Chimia 53 (1999) 613–616
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ISSN 0009–4293

# The Development of Enzymes for the Preparation of Chemicals

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*Abstract.* The use of enzymes as catalysts for the preparation of novel organic molecules is becoming more widespread every year. The science of biotransformation has matured, and biocatalysts now stand alongside other forms of catalysts to be considered as viable options for the promotion of a particular reaction. Of great importance for the breakthrough of biocatalysts was the availability of enzymes and the increasing demand for enantiomerically pure compounds. Especially, hydrolytic enzymes, lyases, and oxidoreductases are of technical importance today. The potential of enzymes for the synthesis of chemical compounds is by no means exhausted, and the enormous diversity of microorganisms is an almost unlimited pool for new enzymes. In this paper, different strategies are discussed to find and develop new enzymes for the application in organic synthesis.

## 1. Introduction

A great challenge for the chemical industry within the next decades is to develop selective and sustainable processes. A contribution to this task is made by biocatalysis, because biochemical conversions are selective and performed under mild conditions. More than 120 processes have been documented in the literature [1]. A few well-known examples are: the production of acrylamide, nicotinamide, optically active amines, and unnatural amino acids, such as D-*tert*-leucine and (*R*)-pantolactone. The major requirements for a biocatalyst in industry are selectivity, activity, and stability. Often, enzymes are used because of their high enantio- and regioselectivity. However, the catalyst has to convert unnatural substrates, and frequently acceptance of a broad range of substrates is desired. From an economic point of view it is most important that the stability against substrates, products, or even against organic solvents is high.

Once a valid target process is identified, one of the challenges is to develop the process in a minimum amount of time. This does not only include the identification and optimization of the enzymes, but also establishing the synthesis of substrates and the product isolation. One also has to keep in mind that the biocatalytic step is often embedded in a series of steps of classical chemical synthesis. Finally, the entire process has to be economically feasible. The less special equipment is needed, the likelier is the overall success.

Today, most products are specialities or high-value products, such as intermediates for the pharmaceutical and agricultural industry. Especially amines, amino alcohols, diols, alcohols, and carboxylic acids are in demand. To access the segment of bulk chemicals, stable biocatalysts are necessary, and easy-to-use robust redox enzymes have to be developed. One example is the dehalogenase developed by *DOW* for the propylenoxide-process.

## 2. How to Find and to Develop an Enzyme

If you are lucky, you find a suitable enzyme in the catalogue of an enzyme supplier or a reference in the literature that a certain microorganism exhibits the desired activity. If you are lucky one more time, the strain is deposited in a strain collection.

However, usually, the desired enzyme is not available and you have to find it yourself (*Fig. 1*). This means one has to screen and select a microorganism with the right activity from nature.

An alternative method is to extend the potential of a known enzyme based on the knowledge of its mechanism by choosing appropriate reaction conditions.

More recent possibilities are rational and evolutionary strategies to improve an enzyme.

The basis for new biocatalysts is the enormous diversity of organisms. Today, about 5'000 species of bacteria are described, but the estimated number is about 1'500'000. From an estimated number of 3'000'000 fungal species, only 70'000 species are described. With traditional techniques, microorganisms were enriched by linking the desired reaction to the metabolism of the organisms, *e.g.*, with a selected nitrogen or carbon source. Thus, microorganisms are only able to grow when they can use the supplied substrate. Once a pure culture is at hand, one has to verify that the desired reaction is performed by an enzyme with the right specificity. This is a time-consuming and tedious task, because quite often, several hundreds of microorganisms have to be isolated and characterized in detail. The next step is either developing the strain into a high-performance strain with a large space-time yield, or purifying, cloning, and expressing the enzyme in a host organism. To obtain a

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biocatalyst, studies on the fermentation of the production strain, immobilization or cross-linking of the enzyme, and process scale-up are necessary. Thus, it can take several years until a biotransformation process is implemented on a production scale.

The following examples illustrate the development of a biocatalyst.

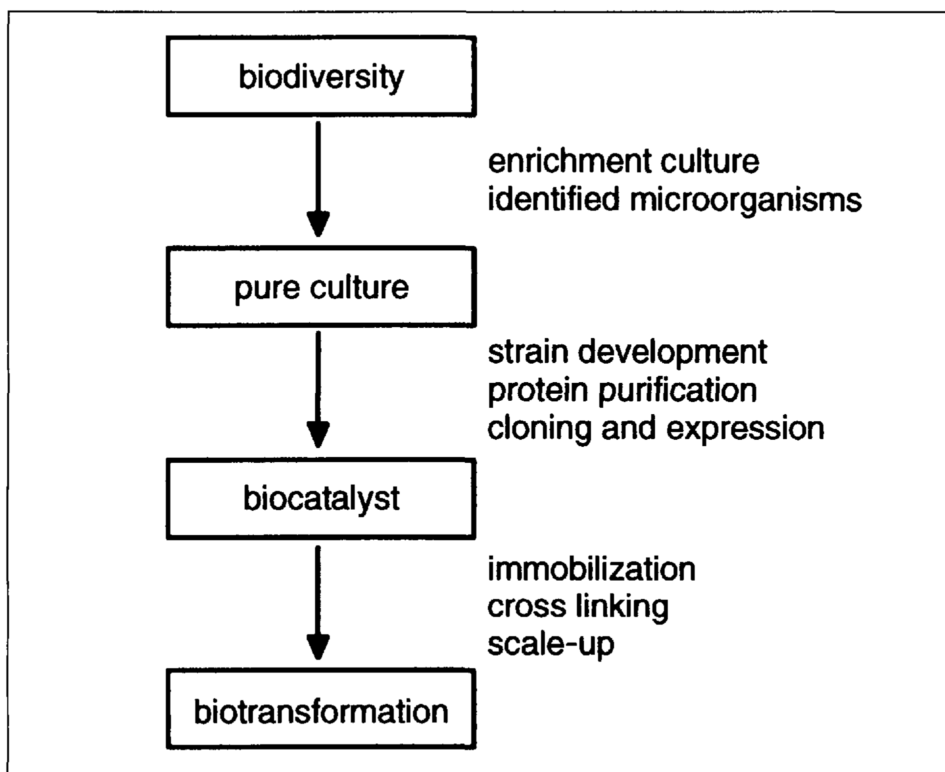
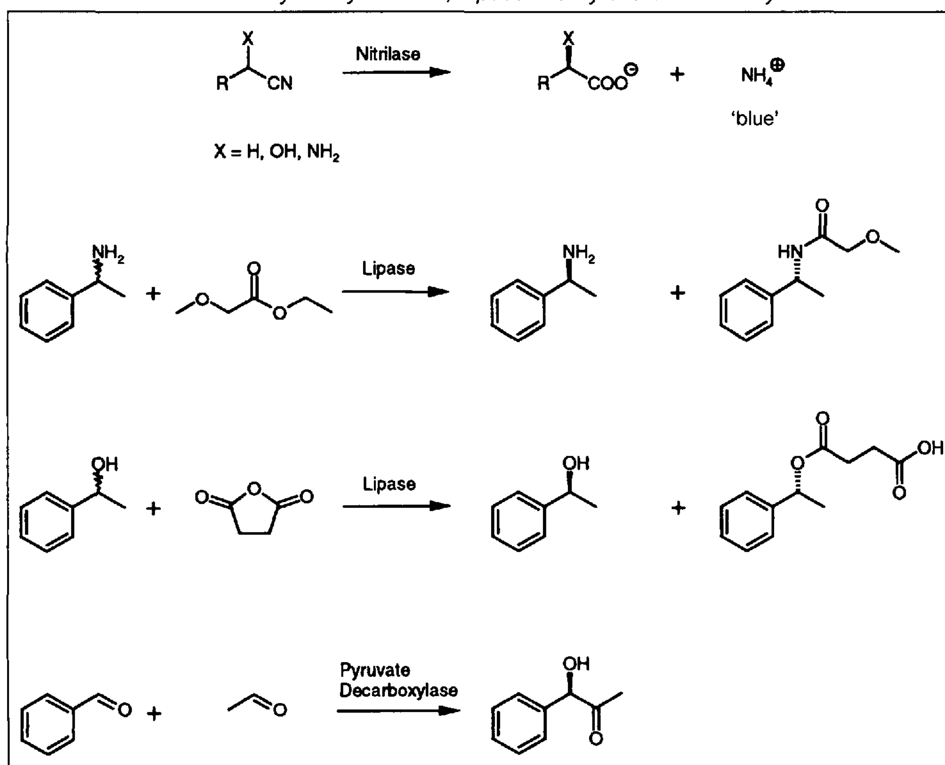



Fig. 1. *Development of a biocatalyst*

Scheme 1. *Reactions Catalysed by Nitrilase, Lipase and Pyruvate Decarboxylase*




### 2.1. *Screening for Nitrilases*

Enantiomerically pure amino- and hydroxycarboxylic acids are interesting building blocks, so-called intermediates.

Such compounds can be synthesized by nitrilases, a class of enzymes, which catalyze the hydrolysis of a nitrile to the corresponding ammonium salt of a carboxylic acid.

As cyanohydrins are known to racemize in aqueous solution by forming an equilibrium with the corresponding aldehyde and hydrogen cyanide, choosing the right pH-conditions can result in a quantitative enzymatic conversion.

The substrates for nitrilases can be synthesized *via* hydrogen cyanide chemistry, and therefore, these enzymes are a line extension giving access to interesting intermediates. Microorganisms having nitrilase activity can be enriched from nature by using nitriles as the only nitrogen and/or carbon source in the growth medium. A problem encountered with this approach is the isolation of false-positive strains, which use the carbon or nitrogen source by means of an undesired enzymatic activity (*e.g.*, a nitrile hydratase), or of strains which do not need a nitrogen source at all, because they are able to fix nitrogen from air. Another frequent problem is the instability of some nitriles. To circumvent this problem, model compounds are used, which are more stable under the enrichment conditions. However, new problems might arise if the reactivity and/or steric hindrance of the model compound is different from the desired substrate. If enrichment is not possible, one has to produce biomass from pure cultures taken from strain collections and test them for enzymatic activity. To do this, an easy and fast assay system is necessary. To find nitrilases, an assay for ammonia can give a hint. The presence of ammonia can be demonstrated with a reaction generating a blue dye (*Scheme 1*). We tested about 5000 strains from enriched cultures and isolates from our strain collection in this way to find three novel nitrilases. Their substrate specificity, selectivity, and kinetic data were determined by HPLC and GC.

### 2.2. *Novel Reactions with Known Enzymes*

The understanding of the catalytic mechanism of an enzyme can be of tremendous value. It opens the door to novel applications of an enzyme in organic syntheses. The best example known today are the lipases [2].

Lipases are hydrolases acting on the ester bonds present in acylglycerols, liberating organic acids and glycerol. The natural substrates are long-chain triacylglycerols. A microorganism secreting a lipase can be identified by the formation a clear hydrolysis zone around the colony growing on turbid, emulsified tributyrate agar.

Understanding the catalytic cycle of lipases was of significant importance to their widespread use in different biotech-

nological applications. Their active site is composed of three different residues: serine, histidine, and aspartate or glutamate. The hydrolysis of an ester involves an acyl-enzyme complex. The catalytic cycle starts by nucleophilic attack of the hydroxy group of the serine side chain on the carbonyl C-atom of the ester bond. At the serine residue, an acyl complex is formed and the alcohol is released. This complex is then hydrolized by nucleophilic attack of water, the fatty acid is liberated, and the enzyme is regenerated.

Since lipases are active in organic solvents, water can be replaced by other nucleophiles, *e.g.*, alcohols. The result of this reaction is a transesterification (*Scheme 1*). In case of a racemic alcohol, only one enantiomer is acylated. Suitable acyl donors are vinyl esters, anhydrides, or diketene. The reaction is irreversible and the separation of the remaining alcohol and the newly formed ester is often simple. There are many examples for the production of enantiomerically pure alcohols based on this principle.

Amines might be used as nucleophiles as well, and we discovered that racemic amines are efficiently resolved using ethyl 2-methoxyacetate as acylating agent [3] (*Scheme 1*). Using ethyl 2-methoxyacetate, the initial reaction rate is more than 100 times faster than with butyl acetate. The reason for this activating effect of the methoxy group is probably an enhanced carbonyl reactivity induced by the electronegative $\alpha$-substituent. Both excellent yields and selectivity are achieved by this process. The products, (*R*)-amide and the (*S*)-amine, can be recovered and separated by distillation. This process is applicable to a broad spectrum of amines (*Scheme 2*).

The lessons we can learn from the success of lipases are of general importance to the development of biocatalysts. Lipases are available in commercial quantities; they are highly stable and active in organic solvents, and the substrate range is quite broad. Besides for the production of alcohols and amines, lipases might be used for other reactions as well. For example, enol esters can be stereoselectivly protonated and even other reactions, as the cleavage of oxime esters, C,C-bond formation, *Michael* reactions and *Diels-Alder* reactions might be envisioned. However, significant improvements are necessary to facilitate those reactions.

### 2.3. Rational and Evolutionary Strategies for Improving Enzymes

Catalysts isolated from nature quite often show limited performance under process conditions. What can be done if neither screening of natural isolates nor applying our knowledge on the catalytic mechanisms of enzymes give us any promising results?

Rational redesign of an enzyme requires not only extensive knowledge of structure and mechanisms, but also determines how to improve the desired feature of an enzyme.

In recent years, evolutionary strategies to optimize enzymes have been developed. By applying sequential cycles of random mutagenesis, recombination, and screening, the enzyme's profile can be changed. Large populations with a high genetic diversity are generated and screened for improved functions (*Fig. 2*). A recent and successful example has been the evolution of an enzyme to catalyze the hydrolysis of a *p*-nitrobenzyl ester of an antibiotic [4]. Other enzymes have been improved in their thermostability, substrate specificity, and enantioselectivity. Evolutionary strategies are still in their infancy, and many new approaches will appear in the coming years [4].

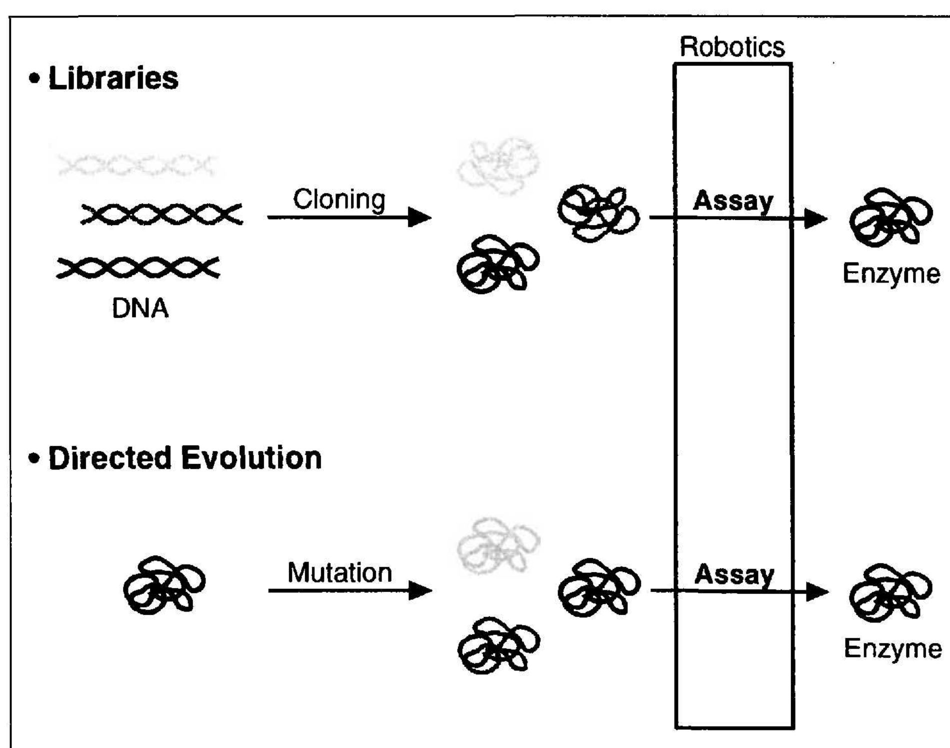



Fig. 2. *New technologies for biocatalyst development*

### 2.4. Improvement of Pyruvate Decarboxylase

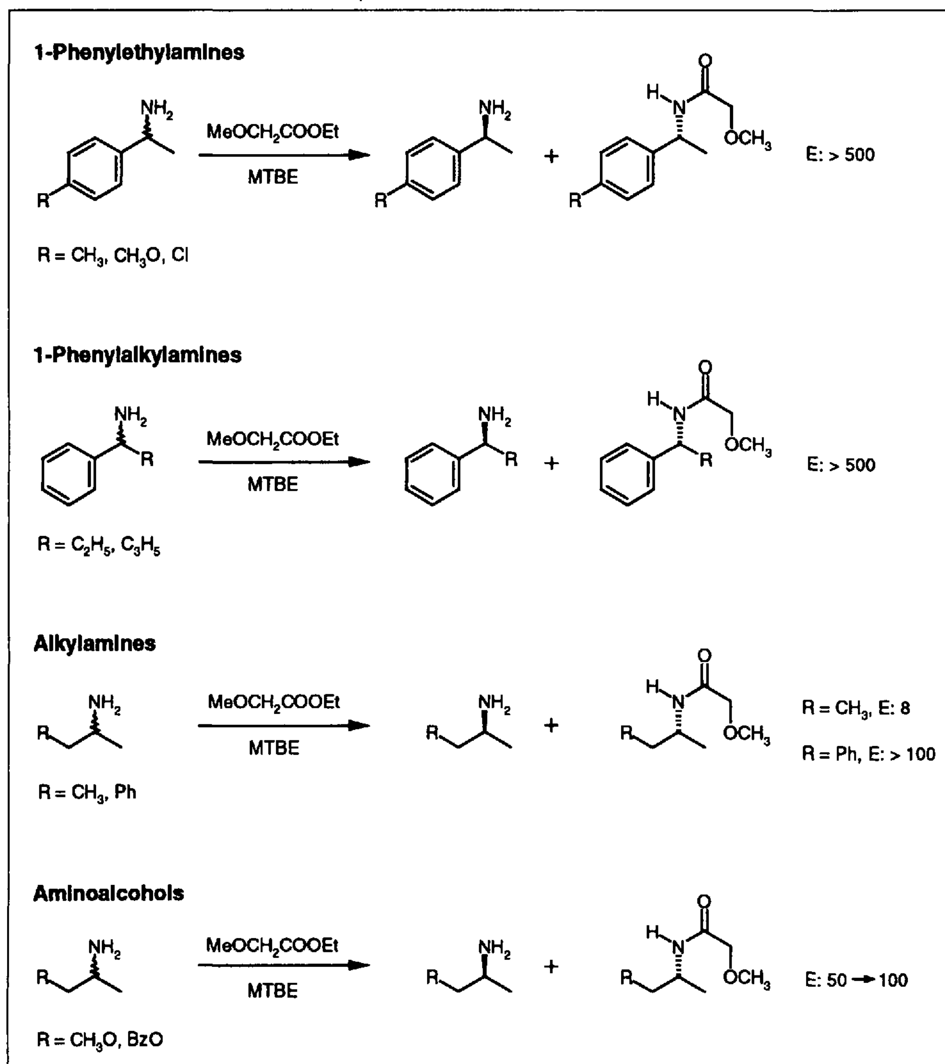
We applied both strategies to improve the pyruvate decarboxylase (PDC). *In vivo*, this enzyme decarboxylates pyruvate to acetaldeyde, but the enzyme of *Saccharomyces cerevisiae* also has a carboligase activity. This was realized as early as in 1921, when *Neuberg* and *Hirsch* showed that yeast cells catalyze the C,C-bond formation in reactions of acetaldehyde with a number of different aldehydes added to the fermentation broth [5]. Upon feeding with benzaldehyde, this carboligation yields (*R*)-1-hydroxy-1-phenylpropan-2-one, an intermediate for the production of ephedrine (*Scheme 1*). This reaction has been exploited in one of the first industrial-scale biotransformations with yeast [6]. However, the isolated yeast enzyme is not stable enough to perform the reaction *in vitro*.

PDC from *Zymomonas mobilis* is a rather stable enzyme, but it has only a very poor carboligase activity [7]. *Pohl* successfully combined stability and carboligase activity in the *Zymomonas* enzyme [8]. By comparing the protein structure, it became apparent that the major difference between the yeast and the *Zymomonas* enzyme is a tryptophan residue at position 392. Replacement of this tryptophan by methionine or alanine resulted in a stable PDC with increased carboligase activity. Quite surprisingly, this enzyme worked not only with pyruvate as a $C_2$-donor, but also with acetaldehyde [9].

For a technical process, however, the enzyme's operational stability was still not high enough. It could be improved by mutating the enzyme and selecting a mutant with increased stability.

Screening a very large number of mutants requires a fast and reliable assay system. In order to obtain a high throughput, chromatographic systems are unsuitable. We exploited the reduction potential

Scheme 2. *Amine Substrates for Lipase*




of (*R*)-1-hydroxy-1-phenylpropan-2-one for the development of a colorimetric assay. (*R*)-1-Hydroxy-1-phenylpropan-2-one reduces tetrazolium salts and similar compounds. This reaction is fairly selective, enabling the detection of (*R*)-1-hydroxy-1-phenylpropan-2-one in the presence of other compounds with redox potential from cell extracts or by-products. Based on this assay, we are able to screen large libraries of mutant enzymes in a very short time.

### 2.5. *Acceleration of the Discovery and Development Cycle*

The potential of enzymes for the synthesis of chemical compounds is by no means exhausted. To take care of all possibilities on the market and also to get results with a given budget, the future demands on biocatalysis are acceleration of the discovery and development of new biocatalysts. New and fast methods to screen and develop enzymes are required. We need faster methods to find a basic activity and to carry out the evolutionary improvement of the enzymes. We also need technical and analytical tools for the efficient handling of large numbers of clones (*Fig. 2*).

At least a small activity is necessary as a starting point for the development of a biocatalyst. We are still not able to develop a novel activity from any given protein scaffold. To improve activity and stability, or to modify the substrate range, evolutionary strategies are the methods of choice. Thus, the discovery of new enzymatic activities has to be accelerated. Enrichment techniques are time-consuming, and so far, only culturable microorganisms are accessible. An interesting novel approach to explore the world of unculturable microorganisms is the sceening of gene-expression libraries of DNA isolated from nature [10][11]. To render this approach successful, several points have to be considered, namely DNA isolation and normalization methods, expression vectors, host strains, and fast and reliable assay systems for the detection of even small amounts of enzymatic activities.

In these new strategies, the technical challenge is the handling and analysis of large numbers of clones. To achieve an efficient parallel operation and a reduction of the analytical cycle time, the automation of these technologies with robotics is the crucial key. One should be able to manage up to 50'000 clones per day using a robot. Libraries of this extent are needed to have a good representation of nature's diversity or to manage the complexity of enzyme variants generated by directed evolution.

To push biocatalysis to its limits, we need not only new and fast methods to screen and develop enzymes, but also the unbiased collaboration between chemists, biologists, and engineers within industry and academia.

Received: October 11, 1999

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