BIOTRANSFORMATIONS

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Contents

Summary

Biotransformations are the basis of life. All natural organic and most inorganic molecules, the building blocks of organic and living matter, are subject to constant change and turnover.

The turnover time is determined by the reactivity and the reaction partners of the respective compounds, and varies from millions of years to microseconds. Fossil hydrocarbons are stored unchanged over millions of years in subsurface environments hostile for microbial enzymatic activities, whereas they are transformed to CO_2 and water within days or only hours in the presence of oxygen or other electron acceptors,

salts, and specialized microbes. The same is true for all other natural and most human-engineered chemicals.

These multistep transformations are catalyzed by enzymes that are mostly specific for the reaction type and starting compound. Yet, very frequently enzymes catalyze not only the reaction they were evolved for by nature over thousands or millions of years, but also the conversion of structurally and/or electronically similar derivatives.

This feature of enzymes can be used for numerous technical purposes like the clean up of contaminated environments (bioremediation) or the production of high value compounds for chemical, agricultural, and pharmaceutical industries.

In this article, we describe biotransformations used for the production of high value chemicals. We give an overview of different aspects such as the discovery and sources of enzyme based catalysts, and their design and application in bioprocesses on not only a small scale but also on an industrial scale.

There is a focus on oxygenenases but most concepts and principles described are also valid for all other enzyme classes.

1. Introduction

Because of the high potential of microorganisms to biodegrade natural organic compounds, they play a fundamental role in the global recycling, and thus in the maintenance of the ecological balance. Some microorganisms can utilize nearly every natural organic compound as a source of energy and/or cell building blocks.

Even many synthetic and non-natural organic compounds, so-called “xenobiotics” (see also *Biodegradation of Xenobiotics*), were shown to be biodegradable, demonstrating the enormous potential of metabolic activities in microorganisms (see also *Microorganisms as Catalysts for the Decontamination of Ecosystems and Detoxification of Chemicals).*

The variety of microorganisms able to degrade natural and synthetic organic compounds can be used for applications in environmental biotechnology as well as in industrial synthetic chemistry. In particular, the latter approach to use enzymes for biotransformations is of growing interest.

Biotransformations are chemical reactions that are catalyzed by microorganisms in terms of growing or resting cells or that are catalyzed by isolated enzymes. Because of the high stereo- or regioselectivity combined with high product purity and high enantiomeric excesses, biotransformations can be technically superior to traditional chemical synthesis.

If these features can be combined with economic benefits, biotransformations become the functional part of new chemical processes for organic synthesis. Further advantages of biocatalytical processes are the mild and ecologically harmless reaction conditions (normal pressure, low temperature, neutral pH), which are one important requirement

for sustainability. Table 1 shows the different enzyme classes and their reaction types used for biotransformations that are applied in the pharmaceutical, agrochemical, chemical, fragrance and flavor, and nutritional industries.

The use of biotransformations for industrial synthetic chemistry is an interdisciplinary, and therefore very exciting, field that needs the close cooperation of microbiologists, molecular biologists, chemists, and engineers.

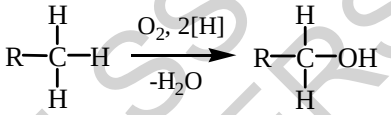
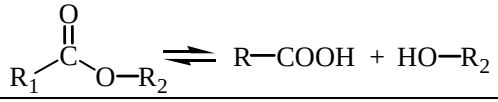
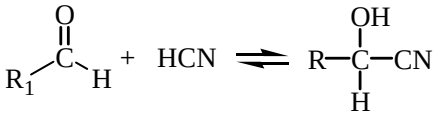
As is shown in Figure 1, several steps are necessary before a biotransformation process can successfully be implemented for an industrial application. After identification of a target reaction, which may be an already existing industrial process that can be substituted by an enzymatic process, finding a suitable biocatalyst is the first crucial step in process development.

Besides classical methods, new technologies including the screening for non-culturable microorganisms (see also *Viable but Non-Culturable Bacteria in the Marine Environment and the Biotechnological Tools to Detect Them*) and high throughput screening techniques are speeding up the discovery of new biocatalysts.

As the next step, the biocatalyst has to be characterized by well-known biochemical techniques in order to identify key parameters like substrate range, reaction conditions, and kinetic data (see also *Microbial Cell Culture*).

This allows a first estimation of the reaction yield and process costs (see also *Process Optimization Strategies for Biotechnology Products: From Discovery to Production*).

Modern technologies allow an improvement of the desired biocatalyst by several engineering tools such as heterologous gene expression and protein and metabolic engineering (see also *Methods in Gene Engineering*, and *Protein Engineering*).

Enzyme class	**Number**		**Reaction type**
	classified (EC-number)	commercially available	
Oxidoreductases	650	90	Oxidation  Reduction  Oxygenation of C-C, C=C, (de)hydrogenation
Transferases	720	90	Transfer of complete groups: —CH_3, —CH_2OH, —CHO, —CH_2-COOH, acyl, sugar, or phosphoryl
Hydrolases	636	150	Hydrolysis or formation of esters, amides, lactones, epoxides, nitriles, anhydides, glycosides, and organohalides 
Lyases	255	35	Addition or elimination of small molecules on C=C, C=N, C=O bonds 

Isomerases	120	6	Isomerization such as racemization, epimerization, and rearrangement
Ligases	80	5	Bond formation or cleavage of C-O, C-S, C-N, C-C under energy consumption

Source: adapted from Faber (2000).

Table 1. Classification of enzymes valuable for biotransformations. Source: adapted from Faber, 2000.

Process development has to meet economic as well as environmental constraints, and mostly includes the following steps: the production of the biocatalyst (whole cells or isolated enzymes) has to be optimized with respect to growth conditions, stability, induction, and storage (see also *Enzyme Production*). In an additional step the actual biotransformation process is optimized**.** Depending on the desired reaction, immobilization of the biocatalyst, enzyme catalysis in organic solvents, two liquid reactions, or cofactor regeneration are considered. Finally, a method for product recovery has to be developed using different extraction techniques, precipitation, crystallization, or *in situ* recovery. The article gives an overview of the state of the art and current developments necessary for developing a biotransformation process for industrial applications (Figure 1). Taking into account that numerous publications are available on the topic (see Bibliography) this article focuses on the screening of biocatalysts and on the use of enzymes involved in degradative pathways of xenobiotics for industrial applications. Additionally, industrial biotransformations that have successfully been applied in the synthetic chemical industry are discussed.

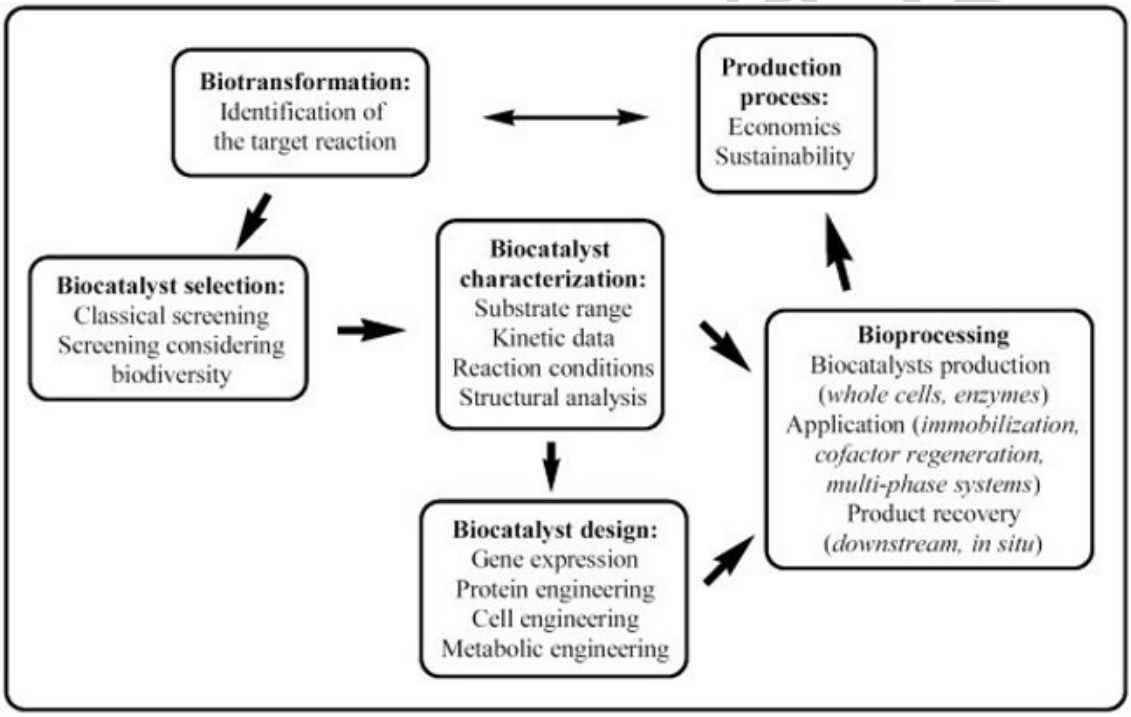


Figure 1. Key steps in developing a biotransformation process

2. Screening of Biocatalysts

Biocatalysts are based on microbes or isolated enzymes. A large number of microbes are available from national type culture collections like ATCC (USA), DSMZ (Germany), NCIB (UK), JCM (Japan) and others (see http://www.dsmz.de/species/abbrev.htm and see also *The Importance of Microbial Culture Collections and Gene Banks in Biotechnology*). Isolated enzymes are also commercially available from various sources (listed, for example, in Faber, 2000). Over 35 000 different reactions catalyzed by enzymes are described and can be easily

screened in databases with respect to substrate, product, productivity, and so on (www.accelrys.com and http://umbbd.ahc.umn.edu/).

On the other hand, finding a powerful new biocatalyst is successful only after parallel screening of large numbers of strains in collections or after screening of mostly environmental samples—despite the impressive number of reactions described in databases. This is because of the often broad, but still limited, substrate spectrum of enzymes, and the difficulties in actually getting individual strains without restrictions for commercial use. In future, the availability of whole genome sequences and the possibility of direct cloning and heterologous expression of genes of interest will simplify this.

2.1. Classical Screening Approaches

The most common approach for isolating bacteria able to degrade or transform a specific organic compound is the simple use of the compound of interest as a substrate for enrichment cultures. For example, a great number of bacteria were isolated by their ability to utilize aliphatic or aromatic hydrocarbons as a growth substrate, which means as the sole source of carbon and energy. Based on the toxic behavior and on the physical or chemical properties of the respective compound, different strategies for offering the substrate are used:

- Gaseous compounds like n-butane have to be supplied via the gas phase. The substrate is continuously dissolved into the aqueous phase (the growth rate is limited by the mass transfer rate of the substrate).
- Volatile and toxic compounds are often placed in a separate glass bulb (for example, benzene or toluene). This provides the bacteria with a continuous supply of the substrate without generating a two-phase system that might kill the bacteria by destroying the functions of their membrane.
- Water insoluble compounds (for instance, naphthalene) are supplied as solids at concentrations well above their water solubility, or are offered in a second organic phase. In both cases the substrate is continuously dissolved into the aqueous phase as it is degraded, and thus the compound is rarely toxic to the bacteria.

In order to overcome problems with toxicity, nitrogen-containing compounds like nitroaromatics or nitriles are often supplied at low concentrations as the sole source of nitrogen in the presence of a readily degradable carbon and energy source. Because this enrichment strategy is often based on partial degradation of the nitrogen-containing compound, it can be particularly helpful for complex molecules.

For biotransformations, the screening by selection can be carried out with direct use of the substrate of interest or by the use of a readily degradable analogue of the substrate (cometabolism).

When screening by selection is impossible, bacteria from strain collections can be screened for the desired biotransformation, using chromometric or fluoremetric detection methods in agar or microplate experiments. Fluorogenic assays are described

for the screening of hydrolases, aldolases, and alcohol dehydrogenases. Remarkably, the latter is even an enantioselective assay. In principle, a more time-consuming screening is possible using GC, GC-MS, LC, or LC-MS methods for the analysis of the biotransformation (see also *Chemical Methods Applied to Biotechnology,* and *Physical Methods Applied to Biotechnology*). In this context it has to be mentioned that two approaches are possible. On the one hand it makes sense to screen a library of microbial catalysts using a wide range of different genera. On the other hand, if an interesting biocatalytic reaction is already described in a certain organism, it is reasonable to screen related genera and families since there are often similarities in enzymatic equipment.

In specific cases, a combination of screening by selection and screening by detection is a promising approach for biotransformations. For example, a *Rhodococcus rhodochrous* strain J1, which is used as catalyst for acrylamide production, was isolated by a combination of these methods. Acrylonitrile-utilizing bacterial strains were enriched and afterwards tested for acrylonitrile-hydrolyzing activity. In a similar manner, a collection of toluene and naphthalene degrading bacteria were screened in order to find a strain able to transform D-limonene to (+)-*trans* carveol.

A miniaturized growth system was recently developed for this purpose because even after preselection large numbers of organisms have to be screened for promising enzyme activities. This system allows maintenance, replication, and growth of microbial strains in microtiter plates without cross-contamination. After sufficient growth of the microbial strains, enzyme activities, determined by product formation, can be measured after two hours of incubation. A successful screen for oxygenases with 2000 microbial strains possessing a wide variety of catabolic activities was achieved using this method.

2.2. Screening by Considering Biodiversity

In the last few years, extreme environments regarding temperature, pH, pressure, and salt concentration are increasingly used as sources for new enzymes. New technologies were developed for cultivating extremophiles, but the favored approach is cloning and expressing genes from such strains into conventional host strains in order to produce enzymes with extremophilic properties.

A crucial disadvantage of the screening by selection approach described above is the fact that most bacteria in the environment are assumed to be nonculturable by using traditional isolation and cultivation techniques. To overcome this drawback a new technology in enzyme screening is becoming increasingly important. DNA extracted from environmental, and thus uncultivated, sources can be cloned and expressed into domesticated host strains. The recombinant clones are screened for new enzyme activities with high-throughput screening methods. This recombinant approach can also be used for enzymes from known microorganisms that are difficult to handle in the laboratory. An increasing number of new enzymes have been found in environmental DNA libraries. DeSantis and coworkers isolated 120 unique nitrilases using this approach. Additionally, Lorenz and coworkers were able to isolate several new lipases, esterases, and metalloproteases as well as oxygenases. Today, this fast availability of new enzymes applies pressure for the development of high-throughput biochemical enzyme characterization methods to learn about kinetics, stabilities, and morphology.

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Biographical Sketches

Hiltrud Lenke, born in 1961, studied chemistry at the University of Wuppertal, Germany. She finished her studies in 1986 with a diploma thesis that dealt with the biodegradation of 2,6-dinitrophenol. She took her Ph.D. at the Institute for Microbiology at the University of Stuttgart, Germany, in the subject of the microbial degradation of polynitrophenols. She continued this work for another year as a post-doctoral student. Between 1991 and 2002 she worked as a group leader at the Fraunhofer Institute for Interfacial Engineering and Biotechnology in Stuttgart, Germany. Initially, her main task was in the area of environmental microbiology. Since 1998, her main emphasis has been in biotransformation and biocatalysis. During the summer of 2001 she finished her habilitation to gain the venia legendi for microbiology. Since January 2002, she has been a visiting lecturer for microbiology at the University of Stuttgart. In 2003, she started work as a consultant for biotechnology at Chemgineering GmbH in Wiesbaden, Germany.

Andreas Schmid, born in 1966, studied Biology with a focus on immunology and microbiology at the University of Stuttgart where he received his Diploma in 1992. During his studies in Stuttgart and at the Swiss Federal Institute of Environmental Science and Technology (EAWAG) in Dübendorf he worked as an academic assistant at the Fraunhofer Society (Stuttgart) and at the Institute of Microbiology (University of Stuttgart) until 1992. In February 1997 he received a PhD in microbiology from the University of Stuttgart. After research activities in applied environmental biotechnology (waste air treatment), at the faculty of civil engineering, University of Stuttgart he joined the Institute of Biotechnology (ETH Zurich) as post doctoral fellow concentrating on the development of microbial biocatalysts for organic synthesis reactions. Since January 1999 he is lecturer and head of the research group 'Technical Enzymology' at the Institute of Biotechnology, ETH Zurich. His research group is specialized on the development and application of bacterial enzymes as biocatalysts with respect to basic biochemistry, protein engineering and process development.