Part I
Principles of Enzyme Catalysis

Enzyme Catalysis in Organic Synthesis, Third Edition. Edited by Karlheinz Drauz, Harald Gröger, and Oliver May.

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Introduction – Principles and Historical Landmarks of Enzyme Catalysis in Organic Synthesis

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1.1
General Remarks

Enzyme catalysis in organic synthesis – behind this term stands a technology that today is widely recognized as a first choice opportunity in the preparation of a wide range of chemical compounds. Notably, this is true not only for academic syntheses but also for industrial-scale applications [1]. For numerous molecules the synthetic routes based on enzyme catalysis have turned out to be competitive (and often superior!) compared with classic chemical as well as chemocatalytic synthetic approaches. Thus, enzymatic catalysis is increasingly recognized by organic chemists in both academia and industry as an attractive synthetic tool besides the traditional organic disciplines such as "classic" synthesis, metal catalysis, and organocatalysis [2].

By means of enzymes a broad range of transformations relevant in organic chemistry can be catalyzed, including, for example, redox reactions, carbon–carbon bond forming reactions, and hydrolytic reactions. Nonetheless, for a long time enzyme catalysis was not realized as a first choice option in organic synthesis. Organic chemists did not use enzymes as catalysts for their envisioned syntheses because of observed (or assumed) disadvantages such as narrow substrate range, limited stability of enzymes under organic reaction conditions, low efficiency when using wild-type strains, and diluted substrate and product solutions, thus leading to non-satisfactory volumetric productivities. However, due to tremendous progress in enzyme discovery, enzyme engineering, and process development, in recent years numerous examples of organic syntheses with (tailor-made) enzymes have been developed that avoid these disadvantages.

The achievements in microbiology and molecular biology have already led to a broad range of widely applicable enzymes showing an excellent performance. Today such enzymes are typically prepared in a highly attractive economic fashion by high-cell density fermentation, and can be used in the form of tailor-made recombinant whole-cell catalysts. This economically attractive access to highly efficient (bio-) catalysts enables an excellent opportunity to realize the development of attractive organic synthetic processes with enzymes as catalysts.

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Benefiting from these achievements in microbiology and molecular biology, organic chemists have applied these tailor-made biocatalysts (as isolated enzymes or recombinant microorganisms, so-called "designer cells") very successfully in a broad range of organic syntheses. Many of those synthetic examples have been found to be suitable even for industrial-scale productions and turned out to be superior to competitive "classic" chemical or chemocatalytic approaches. In particular this is true for the production of chiral compounds used as drug intermediates.

A further aspect that makes the research area *enzyme catalysis in organic synthesis* both highly interesting and challenging from a scientific perspective is the high interdisciplinarity of this field, which requires competencies from a broad variety of disciplines, comprising, for example, microbiology, genetics, molecular biology, organic synthesis, and reaction engineering. The "hybridization" of such competencies is certainly a key factor in the successful development of efficient biocatalytic processes.

The following gives an overview of enzymes typically applied in organic synthesis as well as some selected landmarks of the impressive development of *enzyme catalysis in organic synthesis* towards a highly recognized synthetic technology in academia and industry [3].

1.2 Potential of Enzymes as Catalysts in Organic Synthesis: Enzyme Reactions Overview

1.2.1 Enzyme Catalysts: Three-Dimensional Structure and General Properties

The unique functions of enzymes as catalytically active proteins are a result of their complex three-dimensional structures and the active site integrated therein [4]. This enables a highly specific recognition of specific substrates, leading to excellent selectivities. Besides chemoselectivity, the stereoselectivity of enzymes is also in general high to excellent and, furthermore, this is typically true for regio-, diastereo-, as well as enantioselectivity. Figure 1.1 shows, as a representative example of the impressive (and beautiful) three-dimensional structures of enzymes, the well known and widely used lipase from *Candida antarctica* [5].

The unique properties of enzymes to stereoselectively recognize a substrate was found by Fischer already at the end of the nineteenth century [6, 7]. Based on these findings he postulated the "lock-and-key" theory, according to which the substrate has to fit into the active site of the enzyme like a key into the lock. A further theoretical milestone was the kinetic analysis of enzyme reactions conducted by Michaelis and Menten a few years later [8]. Their theory is based on the formation of an enzyme–substrate complex, and subsequent product formation and release of the enzyme for the next catalytic cycle after the reaction has been conducted. In later years this kinetic model has been further refined and today kinetic analysis [9] of enzymatic reactions and characterization of the enzyme with such methods is a key feature in biocatalytic research projects.

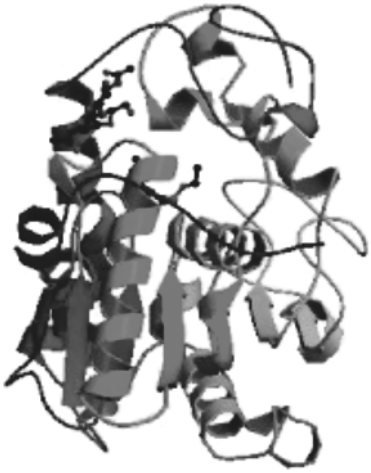


Figure 1.1 Three-dimensional structure of a lipase from *Candida antarctica*; image from the RCSB PDB (www.pdb.org) of PDB ID 1LBS (Ref. [5])

In addition to the substrate the reaction conditions also play a very important role in enzyme catalysis. It is difficult, though, to define properties under which in general enzymes are able to operate as a catalyst. At the same time, however, it is evident that enzyme catalysis requires specific suitable reaction conditions such as pH, temperature, and solvent, which have to be considered in (bio-)process development. In the following, some selected reaction parameters of specific importance for enzymatic reactions are briefly discussed.

For enzymes pH and temperature are certainly highly important reaction parameters in terms of both activity and stability. Typically, enzymes operate in a more or less neutral or weakly basic/acidic pH range, usually between pH 5 and 10, although exceptions are known. The natural reaction environment for enzymes is water. Interestingly, however, water as a reaction medium is not necessarily required and many enzymatic transformations (including industrial processes) are run in organic reaction medium. The factors affecting enzymatic reactions are described in more detail below.

There are several ways to describe enzymatic activity; popular and widely used criteria are the maximum reaction rate (v_{max}, measured, for example, in U mg^{-1}) and the K_m value.

1.2.2
Overview of Enzyme Classes (EC Numbers) and Related Reactions

Enzymes are typically classified according to the types of reactions they catalyze. In the *Enzyme Nomenclature* classification [10] they are subdivided and categorized into six main enzyme classes corresponding to the type of reactions such enzymes catalyze. Table 1.1 gives an overview of this categorization, in particular the main enzyme classes.

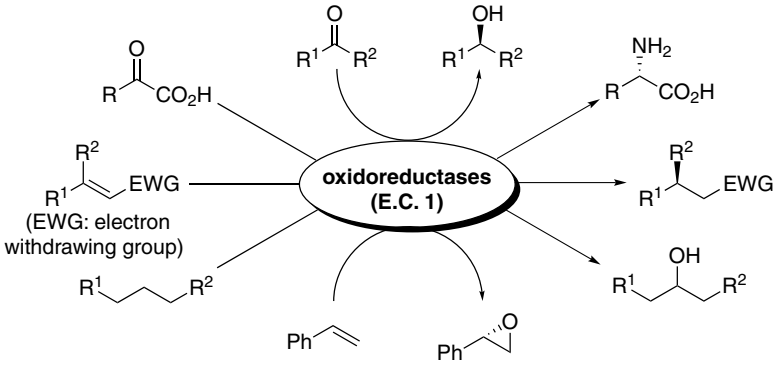
Table 1.1 Categorization of enzymes according to the general type of reactions they catalyze.

Enzyme class	EC number	Selected reactions
Oxidoreductases	1	Reduction of C = O and C = C; reductive amination of C = O; oxidation of C-H, C = C, C-N, and C-O; cofactor reduction/oxidation
Transferases	2	Transfer of functional groups such as amino, acyl, phosphoryl, methyl, glycosyl, nitro, and sulfur-containing groups
Hydrolases	3	Hydrolysis of esters, amides, lactones, lactams, epoxides, nitriles, and so on, as well as the reverse reactions to form such functionalities
Lyases (synthases)	4	Addition of small molecules to double bonds such as C = C, C = N, and C = O
Isomerases	5	Transformation of isomers (isomerizations) such as racemizations, epimerizations, and rearrangement reactions
Ligases (synthetases)	6	Formation of complex compounds (in analogy to lyases) but enzymatically active only when combined with ATP cleavage

With respect to applications of enzymes in organic synthesis, enzymes from nearly all enzyme classes play an important synthetic role in organic chemistry. As an exception, at least in part, one might regard enzymes from enzyme class 6 (ligases). Since *in situ* regeneration of the cofactor ATP is still a challenge, ligases have found limited use as catalysts for *in vitro* applications in organic syntheses. In contrast, enzymes from enzyme classes EC 1–5 turned out to be highly efficient catalysts for a broad range of organic synthetic transformations that, in part, are also suitable for technical-scale applications.

With oxidoreductases (EC 1) many successful reduction and oxidation processes have been realized.[1)] Scheme 1.1 summarizes selected oxidoreductase-catalyzed reaction types that have gained broad interest in organic chemistry. With respect to (asymmetric) reductions as a synthetically important reaction in organic chemistry, the reduction of a carbonyl moiety to an alcohol (when using, for example, alcohol dehydrogenases or α-hydroxy acid dehydrogenases as catalysts) or amino functionality (when using α-amino acid dehydrogenases in reductive aminations) has already found a wide application range in organic chemistry as well as industrial applications. A more recent trend is the increasing tendency to apply enzymes also for C=C double bond reductions. Although pioneering work in this area with so-called "old yellow enzymes" was carried out many decades ago, expansion of the synthetic range as well as the "pool" of available (robust and stable) enzymes has been a main focus of recent research [11a]. Notably, commercialization of this technology also has been reported recently [11b]. A further recent and current "hot topic" is the field of oxidation reactions using suitable oxidoreductases. Key advantages of using enzymes as

1) Enzymatic organic syntheses with oxidoreductases, both academic and industrial contributions, are covered in detail in, for example, Chapters 26–38.



Scheme 1.1 Overview of selected reactions catalyzed by enzymes from EC 1 (oxidoreductases).

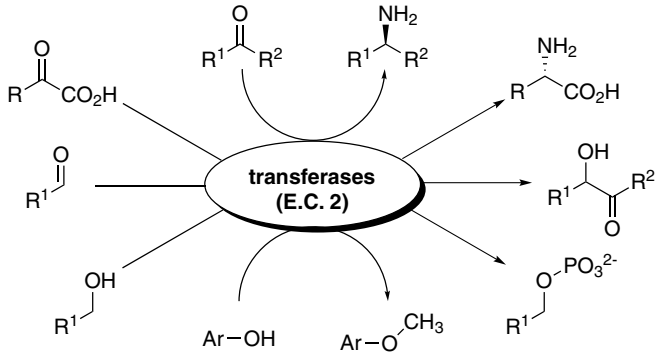
catalysts in redox processes are (i) the excellent selectivity even when using non-functionalized compounds, as, for example, demonstrated for alkanes and cycloalkanes as substrates and (ii) the use of molecular oxygen as a cheap and sustainable oxidizing agent. Oxidative selective functionalization of alkane moieties is still a challenge for chemocatalysts and a limited number of efficient catalysts exist for such transformations. In contrast there is a high demand for this reaction type, also from an industrial perspective. Given the excellent stereoselectivity of enzymes, it is no surprise that today hydroxylation of steroids is industrially carried out by means of biocatalytic hydroxylation instead of chemical methods [12]. A remaining challenge for enzymatic oxidations, however, can be seen in the limited activity of some enzymes, which is often below 1 U mg^{-1}, such as, for example, in case of P450-monooxygenases. As well as hydroxylation other oxidative processes with enzymes are also of interest in organic syntheses, such as, for example, reactions with Baeyer–Villiger monooxygenases (for Baeyer–Villiger oxidations leading to lactones from ketones) and styrene monooxygenases (for epoxidation of styrenes). In summary, oxidoreductases are the second most used enzyme types in organic synthesis; only the representatives of enzyme class EC 3 show more synthetic applications.

Representatives of enzyme class EC 2, so-called transferases, are further versatile catalysts for organic synthetic transformations.[2)] In particular, transaminases have attracted widespread attention with interesting applications for the synthesis of amino acids and amines. Industrial applications have been reported as well. As a starting material the corresponding carbonyl compounds are required. Scheme 1.2 gives an overview of reactions with transaminases and other transferases.

Without doubt, the most popular and most frequently applied enzymes in organic chemistry are hydrolases (EC 3).[3)] In particular this is due to (i) the fact that many hydrolases are commercially available, often in an attractive price range (e.g., in the

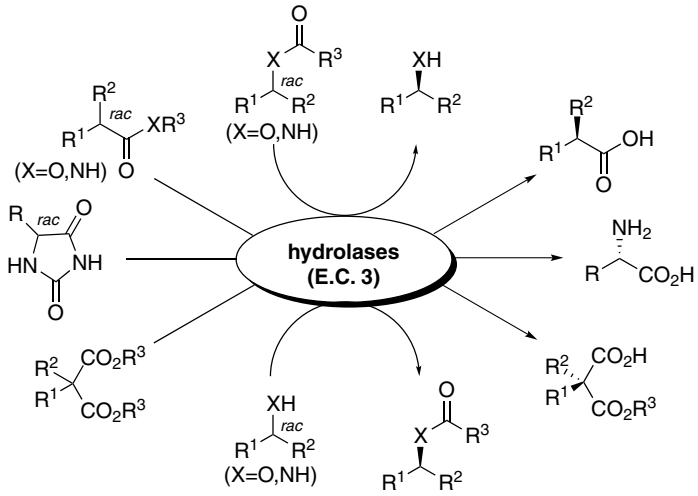
2) Enzymatic organic syntheses with transferases, both academic and industrial contributions, are covered in detail in, for example, Chapters 19 and 20.

3) Enzymatic organic syntheses with hydrolases, both academic and industrial contributions, are covered in detail in, for example, Chapters 8–10, 12, 14–17, 20, and 25.



Scheme 1.2 Overview of selected reactions catalyzed by enzymes from EC 2 (transferases).

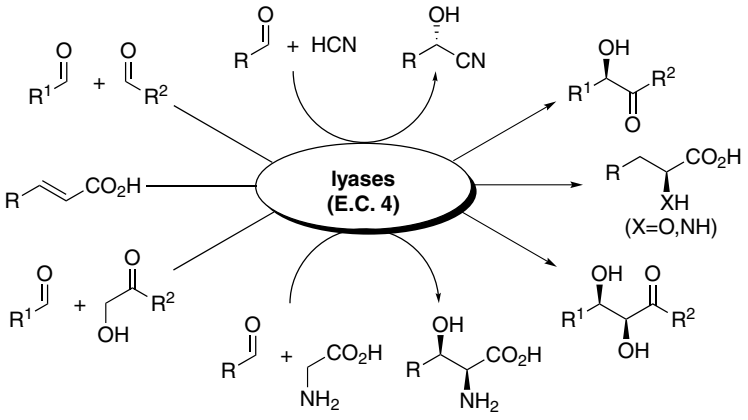
case of applications in the food and laundry detergent industries) [13], (ii) their direct and often simple use without the need for, additional cofactor and cofactor regeneration methods, (iii) numerous synthetic applications through modifications of the carboxyl moiety, for example, in resolution and desymmetrization processes, and (iv) their suitability (at least in part, particularly in the case of lipases) to use hydrolases in an organic solvent as a reaction medium, which is often favored by organic chemists [14]. Representative examples for hydrolases frequently used in organic synthesis are proteases, lipases, and esterases. Typical transformations include the hydrolysis of esters and amides, and their reverse reactions, namely esterification and amidation. Scheme 1.3 gives an overview of selected examples of hydrolase-catalyzed (stereoselective) processes. Often, hydrolases are used in resolution processes since many acids and functionalized derivatives thereof are easily accessible in racemic form by means of "standard chemical methods". A further



Scheme 1.3 Overview of selected reactions catalyzed by enzymes from EC 3 (hydrolases).

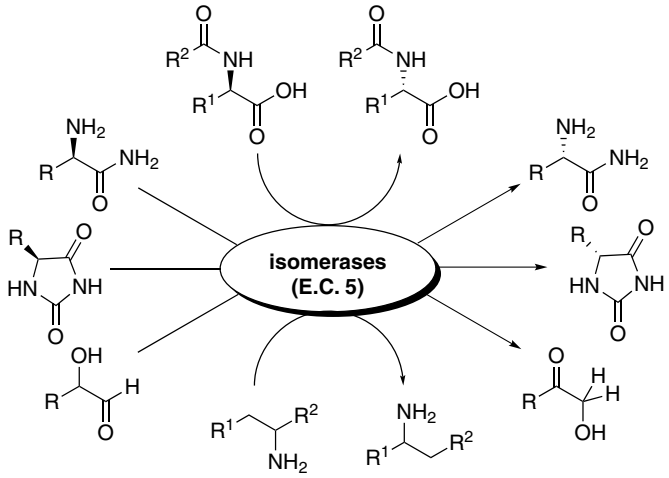
stereoselective process type of interest are desymmetrization reactions, for example, of prochiral or *meso*-type diesters. Furthermore, non-stereoselective applications have been reported as well, for example, cleavage of unwanted acid side-chains under smooth hydrolytic conditions, thus avoiding the harsh chemical reaction conditions of alternative chemical hydrolytic processes. This option has been particularly used for derivatization of easily accessible natural products (e.g., in the synthesis of antibiotics such as 6-aminopenicillinic acid). To date, hydrolases have also attracted a lot of industrial interest and numerous examples of technical applications of hydrolases have been demonstrated. Notably, representatives of this enzyme class, in particular lipases, are also suitable for reactions in pure organic media and this technology is used, e.g., for the large-scale production of fatty acid esters starting from the fatty acid and an alcohol moiety [15–17].

Lyases, which are summarized in enzyme class EC 4, are characterized by the formation of a new C(Nu)-XH bond (X=C,N,O; Nu^-: nucleophile) by an addition reaction of a small molecule Nu-H to a C=X double bond.[4)] Such a "small molecule" can be, for example, water, ammonia, as well as a carbon nucleophile. Enzymes for addition of water to activated C=C double bonds are called hydratases, and ammonia lyases are enzymes capable of adding ammonia to an enoate (typically in a highly enantioselective fashion, albeit the substrate spectrum often is narrow). In addition, the use of carbon nucleophiles enables the (stereoselective) formation of new C–C single bonds. Common carbon nucleophiles such as, for example, cyanide in hydrocyanation of aldehydes as well as aldehydes in umpolung reactions such as the benzoin condensation have been widely used. Scheme 1.4 gives an overview of typical (selected) reactions based on catalysis with lyases. Although the overall number of reactions with lyases used in organic synthesis up to now is still not very broad (when compared with the huge number of known "classic" organic C–C bond formation reactions), those



Scheme 1.4 Overview of selected reactions catalyzed by enzymes from EC 4 (lyases).

4) Enzymatic organic syntheses with lyases, both academic and industrial contributions, are covered in detail in, for example, Chapters 11–13, 18, 21, and 24.



Scheme 1.5 Overview of selected reactions catalyzed by enzymes from EC 5 (isomerases).

reactions known can be carried out highly efficiently. This is underlined by, for example, technical applications of addition reactions of water and ammonia to enoates as well as hydrocyanation and umpolung reactions.

Enzyme class EC 5 consists of those enzymes capable of catalyzing isomerization reactions.[5)] The types of isomerizations are diverse, consisting of, for example, racemizations, 1,2-migrations of functional groups (e.g., of amino functionalities) and *cis–trans* isomerizations. Scheme 1.5 gives an overview of selected isomerization catalyzed processes employed in organic chemistry. Interestingly, the largest biocatalytic application today is based on the use of an isomerase, namely, the production of high fructose corn syrup via enzymatic transformation of glucose into fructose [18], which is carried out on a >1 million tons scale. In organic chemistry, the use of racemases has attracted most interest within the enzymes of EC 5, since the combination of a racemase with a further biocatalyst for a resolution step enables the development of dynamic kinetic resolution processes. Typically, such resolution processes to be combined with racemases are reactions catalyzed by hydrolases, and such resolutions run either in the hydrolytic or acylation direction.

Whereas enzymes from enzyme classes EC 1 to EC 5 are already widely used as catalysts in organic synthesis and have enabled a broad range of highly efficient synthetic processes (running, in part, already even on an industrial scale), the application range of enzymes from EC 6 (ligases) is sill narrow. At first glance this might sound surprising due to the numerous interesting reaction types these enzymes can catalyze. However, these reactions require ATP as a cofactor, which is efficiently regenerated in living cell processes, but its cofactor regeneration *in situ* under *in vitro* reaction conditions remains a challenge. Although some methods have been developed, applicability in organic syntheses (in particular with respect to

5) Enzymatic organic syntheses with isomerases, both academic and industrial contributions, are covered in detail in, for example, Chapters 39 and 40.

large-scale processes) is still limited. Certainly, development of efficient and economically attractive methods for the *in situ* regeneration of ATP is an attractive goal in future research activities.

1.2.3
Overview of Coenzymes and Cofactors and Applications in Organic Synthesis

Cofactors are non-proteinogenic compounds that are required for the catalytic activity of enzymes and which can bind to the enzyme either in a covalent or non-covalent mode [4, 9]. A broad variety of cofactors are known, consisting of organic molecules and inorganic ions. In the covalent mode, when the cofactor is permanently bound to the enzyme, the cofactor is called a prosthetic group. In case of a non-covalent binding of the cofactor to the enzyme it is called a coenzyme. Since the coenzyme is modified during the catalytic process (by transferring electrons or chemical groups to the substrate), its regeneration in a subsequent reaction is a key issue in order to use the cofactor in catalytic amounts. Thus, the cosubstrate required for the cofactor's regeneration is needed in stoichiometric amounts. Figure 1.2 shows selected cofactors that are often applied in organic synthetic processes with enzymes.

With exception of hydrolases (EC 3), members of all other enzyme classes (or at least a part thereof) show a cofactor dependency, although in some cases (e.g., in case of lyases) cofactors are not necessarily involved in the catalytic process. For most enzymes belonging to enzyme classes EC 1–5, however, cofactors are involved in the

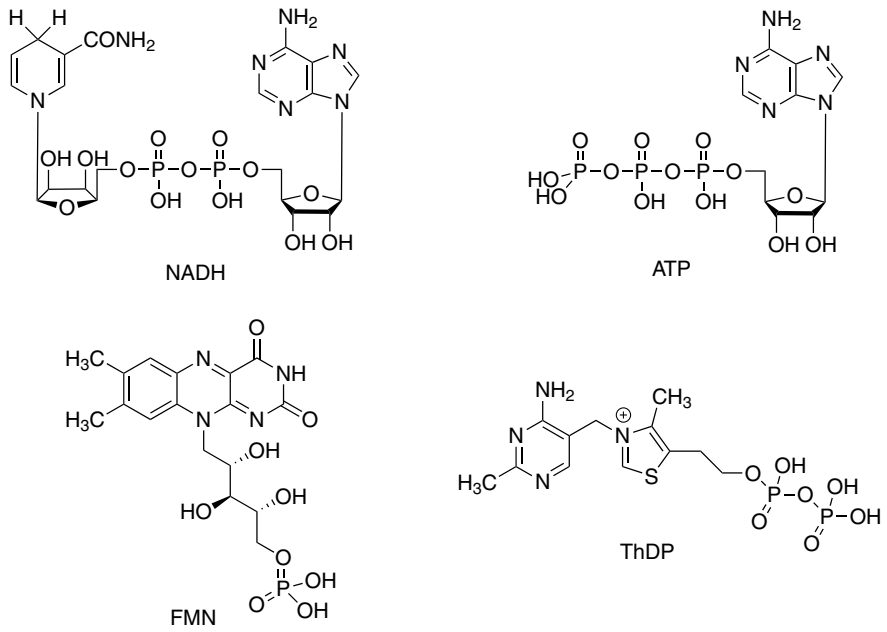
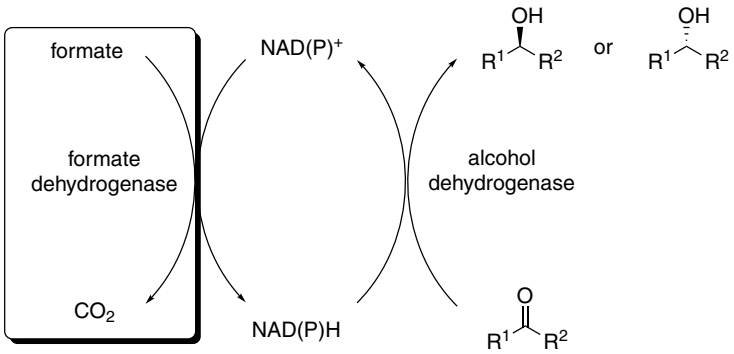


Figure 1.2 Cofactors often applied in enzymatic organic synthesis.

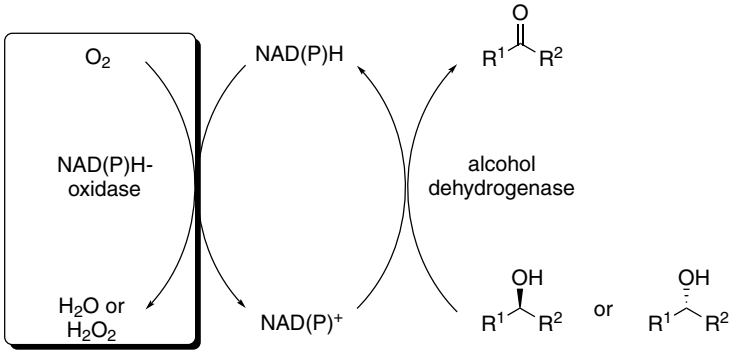
catalytic process. Furthermore, the availability of an opportunity to regenerate such cofactors efficiently under the chosen organic reaction conditions often decides whether this method can be developed towards an attractive synthetic process.

In the following this shall be exemplified for the regeneration of a cofactor in its reduced and oxidized form, namely, NAD(P)H and NAD(P)$^+$, which are used in enzymatic redox processes (Scheme 1.6) [19]. Since the reducing agent for the oxidoreductase in a reduction process is NAD(P)H, in total a stoichiometric amount of such a reducing agent is need (as in "classic" organic chemistry a stoichiometric amount of molecular hydrogen or borane or sodium borohydride is used). Taking into account both the very high molecular weight and price of cofactors such as NAD(P)H, the use of a stoichiometric amount of such molecules would not enable any synthetically useful process. Thus, *in situ* cofactor regeneration of such cofactors, enabling their use in catalytic amounts, is a prerequisite to conduct such biocatalytic redox processes in a synthetically useful and attractive fashion. Such *in situ* cofactor regeneration can be achieved through combination with a second enzymatic trans-

(a) "Reductive" cofactor recycling mode based on the use of a formate dehydrogenase



(b) "Oxidative" cofactor recycling mode based on the use of an NAD(P)H-oxidase



Scheme 1.6 Selected regeneration processes of cofactors in their (a) reduced and (b) oxidized form, exemplified for NAD(P)H and NAD(P)$^+$.

formation, which regenerates the cofactor. To make the cofactor regeneration economically attractive it is important that the substrate consumed in this second enzymatic process is cheap and readily available, since this substrate (the so-called cosubstrate) is required in stoichiometric amount. From the perspective of the reaction formula, this cosubstrate represents the reducing or oxidizing agent required in stoichiometric amount. For example, in the selected cofactor regeneration methods shown in Scheme 1.6, the stoichiometric reducing agent is formate (which is oxidized to carbon dioxide; process 1) and the stoichiometric oxidizing agent is molecular oxygen (which is reduced to water; process 2).

To date, a broad set of cofactor regeneration methods have been successfully developed. Notably, besides enzymatic cofactor regenerations, electrochemical and chemocatalytic cofactor regenerations have also been reported. Processes with *in situ* cofactor regenerations can be conducted using isolated enzymes as well as permeabilized whole-cell catalysts. In addition, in fermentation-like processes with intact microorganisms cofactor regeneration is carried out within the metabolism in the cell. In organic chemistry, all of these options for *in situ* regeneration of cofactors have been realized in enzymatic syntheses with cofactors.

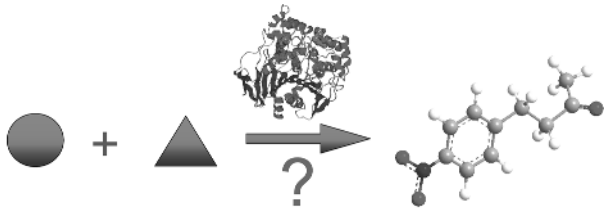
1.2.4
Factors Affecting Enzymatic Reactions

As with chemocatalysts, enzymes also have a typical application range with respect to reaction parameters, which have to be considered in those transformations. These "typical" reaction parameters are in general related to physiological conditions under which the corresponding enzymes work. In particular, the pH and temperature profile of enzymes should be determined prior to use in organic synthesis. For most enzymes a pH in the range 6–10 and temperatures of 20–50 °C are preferred although many exceptions are known. Notably, enzymes suitable for catalyzing the reactions at very low or high pH and also at elevated temperature exceeding 80 °C have been found. A typical natural source of such types of enzymes is the group of so-called extremophilic microorganisms. For example, thermophilic microbial strains from hot springs are an interesting source of enzymes that can be impressively active at high temperatures.

A further important criteria when setting up an organic synthesis with (bio-) catalysts is the choice of reaction medium. The preferred reaction media for enzymes – when taking into account their natural function – are aqueous (buffered) solutions. However, notably, many enzymes are highly tolerant towards the presence of organic solvents [14]. This has been demonstrated in particular for lipases as catalysts. The reaction medium of choice for most of enzymes is nevertheless water (or related buffer solutions). Since, however, organic substrates are often hydrophobic, water-miscible and water-immiscible organic solvents have been added in biotransformations to ensure sufficient solubility of the substrate. Notably, many enzymes turned out to be stable under such conditions, thus allowing the development of efficient processes in aqueous-organic one-phase or two-phase solvent systems.

1.2.5
Why Use Enzymes in Organic Synthesis? Factors Affecting Enzymatic Reactions, Advantages and Drawbacks

Before discussing the advantages and drawbacks when using enzymes as catalysts in organic synthesis, a brief overview is given of selected criteria for choosing a specific synthetic route (based on, for example, chemo- or biocatalysts or classic resolutions to form diastereomeric salt pairs). Scheme 1.7 summarizes selected criteria that are relevant also for biotransformations. In general high conversion and enantioselectivity (and/or regioselectivity/diastereoselectivity) are desirable. A high, ideally quantitative (product-related) conversion has not only the advantage of consuming the maximal amount of substrate (thus contributing to a decrease in substrate costs) but also simplifies downstream-processing. This is particularly true for reactions in which substrate and product show similar properties, for example, similar boiling points, which make separation tedious. With respect to enantioselectivity, typically a high enantiomeric excess of >99% e.e. (as required from the FDA for chiral drugs) for the resulting product is desirable. Besides conversion and selectivity issues, substrate and product concentrations as well as volumetric productivities are further important criteria for (bio-)transformations in organic chemistry. As a "rule of thumb," a substrate input of $>100\,g\,l^{-1}$ (in total, optional added in portions) is desirable to reach economically attractive volumetric productivities in technical production processes. Among further important criteria for realizing an efficient synthetic process are an attractive access to the (bio-)catalyst component and the technical feasibility of the process.



Prerequisites for an efficient biocatalytic production process:

- Atom economy of the process?
- Sustainability of the process?
- Availability of the required substrate?
- Enantioselectivity of the process? → **>99% ee**
- Availability and economical data of the catalyst?
- Stability of the catalyst / Recycling?
- High conversion / yield? → **>95% conversion**
- High volumetric productivity? → **>100 g l^{-1} substrate input**

Scheme 1.7 Criteria for efficient (bio-)transformations in organic synthesis.

A major advantage of enzymes as catalysts in organic synthesis, which is often regarded as *the* major advantage of biocatalysts, are the excellent selectivities enzymatic reactions typically show. For numerous asymmetric reactions starting

from prochiral compounds the desired products are formed in excellent enantiomeric excess of >99% e.e. High enantioselectivities are also typically observed in enzymatic resolution processes, with enantioselectivities often exceeding *E*-values of 100. High to excellent stereoselectivities have also been observed in regio- and diastereoselective enzymatic reactions, respectively. Even when unsatisfactory stereoselectivities are observed for wild-type enzymes, several protein engineering methodologies are available that have already turned out to be suitable for optimization of enzymatic performance in many examples.

Whereas chirality and a defined absolute configuration is also an important criterion in nature, high volumetric productivities and a substrate input of, for example, $>100\,g\,l^{-1}$ is a desirable feature for organic syntheses, but not for processes in living organisms. Thus, unsurprisingly, for many enzymes the development of synthetic processes running at a high substrate input and leading to a high space–time yield turned out to be a challenging task (but at the same time it should be added that many enzymes turned out to be able to do so efficiently!).

In addition, optimization of the specific activity of enzymes by means of protein engineering is a further challenge in order to make the biocatalyst attractive for synthetic purposes. To be suitable for organic syntheses, in general (as a rule of thumb) specific activities exceeding $1\,U\,mg^{-1}$ are desirable. However, it also should be mentioned that numerous enzymes show as wild-type enzymes excellent activity data exceeding $100\,U\,mg^{-1}$ for specific substrates. Needless to say, such enzymes are highly interesting catalysts, fulfilling a key prerequisite towards realizing technically feasible and economically attractive biocatalytic processes.

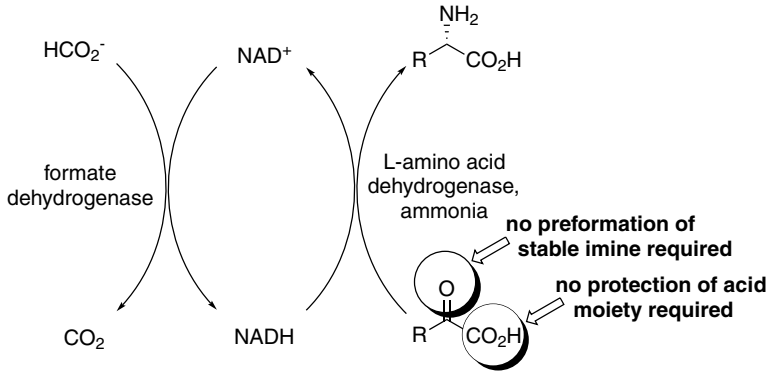
Besides the specific activity, a further key feature for attractive biocatalysts is an economic production method for their preparation. Certainly, this criterion has long been a limiting factor and drawback in enzymatic chemistry when regarding the decade long biotransformations with wild-type organisms. The use of wild-type organisms has major drawbacks, for example, because the expression of the desired protein is very low, thus requiring a large amount of biomass for the biotransformation. Often substrate loading is below $1\,g\,l^{-1}$, accompanied by a high biomass loading of $>25\,g\,l^{-1}$ in such biotransformations (as can be seen, for example, in many reactions with baker's yeast as a biocatalyst). A further consequence of low protein expression of the desired enzyme in wild-type microorganisms is the significant impact of side-reactions. Owing to impressive advances in molecular biology related to protein engineering [20] today enzymes can be (mostly) made available in recombinant form. Overexpression in host organisms such as, for example, *Escherichia coli* often exceeds 20%, which not only contributes to a high biocatalyst amount in the recombinant whole-cell but also to suppressing side reactions due to the favored ratio of desired enzyme over other enzymes catalyzing for competing side-reactions.

Furthermore, tremendous advances in bioprocess engineering have been made that allows the production of such recombinant whole-cells with impressive biomass concentrations. Thus, by means of high-cell density techniques biomass concentrations of >200 g per litre of fermentation broth can now be reached for the required recombinant cells bearing the desired enzyme in overexpressed form. Accordingly, overexpression jointly with high biomass concentration in the fermentation process

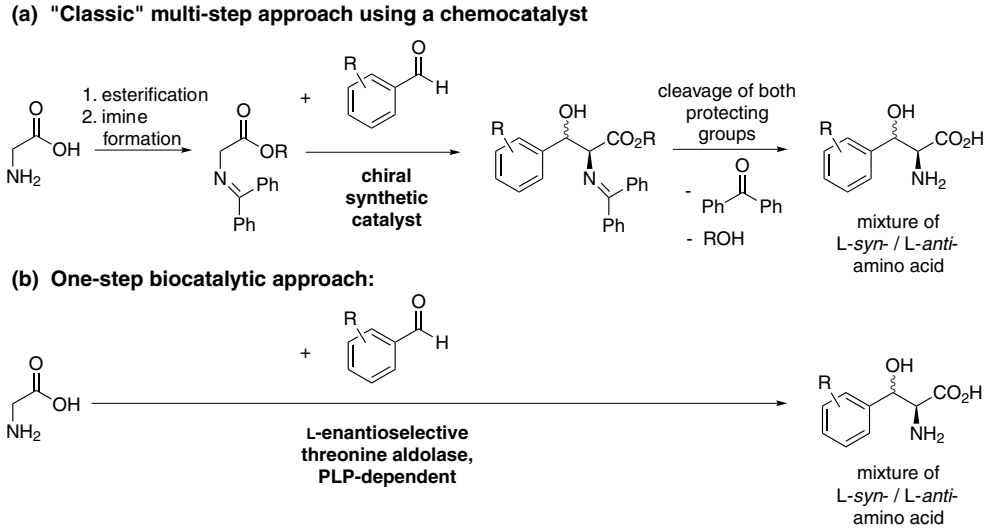
represents a valuable access to the (bio-)catalyst. When these criteria are fulfilled such an economically highly attractive access to the biocatalyst is certainly a major advantage.

Dependent on the process, such recombinant whole-cells can be used directly in the biotransformation or, alternatively, after cell disruption and optional purification the enzymes are used as biocatalyst component in "free" or immobilized form.

A further major advantage of biocatalysis over chemocatalysis is the possibility of using substrates often without the need for protecting groups, which is due to the high selectivity of enzymes for specific functional groups. This is underlined by two examples in asymmetric synthesis, which are visualized in Schemes 1.8 and 1.9:



Scheme 1.8 Protecting group-free biocatalytic strategy for reductive amination.



Scheme 1.9 (a) Multistep chemical and (b) protecting group-free biocatalytic strategies in an aldol reaction [21].

The first example is the biocatalytic reductive amination of α-keto acids (Scheme 1.8; see also Chapter 28). Notably, this substrate can be used directly in the enzymatic reductive amination process without the need to either protect the carboxylate moiety as an ester or to prepare a stable imine prior to the reduction step. Such additional steps might have to be considered when using a potential classic chemical process as an alternative (based on, for example, the formation of an imine with a chiral auxiliary and a diastereoselective reduction, followed by further steps for protecting group cleavage).

The second comparison reflects the situation for an aldol reaction for the asymmetric preparation of β-hydroxy α-amino acids (Scheme 1.9; [21]; also Chapter 21). Whereas in the biocatalytic step glycine can be used directly as a donor, in a chemocatalytic reaction totally protected glycine is required. This requires two steps prior to the asymmetric chemocatalytic key step, conducted, for example, by means of a phase-transfer catalyst or a metal catalyst, as well as two cleavage steps after the reactions. Thus, biocatalysis offers a straightforward access, requiring only one synthetic step compared to five steps in the chemical approach [21]. In this case, however, a challenge for biocatalysis is still the limited diasteresoelectivity of the process, whereas enantioselectivity is excellent.

These examples demonstrate that biocatalysis offers many unique advantages over chemical alternatives, thus representing an exciting complementary alternative to the pool of "classic" chemical and chemocatalytic synthetic methods available to the organic chemist today.

1.3
The Early Steps: From Fermentation to Biotransformations Using Wild-Type Whole Cells

1.3.1
Historical Development of Fermentation and First Microbial Transformations

The finding of the production of ethanol from glucose is regarded as one of the first discoveries of human beings in the field of biotransformations. In such processes whole cells of microorganisms such as the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* and the bacterial producer *Zymomonas mobilis* were used. In addition, the use of α-amylase for saccharification of starch began at least 5000 years ago in Mesopotamia or Egypt, applying this process for the production of beer. Another earlier enzyme-catalyzed process is the production of cheese from milk when kept in the stomach of sheep. Although the presence and function of this enzyme "chymosin" was not understood at that time, people could use it for this application. Another early product produced by biotechnology is vinegar (acetic acid), which is produced by oxidation of ethanol by *Acetobacter aceti* or *Gluconobacter suboxydans* [22]. Acetic acid has been produced at the surface of static cultures of *Acetobacter aceti* or *Gluconobacter suboxydans*. *Acetobacter* has been also used in a large-scale production of acetic acid in an aerobic reaction tower filled with a number

Table 1.2 Milestones in the history of applied microbiology.

Age (year)	Development in microbiology	Typical products
Prehistoric		Alcohol
Middle Ages		Vinegar, pickles, miso, soy sauce
First half of the twentieth century	Surface culture	Organic acids
	Static culture	Acetone, *n*-butanol
Second half of the twentieth century	Aerobic culture	Penicillin
1955	*Screening*	Antibiotics
	Biochemical auxotrophs	Amino acids, nucleic acids
1965	Analog resistant mutants	
1975	*Screening for enzymes*	Steroids
	Microbial transformation	Natural and unnatural amino acids
	Screening for substrates	
	Petroleum microorganisms	Long-chain dicarboxylic acids
1985	C1 microorganisms	Microbial protein
	Recombinant DNA	Interferon, human growth hormone, and so on
	Cell fusion	Interleukin
	Bioreactor	Acrylamide
1995	Genome science	Various

of different packing materials such as ceramics, hollow fibers, charcoal pellets and so on shavings, onto which ethanol is sprayed. Table 1.2 gives an overview of milestones in the history of applied microbiology.

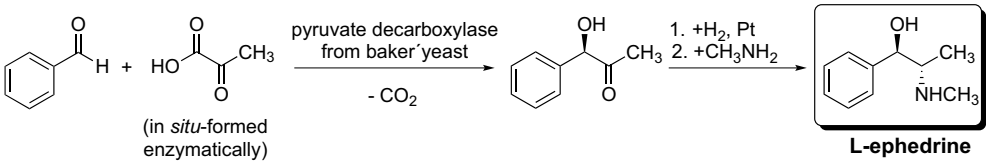
The historical development of enzymatic synthesis is greatly related to the progress of microbiology, because microorganisms have been the main sources of the enzymes. One of the first questions to be asked was what does the mechanism of alcoholic fermentation looks like. It is no exaggeration to say that biochemistry was born to answer such questions. Notably, there was a big paradigm shift from anaerobic culture to aerobic culture around middle of the twentieth century, caused by an engineering development to cultivate the microorganisms aerobically by shaking cultures and aerobic bioreactors, resulting in the discovery of varieties of new abilities of wild-type microorganisms, enabling large-scale production of the products, as compared with the static cultures that had only produced beer, sake, vinegar, yogurt, miso, pickles, and so on. Another big lesson in the history of applied microbiology is the notion of screening or microbial diversity, as evidenced by the fact that wide varieties of new antibiotics were isolated by changing the microbial producers, and also in the way some biochemists always start their research by finding the best producers of the enzyme, to make the purification and characterization of the enzyme much easier. Over the years, there have been many successful examples of microbial biotransformations (Table 1.3). Some selected processes thereof are described in more detail in the following to underline the potential and (early) industrial achievements of microbial biotransformations with wild-type strains.

Table 1.3 Overview of selected milestones in industrial microbial biotransformations with wild-type strains.

Product	Biocatalyst	Operating since	Company
Acetic acid	Bacteria	1823	Various
L-2-Methylamino-1-phenylpropan-1-ol (ephedrine)	Yeast	1930	Knoll, Germany
L-Sorbose	*Acetobacter suboxydans*	1934	Various
Prednisolone	*Arthrobacter simplex*	1955	Schering, Germany
L-Aspartic acid	*Escherichia coli*	1958	Tanabe, Japan
7-ADCA	*Bacillus megaterium*	1970	Asahi Chemical, Japan
L-Malic acid	*Brevibacterium ammoniagenes*	1974	Tanabe, Japan
D-*p*-Hydroxy-phenylglycine	*Pseudomonas striata*	1983	Kaneka, Japan
Acrylamide	*Rhodococcus* sp.	1985	Nitto (Mitsubishi Rayon), Japan
D-Aspartic acid and L-alanine	*Pseudomonas chlororaphis* and *Pseudomonas dacunhae*	1988	Tanabe, Japan
L-Carnitine	*Agrobacterium* sp.	1993	Lonza, Switzerland
2-Keto-L-gluconic acid	*Acetobacter* sp.	1999	BASF, Germany

1.3.2
Development of Practical Synthesis of Chemicals via Transformations Using Wild-Type Whole Cells in Non-Immobilized Form

An early example of a successful application of microbial-based biotransformations is the synthesis of an intermediate for L-ephedrine ((1*S*,2*S*)-2-methylamino-1-phenylpropan-1-ol), established in the 1930s industrially by Knoll AG (Scheme 1.10) [23, 24]. The compound L-ephedrine and its diastereomer, pseudoephedrine, are pharmaceuticals used as decongestants and anti-asthmatics. The biocatalytically synthesized intermediates by means of microorganism are L-phenylacetylcarbinol and its D-enantiomer. Their synthesis is based on a condensation of an "active acetaldehyde" derived from pyruvic acid and externally added benzaldehyde, and as microorganisms yeasts such as *Saccharomyces cerevisiae* and *Candida utilis* have been used. This type production technology, is which still applied today, is shown schematically in Scheme 1.10.

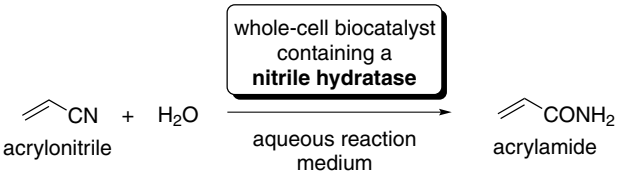


Scheme 1.10 Microbial biotransformation as a key step in the production of L-ephedrine.

1.3.3
Development of Practical Synthesis of Chemicals via Transformations Using Wild-Type Whole Cells in Immobilized Form

Development of practical synthesis of chemicals via transformations using wild-type whole cells has often been achieved based on the concept of treating microbial cells as just a "bag of enzymes" for the use in organic synthesis. This research field led to tremendous synthetic applications, often applied on an industrial scale [1], in particular when immobilized microorganisms were used as biocatalyst. This technology of using (immobilized) whole microbial cells was made possible by combining the notions of screening unknown microorganisms, finding new enzymes from nature, induction phenomenon of the enzymes, the uses of precursors or analogues as substrates, the development of immobilization of whole microbial cells, and biochemical engineering technologies.

One of the most successful achievements in biocatalytic organic synthesis is the transformation of acrylonitrile into acrylamide catalyzed by a nitrile hydratase [25, 26]. This biocatalytic transformation, running in the presence of an immobilized microbial catalyst, led to an industrial production process that is now estimated to produce more than 400 000 tons of acrylamide annually worldwide. Notably, when the enzymes were fully induced in the microbial cultures, as in the case of nitrile hydratase, they consisted of up to 50% of the protein of the whole cells of *Rhodococcus rhodochrous* J-1. Scheme 1.11 shows this process concept.

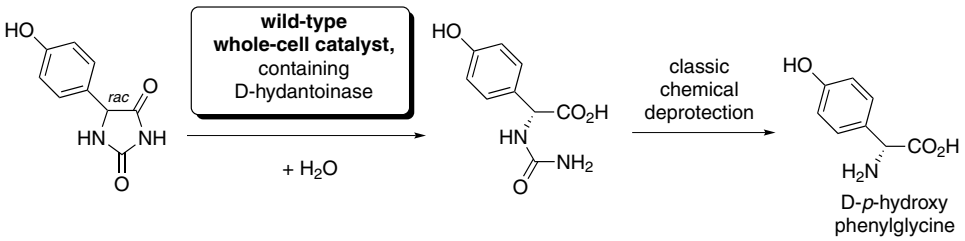


Scheme 1.11 Nitrile hydratase-catalyzed transformation of acrylonitrile into acrylamide.

The historical development of this microbial process is also of interest since it underlines that careful identification of metabolic steps and identification of the individual enzymes involved has been required to develop an efficient, selective biotransformation for acrylonitrile hydration: Based on earlier work, studies on microbial degradation of polyacrylonitrile was started and further extended to screening for microorganisms that degrade various low molecular weight nitrile compounds. The microbial hydrolysis of nitriles was found to be catalyzed by two enzymes, nitrile hydratase and amidase, or by a single enzyme nitrilase. Furthermore, the ability of microbial enzyme to synthesize acrylamide in a very high concentration was discovered for the first time. By an enrichment culture technique, *Rhodococcus rhodochrous* (formerly *Arthrobacer* sp.) J-1 and *Pseudomonas chlororaphis* B23, which have both become industrial strains, were isolated as acetonitrile and isobutyronitrile utilizers, respectively. *P. chlororaphis* B23 was

found to catalyze the synthesis of acrylamide, forming up to 400 gl^{-1} of acrylamide from acrylonitrile [27]. From *R. rhodochrous* J-1, nitrile hydratase was discovered, purified, and characterized.

Since only proteinogenic amino acids are produced by fermentation, attempts were made at an early stage to produce non-proteinogenic amino acids also by means of biotransformations with microorganisms starting from chemically synthesized substrates. An elegant example of using (immobilized) wild-type microorganisms for the production of non-proteinogenic amino acids is the biocatalytic synthesis of the semisynthetic β-lactam side-chain D-*p*-hydroxyphenylglycine [28, 29]. This compound is required for the preparation of amoxicillin, which is one of the commercially most successful semisynthetic antibiotics and has a structure that consists of an amide compound based on D-*p*-hydroxyphenylglycine and 6-APA (6-aminopenicillanic acid). When the action of enzyme with the whole cells of microorganisms were screened with substituted hydantoin compounds as substrates, D-stereoselective hydrolysis of the hydantoins was, surprisingly, observed together with a simultaneous racemization of the substrate in slightly alkaline pH, thus enabling dynamic kinetic resolution of the synthetic substituted hydantoins to form, for example, *N*-carbamoyl D-*p*-hydroxyphenylglycine (Scheme 1.12). The enzyme responsible for the D-stereoselective hydrolysis of the hydantoin was characterized as a hydantoinase (dihydropyrimidinase). The product of the reaction, namely, *N*-carbamoyl D-*p*-hydroxyphenylglycine, can then be decarbamylated chemically (or as an alternative enzymatically) to form D-*p*-hydroxyphenylglycine. The production process for this D-amino acid was established at Kanegafuchi Chemical (now: Kaneka Corporation) and runs highly enantioselectively in the presence of immobilized whole-cells of a *Bacillus brevis* strain (Scheme 1.12). Notably, quantitative conversion is achieved, and the production volume has been in the range 300–700 tons annually. The condensation reaction of D-*p*-hydroxyphenylglycine, obtained after further cleavage of the *N*-carbamoyl moiety by chemical treatment with sodium nitrite, and 6-APA can be carried out by enzymatic process using a penicillin acylase from *Klebsiella citrophila*.



Scheme 1.12 Microbial production of *N*-carbamoyl D-*p*-hydroxyphenylglycine from the corresponding racemic hydantoin and subsequent classic chemical deprotection.

In summary, by means of (non-genetically engineered) wild-type strains a range of efficient microbial transformations have been realized that, in part, turned out to be suitable for industrial application.

1.4
Chemical Processes with Isolated Enzymes: The Impact of Process Engineering

1.4.1
Historical Development of Transformations with Isolated Enzymes

The historical development of biotransformations in organic synthesis has been mainly driven from two perspectives. The first is based on earlier available fermentation methods applied in food industry and this experience was then used for the production of chemicals. Such microbial processes and their historical development towards biotransformations for synthetic purpose are described and summarized in the previous section. Notably, based on these earlier biotechnological production processes, which have been established, an awareness of the occurrence and function of enzymes arose around the nineteenth century. In 1811, Kirchhoff found that maltose is formed from starch by the action of barley extract [30], and in 1833 Payen and Persoz named the ethanol precipitate active toward starch as "diastase," which means "separation" in Greek [31]. In1836, Schwann named a substance in the human stomach as "pepsin," which hydrolyzes meat [32]. 1860, Berthelot found an invertase activity in the cell-free extract of yeasts. The term "enzyme" was coined in 1876, by Kühne, meaning "in" (en) "the yeast" (zyme) [33].

Thus, besides the microbial-based biotransformation based on an increasing understanding of the occurrence and function of enzymes as molecular catalysts, a second trend began with the development of biotransformations using isolated enzymes. This trend, however, is also a result of the perspective of the chemical industry and in general organic chemists in developing and designing catalytic synthetic processes. A key issue in the development of chemocatalytic processes is the use of a single molecule or salt pair as a catalytic component. Such catalytically active molecules or salt pairs are typically used in isolated and purified form. Accordingly, the development of enzymatic processes based on such a philosophy is based on the use and understanding of single isolated enzymes as "molecular catalysts" rather than microbial systems (being aware that, of course, also in microbial systems the same enzymes are responsible for the catalytic reaction). However, compared to microbial systems, access to isolated enzymes is connected with additional purification efforts that represent a costs factor. Whereas fermentation of biomass as a catalyst is a cheap process, purification of enzymes (dependent on the purification degree) makes the enzyme component increasingly expensive. Owing to such higher costs for manufacturing isolated (purified) proteins over microbial whole-cells obtained as biomass directly from the fermentation, during process development there has been great interest in immobilization and recycling of isolated (purified) enzymes and reaction engineering issues to attain an economically attractive biocatalyst and economically favorable data for the biotransformation (in particular) on a large scale [34].

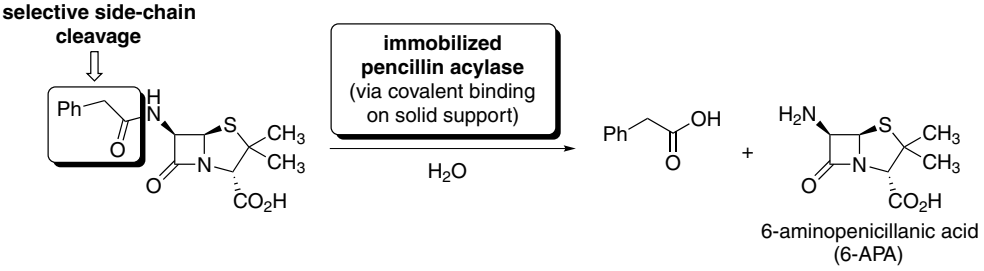
The following describes selected concepts for recycling of isolated enzymes, which enabled the realization of industrial manufacturing processes known today as "landmark processes" in industrial biocatalysis. More recently, due to the tremendous progress in molecular biology and the availability of efficient recombinant

whole-cells as production strains as well as high-cell density fermentation methods, the production of isolated enzymes has also become economically very attractive, at least when using them as a crude extract or in only partially purified form (which often is suitable for biotransformations in contrast to diagnostic purposes). Thus, dependent on the type of recombinant enzyme and degree of purification, even biotransformations without recycling might represent an attractive option for today's large-scale biotransformations. Selected examples of such types of biotransformations with "free," non-immobilized enzymes are described below.

1.4.2
Development of Practical Synthesis of Chemicals via Transformations Using Isolated Enzymes in Immobilized (Solid-Supported) Form

The option of recycling a catalyst has, in general, often been realized by means of immobilization of the catalyst on a solid support, which enables simple separation of the heterogeneous catalyst from the reaction mixture and its synthetic re-use [35, 36]. However, it also should be mentioned that many other techniques for catalyst immobilization have been developed.

As for enzymes, one example of a very successful application of this concept of heterogeneous enzyme catalysis is the established biocatalytic synthesis of 6-amino penicillanic acid (6-APA) [37–39], which is applied with an annual production volume exceeding 10 000 tons per year. The catalytic concept is shown in Scheme 1.13. Compared with the alternative chemical route, the use of an immobilized Pen G acylase enables cleavage of the unwanted side-chain without the need for significant amounts of a range of hazardous chemicals. As a solid support, Eupergit beads turned out to be highly efficient for the Pen G acylase catalyst. Notably, the immobilized enzyme catalyst can be re-used more than 850 times, thus delivering a highly efficient production process and very low overall enzyme loading of the heterogenized enzyme catalyst per kg of 6-aminopenicillanic acid [40].



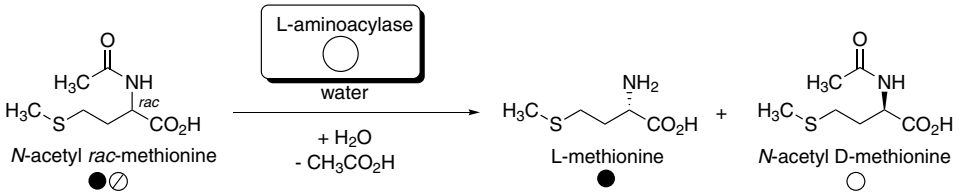
Scheme 1.13 Immobilized penicillin G acylase (Pen G acylase) in the production of 6-APA.

Furthermore, heterogeneous enzyme catalysis has also been carried out very successfully in organic reaction media. For example, when using immobilized lipase, direct ester formation starting from an acid and an alcohol enables efficient formation of the ester in a solvent-free medium. Such a process technology has been industrially established for fatty acid ester manufacture, for example, at Unichema Chemie and Degussa AG (now: Evonik Degussa GmbH) [15–17]. In the

field of racemic resolution, the BASF process for the production of chiral amines is based on an enantioselective acylation when starting from a racemic amine in the presence of an immobilized lipase [41–43]. The production volume of this process technology is in the >1000 tons per year range.

1.4.3
Development of Practical Synthesis of Chemicals via Transformations Using Isolated Enzymes in "Free" Form

The use of solid-supported catalysts, however, also means that there is a switch from a catalytic reaction in a homogeneous reaction medium (as in case of "free" enzymes) towards a heterogeneous reaction medium. Thus, it has been a challenge to develop "immobilization-like" systems that nonetheless enable both (i) the simple separation and re-use of the enzyme component and at the same time (ii) the enzymatic reaction to be carried out under homogeneous reaction conditions. This has been achieved, for example, by means of a so-called enzyme-membrane reactor (Figure 1.3) [44–47]. In an EMR, the enzyme reacts as a "free" enzyme but is prevented from leaving the reactor by a membrane. This membrane has a specific molecular-weight cut-off, that



Process concept of the enzyme membrane reactor for L-amino acid synthesis

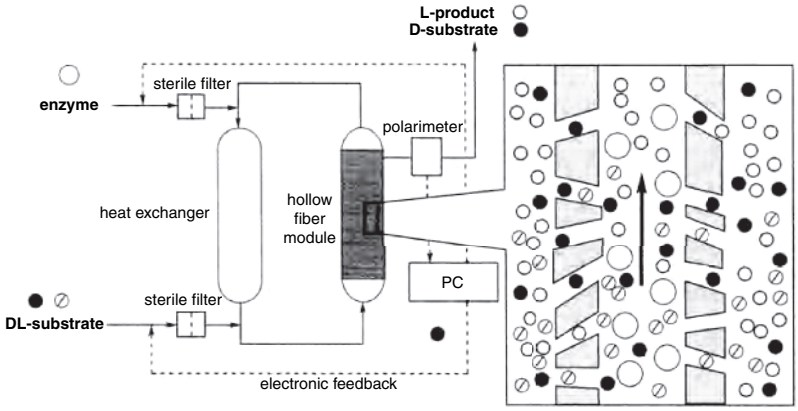
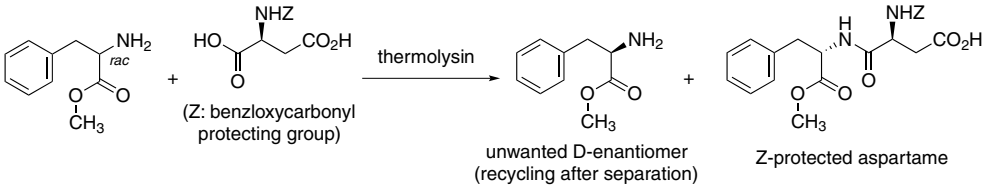


Figure 1.3 Production of L-methionine in an enzyme-membrane reaction (EMR) [47].

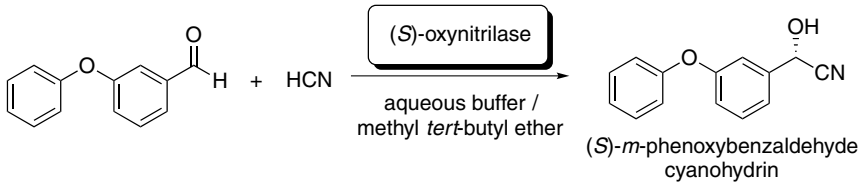
is, only molecules that have a molecular weight below the cut-off can cross the membrane. These membrane-permeable molecules are typically substrate and product. The structure and concept of such an enzyme-membrane reactor is shown in Figure 1.3 [47], exemplified for the synthesis of L-methionine by means of an aminoacylase. Notably, the EMR runs also very successfully on an industrial scale, producing L-methionine on a multi-hundred tons scale annually [45–47]. This industrial process has been developed and established jointly by the Wandrey group and researchers at Degussa AG (now: Evonik Degussa GmbH).

A further elegant example of the combination of organic chemistry with a biocatalyst used in the form of an isolated enzyme is the synthesis of the artificial sweetener aspartame in homogeneous reaction medium (Scheme 1.14) [48, 49]. This process also represents an efficient and well-established industrial process running at about the 2500 tons scale annually. The condensation reaction of the protected aspartate and phenylalanine methyl ester is catalyzed by thermolysin from a *Bacillus* strain in aqueous homogenous medium. In the downstream processing a filtration step is included that ensures enzyme separation and recovery for re-use. The reaction, which proceeds with excellent selectivity (>99.9%), originally had much advantage as an efficient resolution of racemic phenylalanine, until the supply of L-phenylalanine was started by the fermentative method.



Scheme 1.14 Enzymatic synthesis of L-aspartame with thermolysin.

A further process option, which is of particular of interest when producing hydrophobic molecules, is to run the reaction in a two-phase reaction medium consisting of an aqueous phase and a water-immiscible organic solvent. This concept is, of course, not only suitable for enzymes but also for whole-cell catalysts. Whereas the enzymatic reaction proceeds in the aqueous phase, product accumulation takes place in the organic phase. After completion of the reaction, simple phase separation enables recovery of the enzyme (dissolved in the aqueous phase and ready for use in the next reaction run) and isolation of the product (dissolved in the organic phase). A prerequisite for such a process with enzyme recycling is a high stability of the enzyme towards the reaction medium and substrates/products, leading to sufficient remaining enzyme activity after the biotransformation and phase-separation steps. Today many enzymes fulfill this prerequisite. Notably, among them are not only hydrolases but also enzymes from other enzyme classes.



Scheme 1.15 Hydrocyanation process with "free," non-immobilized enzymes.

A selected example of such a process concept with non-immobilized enzymes is the hydrocyanation of *m*-phenoxybenzaldehyde in the presence of a recombinant oxynitrilase from *Hevea brasiliensis* (Scheme 1.15) [50–52]. This biotransformation, leading to an asymmetric C−C bond formation, which has been developed by the Griengl group, has found an industrial application at DSM, running at a hundred tons scale per year. Notably, an impressive space–time yield of 1 kg l^{-1} day^{-1} has been achieved. The enantioselectivity is also excellent, leading to the desired *(S)*-cyanohydrin with 98.5% e.e.

Thus, today, various options are readily available for the development of efficient biotransformations with isolated enzymes. These methodologies consist of the use of enzymes in immobilized, solid-supported form or as "free" enzymes. In the latter case, enzyme separation from the reaction mixture and re-use for the next synthetic cycle can be realized by means of, for example, enzyme-membrane reactor technology or the use of two-phase systems and a phase-separation after the reaction is completed.

1.5
Towards Tailor-Made Enzymes: Principles in Enzyme Screening and Protein Engineering Methodologies

In the following, recent remarkable examples in the screening for new enzymes and enzyme evolution are introduced and their merits discussed. In particular, the history of enzyme discovery will be covered, consisting of screening strategies for enzymes, for example, via enrichment culture technique, stock cultures, *in silico* screening, and modeling of proteins. In Figure 1.4 a flowchart of the use of tools for the discovery and development of industrial enzymes is shown.

1.5.1
Tools for Enzyme Discovery

The success in microbial transformation has been based on the screening for microbial enzymes catalyzing new reactions or by screening known enzymes for an unknown activity with synthetic substrates. Taking into account the advantages of using enzymes in organic synthesis (which have been in part been described in the sections above and which are illustrated in detail in the individual chapters of this

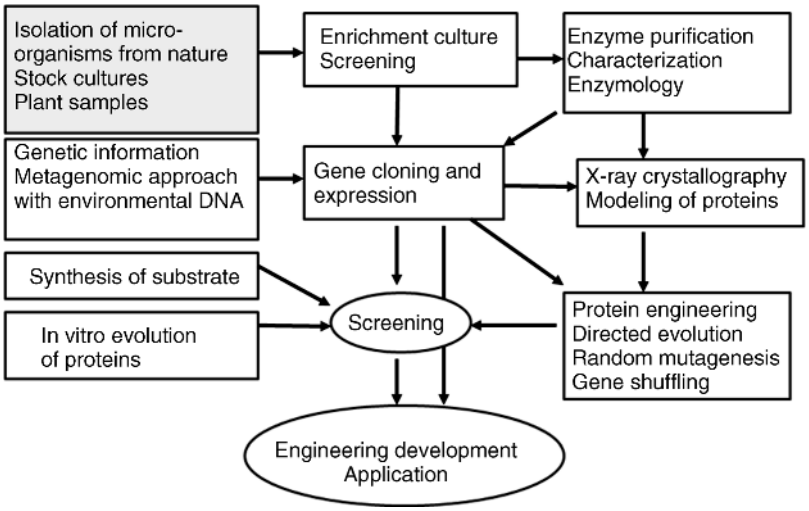


Figure 1.4 Flowchart of the use of tools for discovery and the development of industrial enzymes.

book), more and more attention has been paid to the systematic exploitation of new enzyme reactions and how to obtain enzymes with desired activities from various enzyme sources and databases. In screening enzymes, it is extremely important to make clear what the purpose of the experiment is. One would screen microorganisms or databases of enzymes for an enzyme when (i) nothing is known about the specific reaction but a homologous enzymatic reaction of the same category is known (substrate specificity), (ii) novelty is necessary and the same enzyme is known only in other biological sources, (iii) improved function, such as a better productivity, heat-, solvent-, pH stability, and so on for practical use is required, and even when (iv) almost no information is available for the desirable reaction.

Different strategies for enzyme screening are conceivable. One can simply buy an enzyme from the suppliers or clone the known gene by polymerase chain reaction (PCR) according to the information given in the genome database on the internet, and express it in a versatile heterologous host, such as *E. coli*. On the other hand, if one would like to establish an entirely new enzymatic industrial process, there should not be a similar case in the literature and therefore the enzyme reaction should be unique. This strategy is extremely interesting because such basic studies often accompany the discovery of unforeseen biological phenomena and new materials hidden in nature. Thus, a successful screening should focus on what is new in the screening: substrate, product, gene, protein, property, screening source, method, and so on [53].

Enzymes need to be made more robust under harsh conditions in order to further expand their use in the varieties of practical chemical reactions. Because several substrates need to be checked for the transformation, the enzymology established with a certain enzyme for a physiological substrate sometimes does not supply enough information. The enantioselectivity shown by the enzyme is one of the most

important properties, although such information has been sometimes limited because of, e.g., the lack of the supply of synthetic substrates and analyses of the products by fine analytical methods.

Enrichment culture is a technique to isolate microorganisms having special growth characteristics. It is estimated that there are 10^8 living microorganisms in a 1-g soil sample. Many industrial strains or new reactions have been discovered with this technique [54]. Some microorganisms grow faster than others in media with limited nutrients, high temperature, extreme pH values, and so on. Microorganisms that grow faster than other species become dominant after several transfers of the culture. An adaptation to a synthetic medium containing a target compound often results in the isolation of microorganisms having a new enzyme to degrade the sources for their growth. For example, industrial acrylamide producers *Pseudomonas chlororaphis* B23 [55] and *Rhodococcus rhodochrous* (formerly *Arthrobacer* sp.) J-1 [56] were isolated by using nitrile compounds as carbon and nitrogen sources, or just as a nitrogen source. Nitrile hydratase was discovered from *Rhodococcus rhodochrous* J-1 [26].

1.5.2
Protein Engineering Methodologies

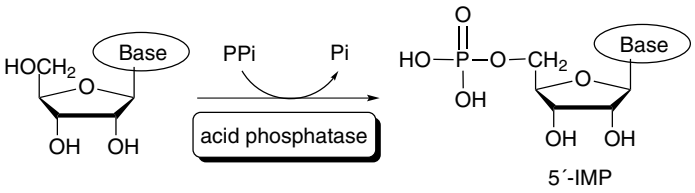
Protein engineering is the term given to the alteration of the primary structure of proteins or enzymes by substituting amino acid residues by changing the codon of its corresponding DNA. It has become possible as recombinant DNA techniques and structures obtained by X-ray crystallography of the enzymes have become widely available. The amount of information on the DNA and primary structures of the proteins and the X-ray structures is so huge that it is now available through databases on the internet. The key residues causing hydrogen bonding, hydrophobic, and electrostatic effects around the active sites are often subjected to alteration to different amino acids. Furthermore, by "saturation mutagenesis," the single residues can be changed to one of 19 other residues. These point mutations are very effective in the analysis of specific residues in proteins by changing their properties, although the effect of the specific changes to the structure and the properties of the enzymes often lack additivity and is still complex and sometimes unpredictable. Therefore, satisfactory results for applications have often been obtained by a combination of mutagenesis and screening of the mutants, similar to the screening that had been made for the wild-type microorganisms or enzymes from the nature. The screening has been extended to high-throughput screening by the development of robotics such as colony pickers, various kinds of distributors, mini-scale cultivation, followed by monitoring by micro plate readers, along with miniaturization of the biochemical reactions and the use of appropriate indicators [57].

Directed evolution has become employed for the mutagenesis of enzymes since the commercial availability of the thermal cycler and DNA polymerase from thermophilic microorganisms for PCR in the late 1980s to early 1990s [58, 59]. The gene for the enzyme is randomly mutated by error-prone PCR, in which the DNA polymerase reaction is staggered by the inadequate reaction conditions such as very

low concentration of some of the substrate dNTP, or using Mn^{2+} instead of Mg^{2+} in the reaction mixture. The mutated genes are expressed in appropriate hosts such as *E. coli*, or *in vitro* by DNA translation system from various biological sources. A number of the *E. coli* transformants are picked up manually or by a machine called a colony picker and assayed by various high-throughput robotic systems, with microtiter plates of 96-wells or more. By choosing the best candidate as a parent, the next cycle of mutagenesis is begun.

In DNA shuffling, which causes much mutation in PCR, the DNA-catalyzed reaction is not started with primers but only with fragmented DNA molecules partly digested with DNase I. Annealing reactions proceed with partially overlapped DNA fragments and, finally, DNA of the same original size is constructed, creating mixtures of DNA with different mutation sites. Stemmer *et al.* described an example of generating TEM-1 β-lactamase that showed 16 000 times resistance (MIC (minimum inhibitory concentration) 320 $\mu g\,ml^{-1}$) against the wild-type enzyme (MIC 0.02 $\mu g\,ml^{-1}$) [60]. DNA shuffling of a family of genes from diverse species accelerates directed evolution. Family shuffling is a more powerful method for making libraries of chimeric genes by random fragmentation of a pool of related genes, by combining useful mutations from individual genes. Moxalactamase activity from four cephalosporinase genes evolved separately from a mixed pool of the four different genes. A single cycle of shuffling yielded 270- to 540-fold improvement from the four genes shuffled together. The best clone contained eight segments from three of the four genes as well as 33 amino-acid point mutations [61].

In the following, as a case study, the successful combination of a classic screening with a subsequent directed evolution as a highly efficient tool for enzyme optimization is shown, and exemplified for a new industrial enzymatic method of selective phosphorylation of nucleosides for the production of, for example, inosine-5′-monophosphate (5′-IMP). This is one of the first examples of the application of directed evolution for an industrial reaction combined with conventional screening for a very selective phosphorylation reaction, catalyzed by an acid phosphatase. Scheme 1.16 illustrates the corresponding target reaction.



Scheme 1.16 Acid phosphatase-catalyzed synthesis of 5′-IMP.

Inosine-5′-monophosphate (5′-IMP) and guanosine-5′-monophosphate (5′-GMP) are important nucleotides because they give foods the "umami" taste. There is no taste in other isomers such as 2′- and 3′-inosinic acids. Two phosphorylation methods have been reported. One is a chemical phosphorylation process that uses phosphoryl chloride ($POCl_3$) and the other is an enzymatic process that uses inosine kinase. To attain a greener and newer enzymatic method to produce 5′-IMP, microorganisms

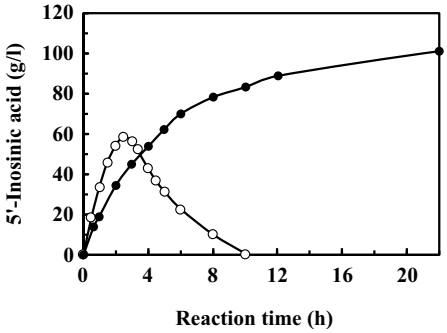


Figure 1.5 Time course of the synthesis of 5′-inosinic acid monophosphate with (o) wild-type enzyme and (•) mutant.

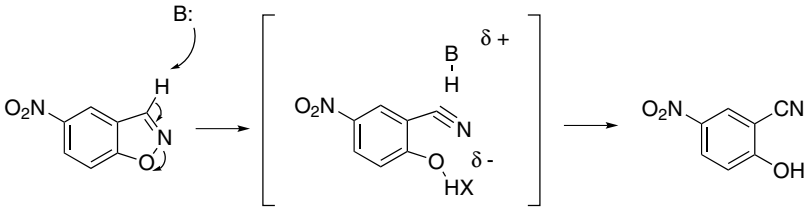
(3000 strains) that phosphorylate nucleosides using pyrophosphate (PPi) as the phosphate donor regioselectively at the 5′-position were screened (Figure 1.5) [62]. Although many of the microorganisms screened were able to phosphorylate inosine, phosphotransferase activity specific to the 5′-position was found to be distributed among bacteria belonging to the family Enterobacteriaceae. *Morganella morganii* NCIMB 10 466 was selected as a 5′-IMP producer.

A selective nucleoside phosphorylating enzyme was purified to homogeneity from *M. morganii* NCIMB 10 466 crude extract. The enzyme appeared to consist of six subunits identical in molecular weight (M_r, 25 000). It phosphorylated various nucleosides at the 5-position to produce nucleoside-5′-monophosphates using PPi as the phosphate source. Energy-rich compounds, such as carbamylphosphate and acetylphosphate, were also very effective phosphate donors. The enzyme also exhibited phosphatase activity, and dephosphorylated various phosphate esters, but had a weak effect on nucleoside-3′-monophosphates. The *M. morganii* gene encoding a nucleoside phosphorylating enzyme was isolated by a shotgun-cloning strategy. It was identical to the *M. morganii* PhoC acid phosphatase gene. Using the purified enzyme, 32.6 mM 5′-IMP was synthesized from inosine with a 41% molar yield, but the synthesized 5′-IMP was hydrolyzed back to inosine due to its phosphatase activity as the reaction time was extended.

To suppress the dephosphorylation reaction and increase the efficiency of the transphosphorylation reaction, a random mutagenesis approach was used. By error-prone PCR, one mutated acid phosphatase that increased the phosphotransferase reaction yield was obtained. With the *E. coli* overproducing the mutated acid phosphatase, 101 g l^{-1} (191 mM) of 5′-IMP was synthesized from inosine in 85% molar yield [63]. This improvement was achieved with two mutations, Gly to Asp at position 92 and Ile to Thr at position 171. A decreased K_m for inosine was responsible for the increased productivity. Ile151Thr caused the improvement of the affinity toward inosine, and the mutation at Gly72Asp caused not only increasing affinity toward inosine together with Ile151Thr, but also lowered the dephosphorylating activity. An X-ray analysis of the crystal of the enzyme revealed two mutation sites located near the active site, and the Thr151 seems to form hydrogen bonds with inosine and enhance the affinity towards

inosine [64]. Now, 5′-IMP and 5′-GMP have been produced in multi-thousand tons per year since 2003 by Ajinomoto Co. Inc., Japan.

A further efficient tool for protein design and engineering is based on computational methods. Here a range of successful modeling work has been done, leading to impressive enzyme properties. Details about rational protein engineering are given in Chapters 3 and 4. In the following the power of rational protein design and engineering is exemplified by the exciting development of a new, *de novo* designed enzyme suitable for the Kemp elimination, a reaction not found in biological systems. This recently reported computationally designed enzyme uses two different catalytic motifs to catalyze the Kemp elimination (Scheme 1.17) [65].



Scheme 1.17 Kemp elimination reaction.

These results demonstrate the power of combining computational protein design with directed evolution for creating new enzymes, and we anticipate the creation of a wide range of useful new catalysts in the future. The first step for designing new enzymes is to assume a catalytic mechanism and then to use quantum mechanical transition state calculations to create an idealized active site, with protein functional groups positioned so as to maximize transition state stabilization. The next step is the use of software to search for protein backbone positions capable of supporting these idealized active sites from already reported high-resolution natural crystal structures. Subsequently, residues surrounding the transition state are redesigned to maximize the stability of the active site conformation. It was found that TIM (triose phosphate isomerase) barrel scaffolds were enriched in the computer search. Eight of the designs showed measurable activity in the Kemp elimination. Scaffolds of indole-3-glycerolphosphate synthase from *Sulfolobus solfataricus* and deoxyribose-phosphate aldolase from *Escherichia coli* were virtually chosen. Furthermore, enzymes with a better activity were generated by an *in vitro* evolution procedure. It represented a rate enhancement of 1×10^6 over the spontaneous reaction in a solution. The structures of the enzymes were solved.

1.6 "Hybridization" of Enzyme Catalysis with Organic Syntheses: New Opportunities for Industrial Production of Chemicals and Drugs

This section presents a selection of more recent trends in enzyme catalysis that already have had or which (presumably) will have an impact on organic synthesis and

technical applications, while being aware that there will be numerous other interesting application areas of enzymes as catalysts in organic synthesis as well (which are also covered in different chapters of this book).

1.6.1 Applications of Tailor-Made Recombinant Whole-Cell Catalysts in Organic Synthesis

The tremendous progress made in molecular biology (as discussed in the previous section) also had a significant impact on process development with biocatalysts. Some trends resulting from these impressive achievements are given here. The opportunity to optimize enzyme properties by protein engineering combined with overexpression in recombinant host organisms and high-cell density fermentation for their production enables access to both economically and synthetically highly attractive catalysts. Thus, there has been an increasing tendency in organic chemistry to directly use such types of tailor-made recombinant whole-cell catalysts, so-called "designer cells" or "designer bugs" in organic (asymmetric) synthesis. Such recombinant whole-cell catalysts have gained a high level of popularity in particular in the field of those processes where more than one reaction is carried out, since two or more enzymes are overexpressed in the recombinant host strain. Thus, only one fermentation process is required to produce the required enzymes. Furthermore, the biomass represents the cheapest form of an enzyme, and costly enzyme purification steps can be avoided. Figure 1.6 give a schematic comparison of process unit operations of a whole-cell biocatalyzed (redox) process and an analogous process in the presence of isolated enzymes [66], exemplified for the biocatalytic redox process in which two enzymes as well as a cofactor are required.

Biocatalytic production processes that require more than one enzyme are, for example, the multistep transformation of a hydantoin into an L- or D-α-amino acid and

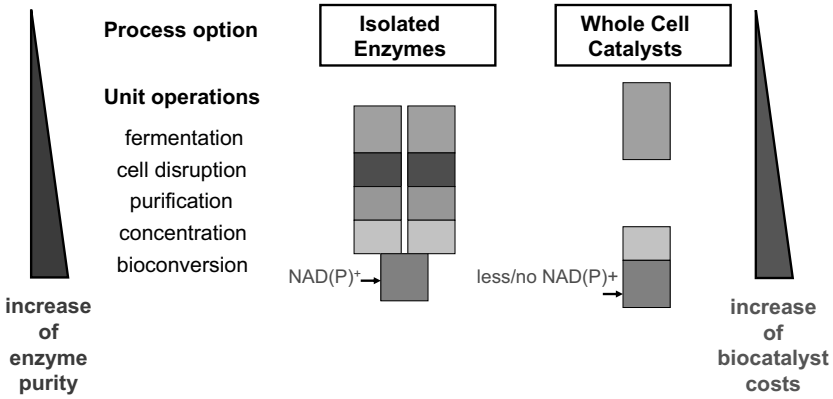
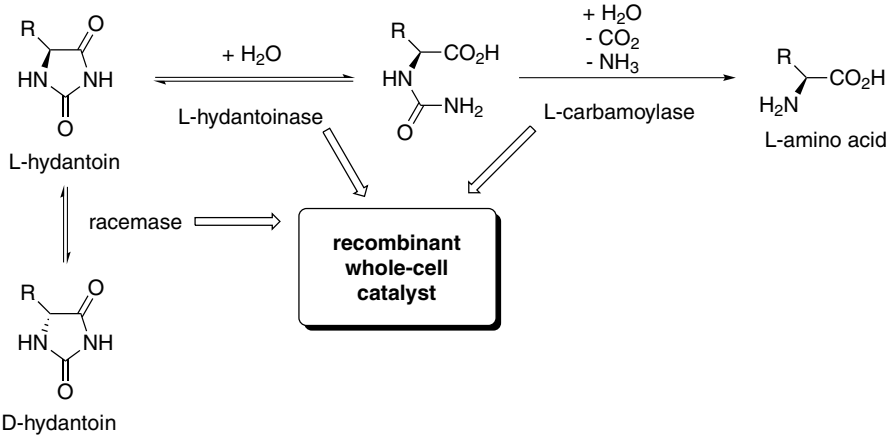


Figure 1.6 Overview of process unit operations when using recombinant whole-cells and when using isolated enzymes in a redox process [66].

the reduction of ketones towards chiral secondary alcohols. Both processes have already been conducted in a very efficient way by means of recombinant whole-cell biocatalysts and in the following these two types of processes are described in more detail.

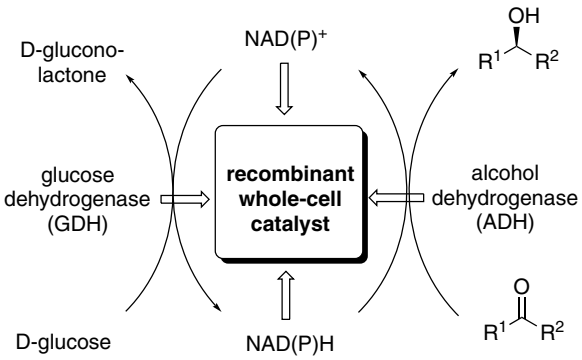
Starting with the biocatalytic dynamic kinetic resolution of hydantoins with a recombinant whole-cell biocatalyst, this process is based on three enzymatic steps, requiring a racemase, hydantoinase, and carbamoylase [67, 68]. Overexpression of all three enzymes in an *E. coli* host organism delivered a highly efficient catalyst for this process, enabling excellent productivity data. Such a process technology, enabling access to L- as well as D-α-amino acids, has been established on an industrial scale at Degussa AG (now: Evonik Degussa GmbH). Scheme 1.18 shows such a process schematically for the synthesis of L-α-amino acids. Since – in contrast to wild-type microorganisms – different strains can represent the original sources of the three enzymes, the most suitable enzyme from all strains for each step can be utilized as preferred enzyme component in overexpressed form in the recombinant host organism.



Scheme 1.18 Dynamic kinetic resolution of hydantoins using a recombinant whole-cell catalyst.

The use of recombinant whole-cell catalysts has also attracted high interest for the asymmetric reduction of ketones, leading to secondary alcohols in enantiomerically pure form (>99% e.e.) [69]. The desired reduction of the ketone is catalyzed by an alcohol dehydrogenase, requiring NAD(P)H as a cofactor and reducing agent. The oxidized cofactor $NAD(P)^+$ is subsequently reduced and recycled to NAD(P)H, which then can be used for the next catalytic cycle. One option for the *in situ* cofactor recycling is based on the use of a glucose dehydrogenase, which oxidizes D-glucose to D-gluconolactone, thus recycling the required cofactor. It turned out that a recombinant *E. coli* strain, containing an alcohol dehydrogenase and a glucose dehydrogenase in overexpressed form, represents a highly efficient, tailor-made biocatalyst. Such whole-cell catalyzed reductions of ketones have been established at, for

example, Kaneka and Degussa AG (now: Evonik Degussa GmbH), and different process options can be used for this type of process (e.g., in the presence or absence of an organic solvent) [69–72]. Notably, such processes run at a high substrate input, exceeding 100 g l^{-1}, and lead to excellent productivity data. The process concept is shown in Scheme 1.19 (and for process unit operations of the overall concept, including access to the biocatalyst, see Figure 1.6 and the discussion above).



Scheme 1.19 Asymmetric ketone reduction using a recombinant whole-cell catalyst.

A range of other types of efficient whole-cell catalyzed processes have been reported as well, for example, the reductive amination of α-keto acids [66, 73, 74] and reduction of C=C double bonds [75, 76] as further technologies applied on a technical scale.

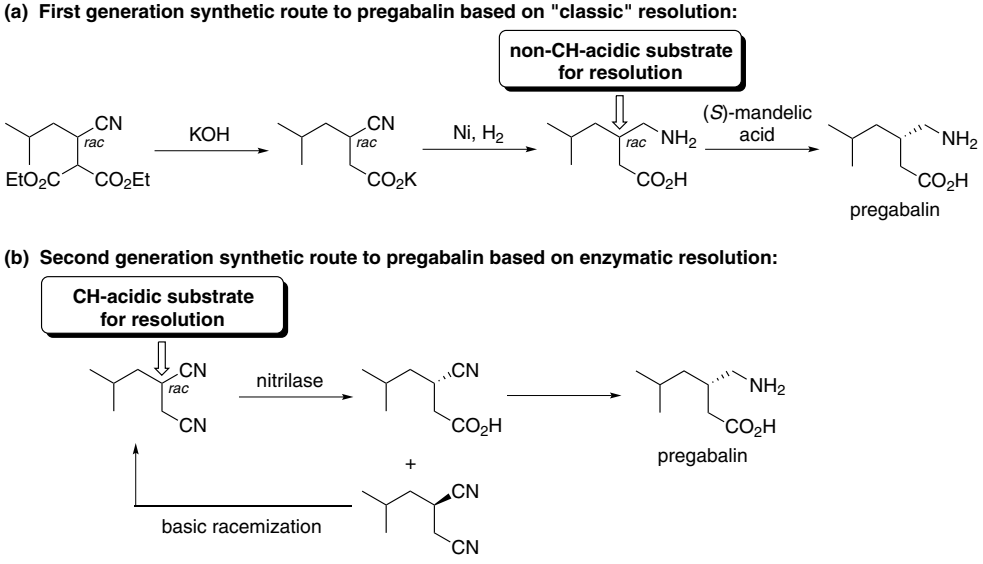
Thus, in such modern biotransformations with recombinant whole-cells one can see a "renaissance" of whole-cell catalysis with high industrial impact, since major limitations when using wild-type strains such as low substrate input, formation of side-products, and limited volumetric productivity can be overcome by means of tailor-made recombinant whole-cell catalysts.

1.6.2
Novel Retrosynthetic Approaches in Drug Synthesis: From Enzyme Catalysis in Chemoenzymatic Multistep Processes towards New Drug Production Pathways in Industry

A major application area of enzyme catalysis is the synthesis of pharmaceuticals and intermediates thereof, since such molecules often are chiral and, at the same time, enzymes often show excellent stereoselectivities [77, 78]. Despite this "ideal fit" of synthetic requirement and enzyme stereoselectivity properties, the number of enzyme-catalyzed processes in organic syntheses has been rather limited for a long time compared with classic chemical or chemocatalytic syntheses. One reason that might represent in part an explanation for this is, on first glance, the surprising observation that retrosynthetic strategies in natural product or drug synthesis have often been designed mainly based on classic organic synthetic routes without

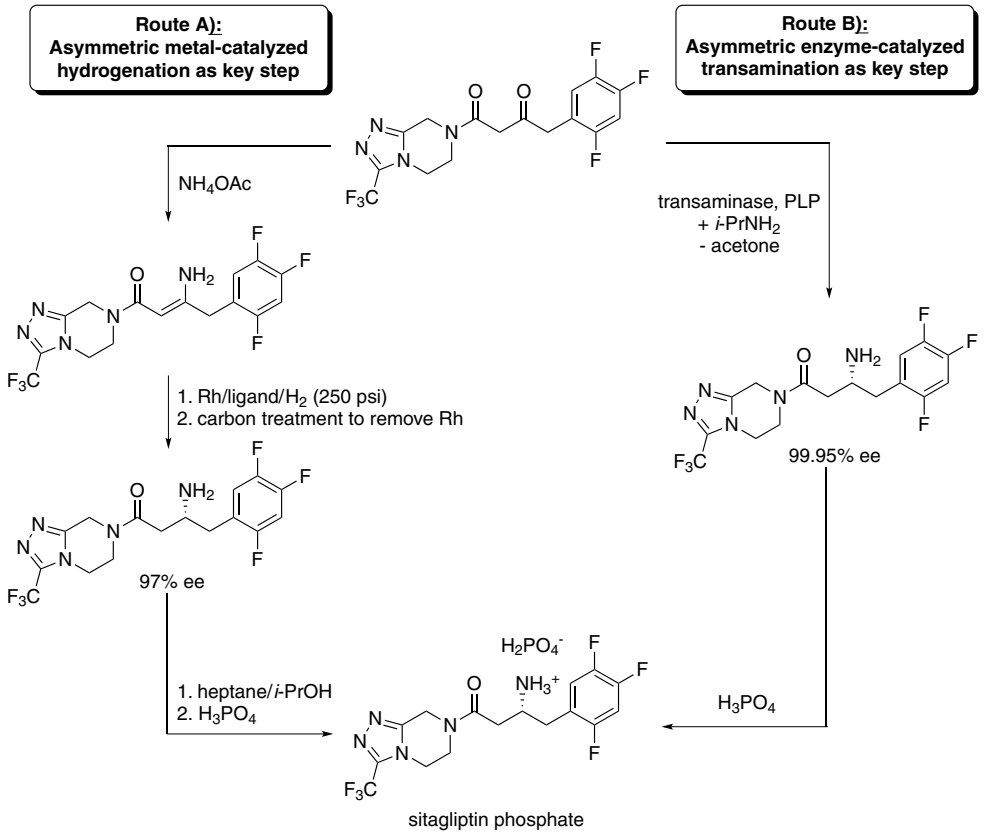
consideration of biocatalytic key steps. This might be because biocatalysis still needs to be more implemented as a "standard synthetic tool" in teaching courses on organic chemistry. Another reason for the limited use of enzymatic steps in natural product and drugs synthesis is that for a long time there was a lack of process efficiency of enzymes despite the excellent stereoselectivities (such as, for example, low volumetric productivities). Although highly efficient processes have long been reported mainly when using hydrolases as catalysts (and in part oxidoreductases), other enzyme classes have been used to a lesser extent with respect to the development of highly efficient production processes.

However, in the last two decades tremendous progress has been made to overcome these two above-mentioned limitations. In particular for pharmaceuticals exciting retrosynthetic approaches have been designed that are now based on enzymatic key steps (a selected example is given below; Scheme 1.20). In addition, process efficiencies have also been increased in a broad range of biocatalytic processes with enzymes other than hydrolases. Today, besides hydrolases-catalyzed syntheses a range of other industrially applied reactions based on the use of oxidoreductases, transferases, lyases, and isomerases are known. A very recent selected impressive example (shown in Scheme 1.21) is also discussed below.



Scheme 1.20 (a) First- and (b) second-generation multistep route to pregabalin.

Starting with the implementation of biocatalytic reactions as key steps in retrosynthetic approaches for drugs synthesis, an impressive contribution has been, as an example, by Tao and coworkers in developing alternative pathways towards pregabalin as the active pharmaceutical ingredient (API) of Pfizer's neuropathic pain drug



Scheme 1.21 Comparison of original metal-catalyzed and new biocatalytic route to sitagliptin.

Lyrica® (Scheme 1.20) [79, 80]. The original synthetic route, shown in Scheme 1.20, starts from a β-cyano-malonate, which is then transformed by classic chemical synthetic steps into the desired racemic γ-amino acid. The final step is a "classic" resolution based on diastereoselective salt formation with a chiral acid. By means of this method pregabalin was obtained in an overall yield of 20%. Its main disadvantage is the lack of a suitable racemization for the unwanted enantiomer, since the β-amino acid is difficult to racemize due to the lack of a C-H-acidic functionality at the β-position. This has been addressed in a "second-generation" route as an improved alternative, which is based on a regio- and enantioselective hydrolysis of isobutyl-succinonitrile as the starting material in the presence of a nitrilase as a biocatalyst, leading to the required intermediate in 45% yield and with >97% e.e. This strategy turned out to be superior to the first route since the resolution process now leads to an unwanted enantiomer that can be easily racemized under basic conditions due to the presence of an α-C-H-acidic functionality at the stereogenic center. Thus, this chemoenzymatic multistep synthesis of pregabalin is a very elegant approach,

combining an efficient enzymatic resolution with the idea of implementing the resolution at an early stage, thus enabling racemization of the unwanted enantiomer. Notably, in addition, this example underlines the huge diversity of enzyme catalysis with respect to resolution processes. Whereas enzymatic resolution of nitriles is catalyzed enantio- and regioselectively by nitrilases, chemical resolution methods for nitriles are less explored (compared with amines and acids).

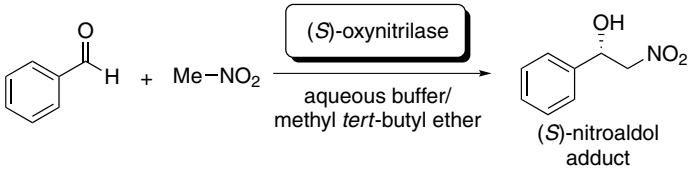
The next example addresses the tremendous progress that has been made to make enzymes suitable for the highly competitive industrial synthesis of complex, pharmaceutically relevant molecules. Without doubt, one of the highlights in recent years has been the development of a chemoenzymatic production process for the drug sitagliptin phosphate by Merck and Codexis researchers (Scheme 1.21) [81, 82]. Notably, this enzymatic process, based on a transaminase as a biocatalyst, has turned out to be advantageous over the previously developed and also industrially established chemocatalytic alternative. In the chemical synthesis, using an asymmetric metal-catalyzed hydrogenation as a key step, first the ketone used as a starting material is transformed into an enamine, which is subsequently hydrogenated enantioselectively. The final step consists of salt formation of the drug sitagliptin phosphate. Despite an excellent hydrogenation process, the whole synthetic route possesses two drawbacks: First, direct transformation of the ketone into the amine instead of a two-step process with an enamine intermediate formation would be more desirable. Even more important, however, is having a heavy-metal catalyzed process nearly at the end of the multistep synthesis; the need to remove metal traces from a drug intermediate at a late stage is tedious and disadvantageous. These drawbacks have been solved by applying a direct enzymatic transformation with a transaminase, allowing direct conversion of the ketone substrate into the desired amine. Furthermore, heavy metals (required as catalyst component in the chemocatalytic asymmetric hydrogenation step) are no longer involved. A major challenge, however, had to be solved to realize this process, namely, the development of a transaminase showing sufficient activity for the sterically demanding ketone substrate. The enzyme optimization carried out also underlined today's tremendous opportunities in protein engineering. Starting from a wild-type enzyme showing negligible activity for the ketone substrate, eleven mutation rounds led to a highly efficient mutant. In the presence of this optimized enzyme, a process has been realized that runs at impressive substrate input and leads to the desired product with the excellent enantioselectivity of 99.95% e.e. A detailed comparison of the chemical and biocatalytic routes, describing the significant advantages biocatalysis offers, has been reported recently [82].

These two recent examples in the field of chemoenzymatic multistep drugs synthesis underline the tremendous potential of enzyme-catalyzed processes for the multistep synthesis of complex molecules such as drugs and natural products. Thus, in future an increasing tendency to integrate biocatalytic key steps into multistep routes to such molecules can be expected, thus contributing to the development of both economically attractive and sustainable production processes.

1.6.3
Recent Aspects of Applications of Enzymes in Organic Synthesis

Furthermore, there is a range of other emerging fields within enzyme catalysis, for example, running biocatalytic processes in non-conventional media, the development of one-pot multistep processes by combining enzymatic reactions or enzymatic and chemocatalytic reactions, and enzymatic promiscuity in organic synthesis. The latter research area is briefly described here as a representative example of the many exciting emerging research areas in biocatalysis by two selected processes.

The term "enzyme promiscuity" is used to describe unusual reactions enzymes are able to catalyze besides their catalytic properties known from natural processes. One type of "enzyme promiscuity" refers to new reaction types, in particular "non-natural" reaction types that an enzyme turned out to be able to catalyze. It has been exciting to see that by means of enzymes organic reactions such as, for example, the nitroaldol reaction or hydroformylation can be conducted. Different strategies to obtain access to such enzymes have been studied. Often, the starting point was an enzyme that catalyzes a reaction with a similar reaction mechanism in nature. The usefulness of this strategy has been demonstrated successfully, for example, by the Griengl group for the development of the first enzymatic nitroaldol reaction, also known as the Henry reaction (Scheme 1.22) [83–85]. As enzymes, oxynitrilases were studied that catalyze the addition of cyanide as a nucleophile to aldehydes. It, however, also turned out that an oxynitrilase from *Hevea brasiliensis* can accept nitromethane and nitroethane as a nucleophile. The mechanism of both reactions is comparable, since both additions are based on activation of the donor by proton abstraction of the α-C-H-acidic donor. Although catalytic activity is lower when using nitromethane compared with hydrogen cyanide, this enzymatic nitroaldol reaction is an elegant example of a successful expansion of an enzymés reaction range based on the rational concept of applying knowledge of the mechanisms of organic reactions.



Scheme 1.22 Enzyme promiscuity in an asymmetric nitroaldol reaction.

A different approach of enzymes is based on rational protein engineering (see also section above). The starting point for such approaches can be, for example, a model based on a reaction mechanism (followed by a subsequent design of the enzyme by molecular modeling) or a three-dimensional structure of the enzyme and subsequent optimization by means of a rational and experimental approach. The latter strategy has recently led to an enzyme capable of catalyzing hydroformylation as an example for an industrially important non-natural reaction, when starting from a carbonic

anhydrase as a starting point in this rational protein engineering study and replacing the zinc center by rhodium as a metal component in the active site [86].

1.7 Summary and Outlook

In conclusion, due to impressive interactions between biology, chemistry, and engineering in recent decades enzyme catalysis has become an attractive synthetic tool in organic chemistry, thus complementing existing classic chemical and chemocatalytic approaches. Today a broad range of organic reactions such as, for example, redox reactions, hydrolytic reactions, and C−C bond formations can be carried out very efficiently by means of enzymes. Furthermore, enzyme catalysis has developed towards a broadly applied production technology in the chemical industry, in particular in the fields of fine chemicals and pharmaceuticals.

In future, it can be expected that we will see many more biocatalytic reaction types running in a highly efficient manner, thus being suitable for industrial-scale applications, too. It also can be expected that besides optimization of known biocatalytic reactions expansion towards new type of reactions types will be possible by means of protein engineering techniques. Another challenge in the future will be the further implementation of biocatalytic reactions into multistep synthesis of (chiral) building blocks such as pharmaceuticals. This field consists of the development of alternative retrosynthetic approaches to drugs based on biocatalytic key steps as well as the development of multistep one-pot syntheses with biocatalytic reactions. Further improvements in molecular modeling of enzyme-catalyzed syntheses can be expected as well, thus also enabling an increased number of biocatalytic processes based on enzymes optimized or designed by rational protein engineering.

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