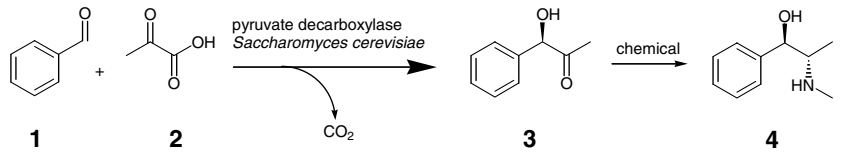


Fig. 1.5 Reactions catalyzed by *Bacterium xylinum*, the vinegar biocatalyst.

In 1897 Buchner [11] reported that cell-free extracts prepared by grinding yeast cells with sand could carry out alcoholic fermentation reactions (breaking down glucose into ethanol and carbon dioxide) in the absence of living cells. This initiated the use of resting cells for biotransformations.

In 1921 Neuberg and Hirsch [12] discovered that the condensation of benzaldehyde with acetaldehyde in the presence of yeast forms optically active 1-hydroxy-1-phenyl-2-propanone (Fig. 1.6).



1 = benzaldehyde
2 = 2-oxo-propionic acid
3 = 1-hydroxy-1-phenylpropan-2-one
4 = 2-methylamino-1-phenylpropan-1-ol

Fig. 1.6 L-Ephedrine production.

DEUTSCHES REICH

AUSGEGEBEN AM
13. APRIL 1932

REICHSPATENTAMT

PATENTSCHRIFT

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Tag der Bekanntmachung über die Erteilung des Patents: 24. März 1932

Knoll A.-G. Chemische Fabriken in Ludwigshafen a. Rh., Dr. Gustav Hildebrandt und Dr. Wilfrid Klavehn in Mannheim

Verfahren zur Herstellung von l-l-Phenyl-2-methylaminopropan-1-ol

Patentiert im Deutschen Reiche vom 9. April 1930 ab

Racemisches 1-Phenyl-2-methylaminopropan-1-ol kann bereits nach verschiedenen Verfahren synthetisch hergestellt werden (vgl. z. B. Nagai u. Kanao, Annalen 470 [1929], S. 157; Patentschrift 469 782; Seita u. Keil, Ber. 62 [1929], S. 1142 ff.; Patentschrift 524 806).

Das racemische 1-Phenyl-2-methylaminopropan-1-ol läßt sich nach bekannten Verfahren (vgl. Nagai u. Kanao, Annalen 470 [1929], S. 157; britische Patentschrift 297 385) in seine optischen Isomeren spalten. Das bei der Spaltung entstehende l-1-Phenyl-2-methylaminopropan-1-ol ist identisch mit dem natürlichen Ephedrin und wird neuerdings therapeutisch erfolgreich verwendet.

Bisher ist jedoch kein Verfahren bekannt geworden, nach welchem l-1-Phenyl-2-methylaminopropan-1-ol auf unmittelbarem Wege dargestellt werden kann.

Es wurde nun gefunden, daß man mit guten Ausbeuten unmittelbar zum l-1-Phenyl-2-methylaminopropan-1-ol gelangt, wenn man links drehendes Phenylpropanolon (Neuberg, Biochem. Zeitschrift 115 [1921], S. 282 ff., und 128 [1922], S. 610 ff.) in Gegenwart von Methylamin der Reduktion unterwirft.

Die Bildung von l-1-Phenyl-2-methylaminopropan-1-ol war keineswegs vorauszusehen, da bekanntlich Abwandlungen von optisch aktiven Verbindungen nicht notwendig zu Verbindungen von optisch gleicher Drehungsrichtung führen müssen, sondern ebensohäufig zu solchen der entgegengesetzten Drehungsrichtung führen können (so entsteht z. B. l-Mandelsäure aus d-Benzaldehydcyanhydrin bei der Verseifung).

Ferner ist hervorzuheben, daß in der vorliegenden Erfindung eine asymmetrische Synthese vorliegt, bei welcher wiederum nicht vorauszusehen war, welche von den möglichen Konfigurationen entstehen würde. Es stand zu erwarten, daß sowohl d- oder l- oder dl-1-Phenyl-2-methylaminopropan-1-ol als auch d- oder l- oder dl-Pseudo-1-Phenyl-2-methylaminopropan-1-ol oder endlich ein Gemisch von mehreren dieser Komponenten entstehen würde. Daß bei der Reduktionskondensation des l-Phenylpropanolons mit Methylamin fast ausschließlich l-1-Phenyl-2-methylaminopropan-1-ol entsteht, war daher durchaus überraschend.

Die Erhaltung der optischen Aktivität war auch deswegen überraschend, weil auf Grund der Neubergschen Beobachtung (Biochem. Zeitschrift 128 [1922], S. 613) l-Phenylpropanolon sich bereits in verdünnt alkalischer Lösung in kurzer Zeit racemisiert. Da bei dem Verfahren der vorliegenden Erfindung die Reduktion in alkalischer Lösung stattfindet, war vorwiegend mit der Bildung von racemischen Basen zu rechnen.

Das Verfahren stellt eine neue Methode dar, um das links drehende Phenylpropanolon in Form von l-1-Phenyl-2-methylaminopropan-1-ol nutzbringend zu verwerten.

Diese unmittelbare Synthese des l-1-Phenyl-2-methylaminopropan-1-ols hat ferner den Vorzug, daß kein therapeutisch wertloses d-1-Phenyl-2-methylaminopropan-1-ol anfällt, wie es bei den bekannten Spaltungsverfahren des Racemkörpers der Fall ist.

Beispiel 1.

120 g des durch Ätherauszug gewonnenen phenylpropanolonhaltigen Gärungsproduktes (vgl. Biochem. Zeitschrift 115 [1921], S. 282 ff.) läßt man ohne weitere Reinigung in eine Lösung von 10 g Methylamin in 500 ccm Äther in Gegenwart von 20 g aktiviertem Aluminium unter Rühren im Verlaufe von 2 Stunden eintropfen. Gleichzeitig läßt man 20 bis 30 g Wasser tropfenweise zufließen. Die sofort heftig einsetzende Umsetzung wird zeitweilig durch Kühlung gemäßigt. Nach beendigter Reduktion wird der filtrierten ätherischen Lösung die entstandene optisch aktive Base mit verdünnter Säure entzogen. Die Aufarbeitung erfolgt in bekannter Weise.

Man erhält das Hydrochlorid des l-1-Phenyl-2-methylaminopropan-1-ols vom F. 214°, welches die aus der Literatur bekannte Linksdrehung zeigt. Die Ausbeute beträgt je nach Art des verwendeten Ausgangsstoffes 25 bis 45 g Hydrochlorid.

Beispiel 2.

360 g des in Beispiel 1 verwendeten phenylpropanolonhaltigen Ätherauszuges werden unter vermindertem Druck destilliert. 300 g der bei 100 bis 150° unter 14 mm Druck übergehenden Fraktion werden in Gegenwart von kolloidalem Platin (70 ccm 1%ige Lösung) und 85 g 33%iger Methylaminlösung der katalytischen Reduktion unterworfen. Es ist vorteilhaft, etwas Äther zuzusetzen. Nach Beendigung der Wasserstoffaufnahme wird die ätherische Lösung mit Salzsäure ausgeschüttelt und das l-1-Phenyl-2-methylaminopropan-1-ol in bekannter Weise abgetrennt.

Das Hydrochlorid schmilzt bei 214° und zeigt die aus der Literatur bekannte Linksdrehung. Die Ausbeute an Hydrochlorid beträgt 110 g.

Beispiel 3.

100 g nach Neuberg (Biochem. Zeitschrift 128 [1922], S. 611) abgetrenntes l-1-Phenylpropan-1-ol-2-on werden in 200 ccm Äther gelöst, mit 75 g 33%iger Methylaminlösung versetzt und etwa eine halbe Stunde lang geschüttelt. Hierbei findet unter Wärmeentwicklung Kondensation statt. Anschließend wird in Gegenwart von 70 ccm 1%iger kolloidaler Platinlösung mit Wasserstoff reduziert.

Die Aufarbeitung geschieht nach Beispiel 2.

Das Hydrochlorid des l-1-Phenyl-2-methylaminopropan-1-ols kristallisiert aus Alkohol in derben Prismen vom F. 214 bis 216°. Der F. der freien Base liegt bei 40°.

Patentansprüche:

1. Verfahren zur Darstellung von l-1-Phenyl-2-methylaminopropan-1-ol, dadurch gekennzeichnet, daß man links drehendes l-Phenylpropan-1-ol-2-on mit Methylamin kondensiert und das Kondensationsprodukt gleichzeitig oder nachträglich mit Reduktionsmitteln, wie aktiviertem Aluminium in Gegenwart von Wasser oder Wasserstoff in Gegenwart eines Platinkatalysators, behandelt.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß man — unter Umgehung der Reindarstellung des l-Phenylpropanolons — Destillate oder Auszüge aus l-Phenylpropanolon enthaltenden Gemischen verwendet, wie sie z. B. bei der Vergärung von Zuckern oder von zuckerhaltigen Produkten in Gegenwart von Benzaldehyd entstehen.

Fig. 1.7 Knoll's patent of 1930.

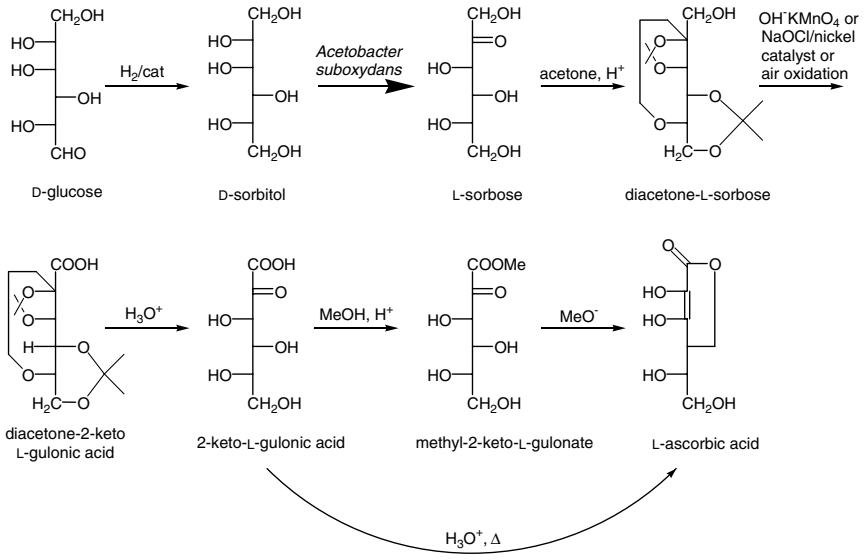

Fig. 1.8 Reichstein–Grüssner synthesis of vitamin C (L-ascorbic acid).

The compound obtained was later chemically converted into L-(–)ephedrine by Knoll AG, Ludwigshafen, Germany in 1930 (Fig. 1.7) [13].

The bacterium *Acetobacter suboxydans* was isolated in 1923 [14]. Its ability to carry out limited oxidations was utilized in a highly efficient preparation of L-sorbose from D-sorbitol (Fig. 1.8).

L-Sorbose became important in the mid-1930s as an intermediate in the Reichstein–Grüssner synthesis of L-ascorbic acid [15].

In 1953, Peterson at al. [16] reported that *Rhizopus arrhius* could convert progesterone into 11α-hydroxyprogesterone (Fig. 1.9), which was used as an intermediate in the synthesis of cortisone.

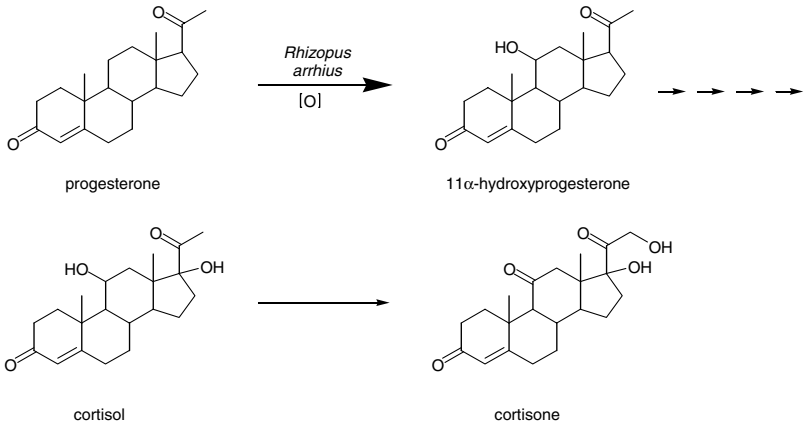

Fig. 1.9 Microbial 11α-hydroxylation of progesterone.

This microbial hydroxylation simplified and considerably improved the efficiency of the multi-step chemical synthesis of corticosteroid hormones and their derivatives. Although the chemical synthesis [17] (Fig. 1.10) from deoxycholic acid developed at Merck, Germany, was workable, it was recognized that it was complicated and uneconomical: 31 steps were necessary to obtain 1 kg of cortisone acetate from 615 kg of deoxycholic acid. The microbial 11α-hydroxylation of progesterone rapidly reduced the price of cortisone from \$200 to \$6 per gram. Further improvements have led to a current price of less than \$1 per gram [18].

In the 1950s the double helix structure and the chemical nature of RNA and DNA – the genetic code for heredity – were discovered by Watson and Crick [19]. Beadle and Tatum [20] received the Nobel Prize in 1958 for concluding that the characteristic function of the gene was to control the synthesis of a particular enzyme. They exposed the red bread mold, *Neurospora crassa*, to X-rays and studied the altered nutritional requirements of the mutants thus produced. These experiments enabled them to conclude that each gene determined the structure of a specific enzyme which, in turn, allowed a single chemical reaction to proceed. A basic hypothesis arose out of this work: one gene specifies the production of one enzyme thus the "one gene–one enzyme" hypothesis. Lederberg [21] shared the Nobel Prize with Beadle and Tatum in 1958 for his discoveries concerning genetic recombination and the organization of the genetic material of bacteria. The Beadle, Tatum and Lederberg discoveries can be regarded as milestones among the main scientific achievements of the 20th century. This led to the synthesis of recombinant DNA and gave a fillip to genetic engineering in the 1970s. In 1973, Cohen and Boyer [22] discovered recombinant DNA technology. They observed that genes from any biological species could be propagated and cloned in foreign cells by linking them to DNA molecules that posses the capacity to replicate in the intended host. Such developments quickly made the recombinant DNA technology part of industrial microbial transformations. Application of this technology for the production of small molecules began in 1983. Ensley et al. [23] reported on the construction of a strain of *E.coli* that excreted indigo, one of the oldest known dyes. They found that the entire pathway for conversion of napthalene into salicylic acid is encoded by the genes of *Pseudomonas putida*. These genes can be expressed in *E. coli*. Their results led to the unexpected finding that a subset of these genes was also responsible for the microbial production of indigo. Moreover, they have shown that indigo formation was a property of the dioxygenase enzyme system that forms *cis*-dihydrodiols from aromatic hydrocarbons. Finally, they proposed a pathway for indigo biosynthesis in a recombinant strain of *E. coli* (Fig. 1.11).

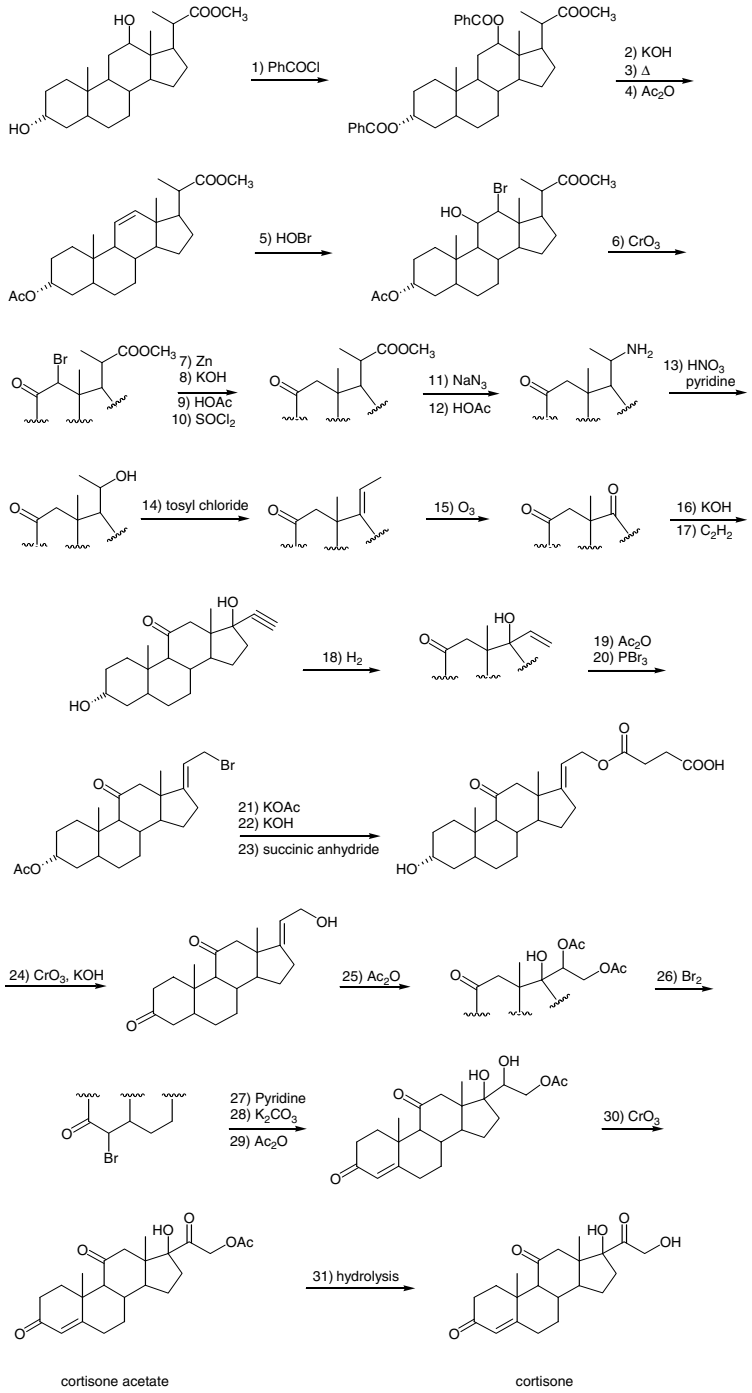

Fig. 1.10 Chemical synthesis of cortisone.

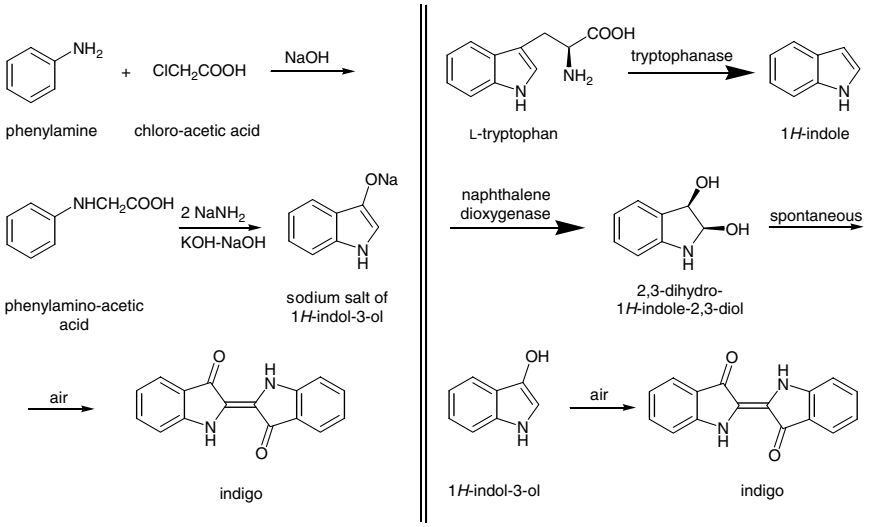

Fig. 1.11 Comparison of the chemical and biological routes to indigo.

Genencor Internationaol is developing a commercially competitive biosynthetic route to indigo using recombinant *E. coli*, which can synthesize indigo directly from glucose [24]. At the neutral pH of the fermentation the indigo precursor indoxyl yields isatin as a significant by-product. An enzyme that hydrolyzes isatin to isatic acid has been identified. After cloning and incorporating the new enzyme in the production strain, the indigo product performed equally well as the indigo produced chemically [25].

In 1984 Novozymes developed the first enzyme from a genetically modified organism for use in the starch industry – a maltogenic amylase, still marketed today under the name Maltogenase® [26].

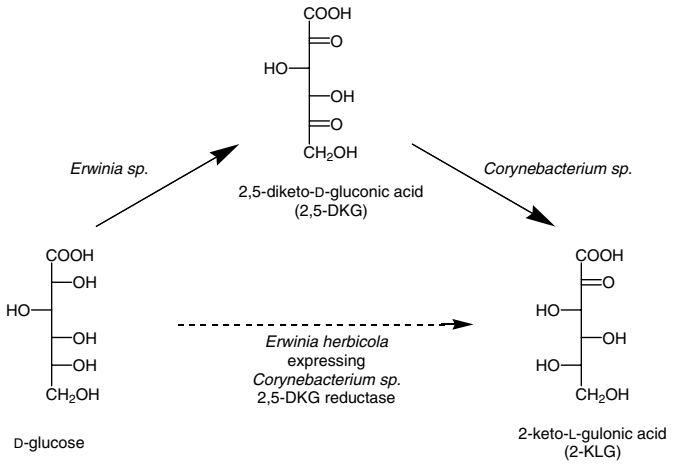

Fig. 1.12 Biosynthesis of 2-keto-L-gulonic acid.

Anderson et al. [27] reported in 1985 on the construction of a metabolically engineered bacterial strain that was able to synthesize 2-keto-L-gulonic acid (Fig. 1.12), a key intermediate in the production of L-ascorbic acid (vitamin C).

BASF, Merck and Cerestar have built a ketogulonic acid plant in Krefeld, Germany. The operation started up in 1999. They developed a new fermentation route from sorbitol directly to ketogulonic acid [28]. This method is probably similar to that described in 1966 [29].

The vision of manufacturing L-ascorbic acid directly by fermentation without the need to isolate 2-keto-L-gulonic acid (2-KLG) has remained elusive. Nevertheless, efforts to this end are ongoing at Genencor International [30].

The Cetus Corporation (Berkeley, CA, USA) bioprocess for converting alkenes into alkene oxides emerged in 1980 [31]. This bioprocess appeared to be very interesting, because of the possibility of replacing the energy-consuming petrochemical process.

There were high hopes that the development of recombinant DNA technology would speed up technological advances. Unfortunately, there is still a great deal of work to be done on the development and application of bioprocesses before the commercial production of low-cost chemicals becomes feasible [32]. The development of some of the flagship bioprocesses of today took between 10 and 20 years: the development of the acrylamide process took 20 years and the Lonza process for L-carnitine 15 years [33]. However, today even the traditional chemical companies such as Dow Chemicals, DuPont, Degussa-Hüls AG, etc., under pressure from investors and because of technological advances, are trying to use microbial or enzymatic transformations in their production processes. This is because they need to establish whether natural feedstocks can provide more advantages than crude oil. One only needs to compare the cost of a barrel of oil to that of corn starch to see that the latter is considerably cheaper [28].

Tepha Inc. (Cambridge, MA, USA) currently produces poly-4-hydroxybutyrate (known commercially as PHA4400) (Fig. 1.13) for medical applications, using a proprietary transgenic fermentation process that has been specifically engineered to produce this homopolymer. During the fermentation process, poly-4-hydroxybutyrate accumulates inside the fermented cells as distinct granules. The polymer can be extracted in a highly pure form from the cells at the end of the fermentation process. The heart of the process is the genetically engineered *Escherichia coli* K12 microorganism, incorporating new biosynthetic pathways to produce poly-4-hydroxybutyrate [34].

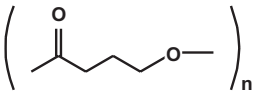

Fig. 1.13 Chemical structure of poly-4-hydroxybutyrate.

A range of polyhydroxyalkanoates with 0–24% hydroxyvalerate have been produced under the trade name of "Biopol" by Zeneca Bio Products and other manufacturers [35]. However, the polyhydroxyalkanoates production price is way above the market price of conventional plastics ($16 per kilogram for "Biopol" against $1 per kilogram for oil-derived plastics). Potentially, the production cost can be lowered by process scale-up, to around $8 per kilogram and by use of recyclable waste material as the substrates [35].

More recently, researchers from the company DSM succeeded in combining enzymatic ring opening polymerization and chemical nitroxide mediated living free radical polymerization. This genuine one-pot reaction is a method for the synthesis of block copolymers in a metal-free fashion. After proving the principle they are extending their chemo-enzymatic polymerization approach to obtain new functional polymers based on new raw materials, and to develop the technology further towards a kinetic resolution polymerization [36].

Acrylamide is one of the most important commodity chemicals with a global consumption of about 200 000 tonnes per year. It is required in the production of various polymers for use as flocculants, additives or for petroleum recovery. In conventional syntheses, copper salts are used as catalysts in the hydration of nitriles. However, this is rather disadvantageous as the preparation of the catalysts is fairly complex. In addition, it is difficult to regenerate the used catalyst and to separate and purify the acrylamide formed. Furthermore, as acrylamides are readily polymerized, their production under moderate conditions is highly desirable. In contrast to the conventional chemical process, there is no need to recover unreacted acrylonitrile in the enzymatic process, because the conversion and yield of the enzymatic hydration process are almost 100%. The removal of the copper ions from the product is no longer necessary. Overall, the enzymatic process – being carried out below 10 °C under mild reaction conditions and requiring no special energy source – proves to be simpler and more economical. The immobilized cells are used repeatedly and a very pure product is obtained. The enzymatic process, which was first implemented in 1985, is already producing about 6000 tonnes of acrylamide per year for Nitto [37, 38]. The use of a biotacalyst for the production of acrylamide may be not the first case of biotransformation being used as part of a biotechnological process in the petrochemical industry. However, it is the first example of the successful introduction of an industrial biotransformation process for the manufacture of a commodity chemical (Fig. 1.14).

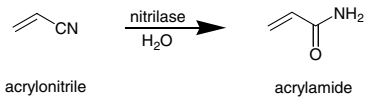

Fig. 1.14 Acrylamide synthesis.

Improvements to the production of 1,3-propanediol, a key component of an emerging polymer business, have been realized. By utilizing genes from natural strains that produce 1,3-propanediol from glycerol, metabolic engineering has enabled the development of a recombinant strain that utilizes the lower cost feedstock D-glucose [39].

Some representative industrial microbial transformations are listed in Table 1.1.

Tab. 1.1 Some representative industrial biotransformations catalyzed by whole cells.

Product	Biocatalyst	Operating since	Company
vinegar	bacteria	1823	various
L-2-methylamino-1-phenylpropan-1-ol	yeast	1930	Knoll AG,Germany
L-sorbose	Acetobacter suboxydans	1934	various
prednisolone	Arthrobacter simplex	1955	Shering AG, Germany
L-aspartic acid	Escherichia coli	1958	Tanabe Seiyaku Co., Japan
7-ADCA	Bacillus megaterium	1970	Asahi Chemical Industry, Japan
L-malic acid	Brevibacterium ammoniagenes	1974	Tanabe Seiyaku Co., Japan
D-p-hydroxyphenylglycine	Pseudomonas striata	1983	Kanegafuchi, Chemical Co., Japan
acrylamide	Rhodococcus sp.	1985	Nitto Chemical Ltd, Japan
D-aspartic acid and L-alanine	Pseudomonas dacunhae	1988	Tanabe Seiyaku Co., Japan
L-carnitine	Agrobacterium sp.	1993	Lonza, Czech.Rep.
2-keto-L-gulonic acid	Acetobacter sp.	1999	BASF, Merck, Cerester, Germany

1.2 From Gastric Juice to SweetzymeT – The History of Enzymatic Biotransformations

Enzymes had been in use for thousands of years before their nature became gradually understood. No one really knows when a calf stomach was used for the first time as a catalyst in the manufacture of cheese.

As early as 1783, Spallanzani showed that gastric juice secreted by cells could digest meat *in vitro*. In 1836, Schwan called the active substance pepsin [40]. The French scientist Payen [41] isolated an enzymatic complex from malt in 1833, naming it "diastase". Diastase, the enzyme that catalyzes the breakdown of starch into glucose, was the first enzyme to be discovered. In 1876, Kühne (Fig. 1.15) presented a paper to the Heidelberger Natur-Historischen und Medizinischen Verein, suggesting that such non-organized ferments should be called *e n z y m e s* [42]. At that time two terms were used: "organized ferment", such as the cell-free yeast extract from Büchner, and "unorganized ferment", such as the gastric juice secreted by cells. Today the terms "intracellular" and "extracellu-

lar" are used. Kühne also presented some interesting results from his experiments with trypsin. The word "enzyme" comes from Greek for "in yeast" or "leavened" [43].

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Ueber das Verhalten verschiedener organisirter und sog. ungeformter Fermente.

Ueber das Trypsin (Enzym des Pankreas).

Von **W. Kühne.**

1876

Ueber das Verhalten verschiedener organisirter und sog. ungeformter Fermente.

Sitzung am 4. Februar 1876.

Hr. W. Kühne berichtet über das Verhalten verschiedener organisirter und sog. ungeformter Fermente. Um Missverständnissen vorzubeugen und lästige Umschreibungen zu vermeiden schlägt Vortragender vor, die ungeformten oder nicht organisirten Fermente, deren Wirkung ohne Anwesenheit von Organismen und ausserhalb derselben erfolgen kann, als *Enzyme* zu bezeichnen. — Genauer untersucht wurde besonders das Eiweiss verdauende Enzym des Pankreas, für welches, da es zugleich Spaltung der Albuminkörper veranlasst, der Name *Trypsin* gewählt wurde. Das Trypsin vom Vortr. zuerst dargestellt und zwar frei von durch dasselbe noch verdaulichen und zersetzbaren Eiweissstoffen, verdaut nur in alkalischer, neutraler, oder sehr schwach sauer reagirender Lösung. Dasselbe wird durch nicht zu kleine Mengen Salicylsäure, welche das Enzym in bedeutenden Quantitäten löst, bei 40° C. gefällt, ohne dabei seine specifische Wirksamkeit zu verlieren. Wird die Fällung in Sodalösung von 1 pCt. gelöst, so verdaut sie höchst energisch unter Bildung von Pepton, Leucin, Tyrosin u. s. w. Nur übermässiger Zusatz von Salicylsäure bis zur Bildung eines dicken Krystallbreies vernichtet die enzymotischen Eigenschaften. Dies Verhalten war kaum zu erwarten, seit Kolbe und J. Müller die hemmende, selbst vernichtende Wirkung kleiner Mengen Salicylsäure auf einige Enzyme hervorgehoben hatten. Die Beobachtungen des Vortr., der ausser dem Trypsin noch das Pepsin eingehender untersuchte, stehen jedoch mit den Angaben von J. Müller, nach welchen Salicylsäure bei einem Gehalte der

Fig. 1.15 W.F. Kühne [42].

Microorganisms synthesize numerous enzymes, each one having a specific function. Intracellular enzymes operate inside the cell in a protected and highly structured environment, while extracellular enzymes are secreted from the cell, thus working in the medium surrounding the microorganism.

The commercial usage of extracellular microbial enzymes started in the West around 1890, thanks to the Japanese entrepreneur Takamine. He settled down in the USA and started an enzyme factory based on Japanese technology. The principal product was called takadiastase. This was a mixture of amylolytic and proteolytic enzymes prepared by cultivation of *Aspergillus oryzae*. In France, Boidin and Effront developed bacterial enzymes in 1913. They found that the hay bacillus, *Bacillus subtilis,* produces an extremely heat-stable α-amylase when grown in still cultures on a liquid medium prepared by extraction of malt or grain [44].

In 1892, in a study of the rate of fermentation of sucrose in the presence of yeast, the British chemist Brown found that the rate seemed to be independent of the amount of sucrose present [45]. He later suggested that this result could be explained if the invertase molecules present in the yeast formed an addition complex with sucrose [46]. This was the first time that the existance of an enzyme–substrate complex had been deduced from the kinetics of an enzyme reaction [47].

As part of his studies on sugars, in 1894 Emil Fischer [48, 49] observed that the enzyme known as emulsin catalyzes the hydrolysis of β-methyl-D-glucoside, while the enzyme known as maltase is active towards the α-methyl-D-glucoside substrate (Fig. 1.16).

This led Fischer to suggest his famous "lock-and-key" theory of enzyme specificity, which he described in his own word as follows: "To use a picture, I would say that enzyme and the glucoside must fit into each other like a lock and key, in order to effect a chemical reaction on each other" [1].

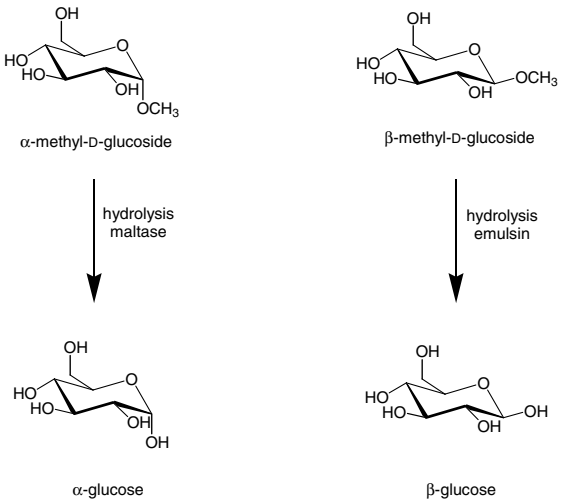

Fig. 1.16 Emil Fischer's substrates.

In 1913, the German biochemist Leonor Michaelis and his Canadian assistant Maud Leonara Menten published a theoretical consideration of enzymatic catalysis. This consideration envisaged the formation of a specific enzyme–substrate complex which further decomposed and yielded the product with the release of the enzyme. They had observed that the effect noted by Brown [45] is only observed at higher concentrations of the substrate. At lower concentrations the rate becomes proportional to the concentration of the substrate. This led to the development of the Michaelis–Menten equation to describe the typical saturation kinetics observed with purified enzymes and single substrate reactions [50]. Some years later a more general formulation of the Michaelis–Menten equation was given by Briggs and Haldane [51]. They pointed out that the Michaelis assumption that an equilibrium exists between the enzyme, substrate and enzyme–substrate complex is not always justified, and should be replaced by an assumption that the enzyme–substrate complex is not necessarily present at equilibrium but in a steady state. With the purification and crystallization of proteins in the 1920s, enzyme kinetics entered a new phase. It became possible to study the interactions between enzyme molecules and their substrate in much more detail. The British physical chemist Butler was the first to carry out kinetic studies with a pure enzyme, trypsin [52].

By 1920, about a dozen enzymes were known, none of which had been isolated [53]. Then, in 1926, Sumner [54] crystallized urease from jack bean, *Canavalia ensiformis,* and announced that it was a simple protein. He later, in 1946, received the Nobel Prize for his work with the enzyme urease, extracted from the jack bean. Urease is an enzyme that catalyzes the conversion of urea into ammonia and carbon dioxide (Fig. 1.17).

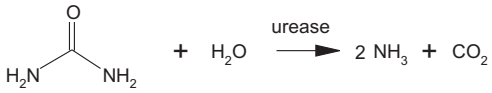

Fig. 1.17 The conversion of urea into ammonia and carbon dioxide.

Northrop and his colleagues [40] soon supported Sumner's claim that an enzyme could be a simple protein. They isolated many proteolytic enzymes, beginning with pepsin in 1930 by applying classic crystallization experiments. By the late 1940s many enzymes were available in a pure form and in sufficient amounts for investigations of their chemical structure to be carried out. Currently, more than 3000 enzymes have been catalogued [55]. The ENZYME data bank contains information related to the nomenclature of enzymes [56]. The current version contains 4309 entries. It is available through the ExPASy WWW server (http://www.expasy.org/enzyme/). Several hundred enzymes can now be obtained commercially [57].

In 1950 there was still no evidence that a given protein had a unique amino acid sequence. Lysosyme was the first enzyme to have its tertiary structure defined (Fig. 1.18), this was in 1966 with the help of X-ray crystallography [58].

However, ribonuclease A was one of the first enzymes to be prepared on a laboratory scale using organic chemistry methods. In 1969, Gutte and Merrifield synthesized its whole sequence in 11 931 steps [59].

By 1970, the complete molecular structures of several enzyme had been established and plausible reaction mechanisms could then be discussed [40].