UDC 577.128.15
ISSN 1330-9862

review

(FTB-1420)

Enzymes from Higher Eukaryotes for Industrial Biocatalysis

Zhibin Liu[1], *Roland Weis*[1] *and Anton Glieder*[2]*

[1]Research Centre Applied Biocatalysis, Petersgasse 14, A-8010 Graz, Austria

[2]Institute of Molecular Biotechnology, Petersgasse 14, A-8010 Graz, Austria

Received: August 6, 2004
Revised version: September 9, 2004
Accepted: November 22, 2004

Summary

The industrial production of fine chemicals, feed and food ingredients, pharmaceuticals, agrochemicals and their respective intermediates relies on an increasing application of biocatalysis, *i.e.* on enzyme or whole-cell catalyzed conversions of molecules. Simple procedures for discovery, cloning and over-expression as well as fast growth favour fungi, yeasts and especially bacteria as sources of biocatalysts. Higher eukaryotes also harbour an almost unlimited number of potential biocatalysts, although to date the limited supply of enzymes, the high heterogeneity of enzyme preparations and the hazard of infectious contaminants keep some interesting candidates out of reach for industrial bioprocesses. In the past only a few animal and plant enzymes from agricultural waste materials were employed in food processing. The use of bacterial expression strains or non-conventional yeasts for the heterologous production of efficient eukaryotic enzymes can overcome the bottleneck in enzyme supply and provide sufficient amounts of homogenous enzyme preparations for reliable and economically feasible applications at large scale. Ideal enzymatic processes represent an environmentally friendly, »near-to-completion« conversion of (mostly non-natural) substrates to pure products. Recent developments demonstrate the commercial feasibility of large-scale biocatalytic processes employing enzymes from higher eukaryotes (*e.g.* plants, animals) and also their usefulness in some small-scale industrial applications.

Key words: enzymes, eukaryotes, industrial biocatalysis

Introduction

Microorganisms and their enzymes are widely exploited in biocatalysis to produce a broad spectrum of fine chemicals, pharmaceuticals and their building blocks, as well as commodity and agrochemicals (*1–4*). The preferred use of enzymes from bacteria and fungi in competitive large-scale bioprocesses stems from their simple

* Corresponding author; E-mail: glieder@glieder.com

Abbreviations: CYP: cytochrome P450; EDA: ethylenediamine; e.e.: enantiomeric excess; FDH: formate dehydrogenase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GDH: glutamate dehydrogenase; GlcNAc: N-acetyl-D-glucosamine; *Hb*HNL: hydroxynitrile lyase from *Hevea brasiliensis*; HLADH: horse liver alcohol dehydrogenase; HNLs: hydroxynitrile lyases; LDH: lactate dehydrogenase; *Lu*HNL: hydroxynitrile lyase from *Linum usitatissimum*; MDL: mandelonitrile lyase; *Me*HNL: hydroxynitrile lyase from *Manihot esculenta*; Neu5Ac: N-acetylneuraminic acid; *Pa*HNL: hydroxynitrile lyase from *Prunus amygdalus*; PDH: phenylalanine dehydrogenase; PLE: pig liver esterase; PPL: porcine pancreatic lipase; rPLE: recombinant PLE isoenzyme; *Sb*HNL: hydroxynitrile lyase from *Sorghum bicolor*; YADH: yeast alcohol dehydrogenase

and quick genetic manipulation (*5*) and also the simple access to large amounts of native enzymes. For comparison, higher eukaryotes provide interesting enzymes (*6–8*), but they usually need to be isolated and purified or cloned and over-expressed in suitable hosts in order to obtain sufficient quantities for biocatalytic applications. In the past years, the use of synthetic host-adopted genes and codon-optimized *Escherichia coli* strains and the development of highly successful eukaryotic expression systems, such as the methylotrophic yeasts *Pichia pastoris* and *Hansenula polymorpha*, have enabled the production of large quantities of eukaryotic enzymes within a short time (*9*).

An increasing experience in molecular modelling (*10,11*), which rationalizes molecule and biocatalyst engineering, as well as recent progress in high-throughput technologies for the expression and engineering of eukaryotic proteins provide great potential to extend industrial applications of eukaryotic enzymes (*12*). Directed evolution of enzymes in the test tube by repeated cycles of random mutagenesis or recombination followed by screening and selection provides a powerful tool if structural or mechanistic information is not available and if efficient screening technology exists (*13*). In addition, this technology gives access to improved enzymes due to mutations that could not be predicted based on knowledge from static enzyme models.

Enzymes from higher eukaryotes have been traditionally used in the food industry, for example, as food additives, in fruit processing, in wine brewing and as pharmaceutical additives. Examples include the extensive use of papain, bromelain, chymosin, ficin, lipoxygenase or pepsin (*14*). However, most of these processes were already developed in the 1980s (*15–17*) and therefore cannot be considered as recent applications, although the products are still widely used.

Oxidoreductases (E.C. 1), hydrolases (E.C. 3), lyases (E.C. 4) and isomerases (E.C. 5) provide the vast majority of examples of higher eukaryotic enzymes for industrial applications; there are no commercial processes reported for plant or animal enzymes from other catalytic groups. In this review, the main focus is on implemented biocatalytic applications of eukaryotic enzymes in industrial processes for the production of fine chemicals, as well as some recent promising developments at pilot scale and for the preparative synthesis of compounds for industrial drug development processes.

Production of Fine Chemicals and Metabolites with E.C. 1 – Oxidoreductases

Mammalian cytochrome P450

Mammalian cytochrome P450 (CYP) monooxygenases comprise a superfamily of hemoproteins with extraordinary catalytic versatility. They catalyze the metabolism of a wide variety of endogenous and exogenous compounds including environmental contaminants, hormones, therapeutic drugs and (pro)carcinogens (*18–20*). The human CYP enzyme system is divided into two large subgroups, the steroidogenic and xenobiotic enzymes. Some extrahepatic P450s have roles in the maintenance of homeostasis and signal transduction. Steroidogenic P450s synthesize steroids and other substances necessary for the maintenance of cell wall integrity and cellular differentiation. The majority of enzymes of the human xenobiotic group are expressed in liver microsomes. They perform a number of physiological functions but their primary role is in the metabolism of xenobiotics. A large number of potential drug candidates fail in later stages of drug development because they are readily degraded or because they interfere with the metabolism of other therapeutics by human cytochrome P450 (CYP) isozymes. An early assessment of drug/drug interactions and isozyme-specific CYP450 induction or inhibition is an essential part of the modern drug discovery process (*20*).

Among these CYP isozymes, the CYP3A enzyme family is responsible for the hepatic metabolism of approximately 60 % of currently available pharmaceutical agents (such as Mephenytoin®, Nevirapine®, Nifedipine®, Nicardipine®, Cyclophosphamide®, Clotrimazole®, Erythromycin® and Fluconazole®) (*21,22*). About 25 % of all drugs used today are substrates for the CYP2D6 isozyme (*23*) and many others inhibit or induce its activity. Approximately 15 % of all drugs used today are metabolized by the CYP1A2 isozyme (*23*).

Industrial application of human P450s evolves into two different directions: (*i*) the production of active pharmaceutical intermediates, and (*ii*) the simple and fast production of metabolites in mg-scale for drug development.

Human cytochrome P450s have been expressed in different systems such as mammalian cells (*24,25*), insect cells (*26*), yeasts (*27–29*) and bacteria such as *Escherichia coli* (*30,31*). *E. coli* expression systems have the advantages of being easier and less expensive to operate and of yielding higher quantities of recombinant proteins. Folding and solubility problems are usually solved by synthesis of optimized genes and N-terminal fusions with other protein sequences that allow correct targeting or increase protein solubility. Dramatic improvement in the levels of reconstitution of activity of human CYP450 in *Escherichia coli* was obtained by coexpression of P450 reductase (*32,33*). Recently, a human mitochondrial cytochrome P450 (P450 aldosterone synthase) was functionally expressed in the fission yeast *Schizosaccharomyces pombe.* No co-expression of P450 reductase was needed for efficient substrate conversion by intact fission yeast cells. Expression was much higher in fission yeast microsomes than in *Saccharomyces cerevisiae* and the transformed yeast showed inducible activity for the *in vivo* conversion of considerable amounts of 11-deoxycortisol to cortisol. Furthermore, 11-deoxycorticosterone could be converted almost entirely to corticosterone, with small amounts of 18-hydroxycorticosterone and aldosterone as by-products (*29*). This study can be regarded as a significant step towards simple and efficient over-expression and characterization of human P450s and represents considerable progress concerning their possible applications in fermentative production processes.

The co-expression of human CYP3A4 and human NADPH-cytochrome P450 oxidoreductase in insect cells (aided by baculoviral infection) was reported by a group of the Merck Research Laboratories in 2000 (*34*). The aim was to establish a platform for both the production and

subsequent purification of milligram quantities of P450-generated metabolites. According to the authors, the large-scale production (which is still in the scale of several milligrams) of metabolites derived from the standard substrates testosterone, diazepam and diclofenac was achieved and could be scaled up further if necessary.

Shaw *et al.* (*35*) demonstrated the development of ready-to-use premixed human enzyme preparations, consisting of CYP3A4, NADPH-P450 reductase, cytochrome b_5 and liposomes, for the large-scale screening of novel P450 substrates or inhibitors. The formulation allows for repeated freeze-thaw cycles as well as prolonged storage time at 4 or –80 °C, without significant loss of activity on the model substrate testosterone. The striking advantage of such a system is the ability to perform large numbers of P450 assays using purified components in a reliable fashion (*35*).

BioCatalytics (Pasadena, California) is commercializing a comprehensive set of ready-to-use liver enzymes, which were expressed in *E. coli*. This provides all six major human liver cytochromes in a kit for simple synthesis of metabolites.

There is a requirement of milligram amounts of P450-generated metabolite(s) for toxicological evaluation and pharmacokinetic studies in many situations during drug discovery and development (*34*).

Glycolate oxidase

Glycolate oxidase is a peroxisomal oxidase that has been isolated from many green plants and mammals, including spinach leaves, pea, pumpkin, cucumber as well as pig liver, rats and humans. An investigation of glycolate oxidase production and substrate specificity to glycolic acid indicated that the enzyme from spinach leaves produced the highest yields and specific activities in terms of glyoxylic acid production (*36*). The spinach glycolate oxidase can be generally used for the oxidation of hydroxy carboxylic acids and it was isolated as a catalytically active tetramer or octamer made up of identical 40-kDa subunits. The crystal structure revealed that the subunits form an eight-stranded alpha/beta barrel (*37*). The biocatalytic oxidation was done in the presence of oxygen and ethylenediamine (EDA), using glycolate oxidase and catalase. These oxidations produced both high yields (>99 %) and high conversions (100 %), resulting in few undesirable by-products (*36*) (Fig. 1).

The efficiencies of the catalases from cattle liver and *Aspergillus* sp. for glyoxylic acid production were compared. This revealed that the fungal enzyme was superior. The technical feasibility of a biocatalytic process for the production of glyoxylic acid was demonstrated by the preparation of 0.50 kg of the acid in a series of 2.0-L batch reactions with 100 % conversion. However, in this process, the biocatalyst was not stable and could not be recovered for subsequent reuse (*36*).

To make the enzyme more stable, the glycolate oxidase isolated from spinach and the catalase from yeast were immobilized on oxirane acrylic beads. Product yields were 93–100 % after 10 batch reactions under the conditions of 200–250 mM substrate concentration, 1.05–1.33 EDA/glycolic acid molar ratios and 0.58 MPa oxygen pressure (*38*).

Nevertheless, purified enzymes from spinach leaves are too expensive and not very suitable for stable enzyme preparations in industrial applications. Therefore the spinach glycolate oxidase gene has been cloned and over-expressed in *Escherichia coli* (*39,40*), *Saccharomyces cerevisiae* (*41*) and *Pichia pastoris* (*42*). When produced in fermentors, more than 250 units of recombinant enzyme per gram of cells (*Pichia pastoris*, wet weight) were produced, which represents roughly 20–30 % of the soluble cell protein. Using permeabilized recombinant *Pichia* cells as biocatalyst, the biotransformation was carried out to completion with 98 % yield of glyoxylic acid after 4.5 h. This was the first example of expression of a plant gene in *P. pastoris* and, more significantly, the first example of engineering *P. pastoris* as a whole cell catalyst for the explicit purpose of commercial bioprocess development (*42*).

The co-expression of spinach glycolate oxidase and catalase T from *Saccharomyces cerevisiae* in *Pichia pastoris* has been investigated in order to enable the efficient simultaneous production of multiple enzymes, and afterwards to develop this system for commercial bioprocesses (*43*). The *Pichia pastoris* double transformant was used for glyoxylate production and also produced high yields of glyoxylic acid (90 %) and low amounts of by-products (*43*).

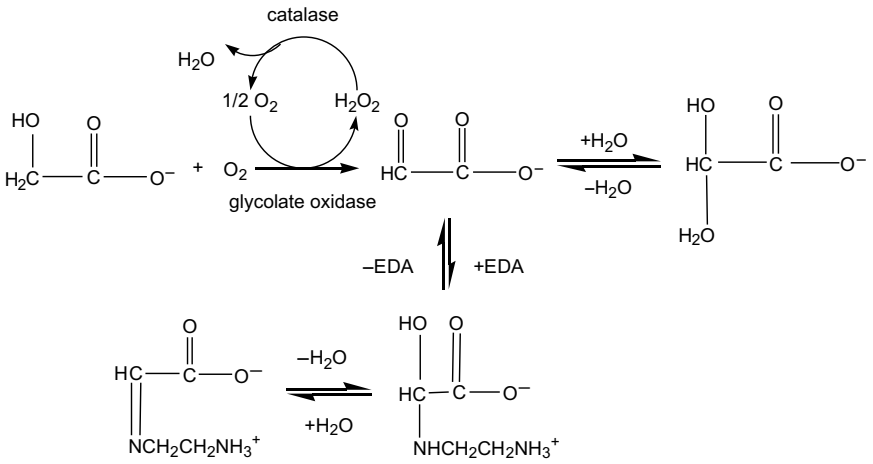

Fig. 1. Biocatalytic oxidation of glycolic acid

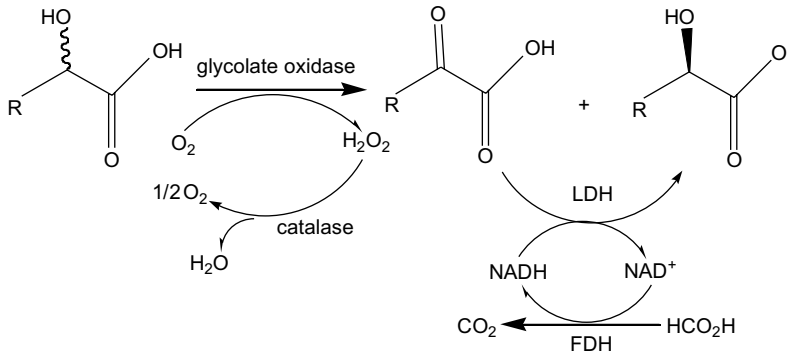

Fig. 2. Resolution of racemic 2-hydroxy acids into (*R*)-2-hydroxy acids (R=Et: e.e.>99 %, conversion 100 %; R=n-Bu: e.e.=67 %, conversion 100 %)

Adam *et al.* reported another commercially interesting process employing this oxidase. Racemic 2-hydroxy acids were enzymatically resolved into chiral hydroxy acids with spinach glycolate oxidase and D-lactate dehydrogenase (*44–46*). Optically active 2-hydroxy acids are important building blocks for the asymmetric synthesis of glycols, halo esters and epoxides. The novel enzymatic transformation revealed that the biocatalytic production of a broad variety of (*R*)-hydroxy acids leads to a high degree of enantioselectivity by preferential oxidation, with glycolate oxidase, of (*S*)-hydroxy acids to 2-oxo acids in racemic mixtures. Following enzymatic oxidation, D-lactate dehydrogenase (LDH) was added together with NADH for enantioselective reduction of the 2-oxo acid to the corresponding (*R*)-2-hydroxy acid. The co-substrate NADH was recycled by the formate dehydrogenase (FDH) system (*45*) (Fig. 2). (*R*)-2-hydroxy acid was produced with an excellent enantiomeric excess (e.e.) value (>99 %, R=Et; 67 %, R=n-Bu) and high yields (100 %) (*46*).

Dehydrogenase

Due to their strong capability to convert carbonyl groups to optically pure alcohols, dehydrogenases became very important catalysts for organic syntheses. Many developments were focused on finding suitable biocatalysts with expected stereoselectivity followed by the design of commercial methods for industrial use. At large scale, whole cell reactions were preferentially performed because whole cells already contain the nicotinamide cofactors ($NAD(P)^+$/NAD(P)H) and the corresponding recycling systems that are essential for commercially viable biocatalytic processes (*47–49*). On the other hand, the use of isolated dehydrogenases also has advantages as, for example, in the elimination of competition with reductases with different stereoselectivities or avoidance of metabolism of substrates and products, a phenomenon that results in lower yields and higher production of by-products (*50,51*). Many important dehydrogenases originate from microbial sources like S*ulfolobus solfataricus* glyceraldehyde-3-phosphate dehydrogenase (GAPDH), yeast alcohol dehydrogenase (YADH), *Candida boidinii* formate dehydrogenase (FDH) and *Escherichia coli* phenylalanine dehydrogenase (PDH). Bovine liver glutamate dehydrogenase (GDH) and horse liver alcohol dehydrogenase (HLADH) are examples from higher eukaryotes.

Bovine GDH reversibly catalyzes the reductive amination of α-ketoglutarate to L-glutamate using NADH as cofactor. The crystal structure of this homohexameric mitochondrial enzyme has been solved at 2.8 Å resolution and some more detailed mechanisms have been demonstrated (*52*). Bovine GDH has mainly been applied for the synthesis of L-6-hydroxynorleucine, which is a chiral intermediate for the synthesis of Omapatrilat® (Fig. 3), an antihypertensive drug acting as an angiotensin-converting enzyme (ACE) and neutral endopeptidase inhibitor (*53,54*).

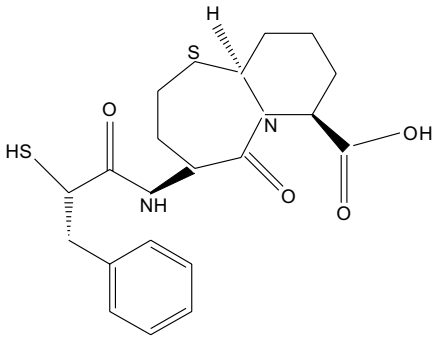

Fig. 3. Structure of Omapatrilat®

L-6-hydroxynorleucine is also an intermediate for the synthesis of C-7 substituted azepinones, which are potential intermediates for the synthesis of other antihypertensive metalloprotease inhibitors (*55*). An enzymatic method using bovine liver glutamate dehydrogenase was developed based on the conversion of 2-keto-6-hydroxyhexanoic acid (synthesized by a chemical method) to L-6-hydroxynorleucine by reductive amination (Fig. 4, Route 1). NADH was regenerated by glucose dehydrogenase from *Bacillus* sp. To avoid the necessity of the chemical synthesis of the ketoacid from the carboxylic acid, an alternative route with a theoretical yield of 100 % was constructed to prepare the ketoacid directly from racemic 6-hydroxynorleucine with porcine kidney or *Trigonopsis variabilis* D-amino acid oxidase and catalase, followed by the same reductive process from bovine liver GDH (*53*) (Fig. 4, Route 2). One method (Route 1) used 100 g/L of substrate for the preparative reaction and was completed in about 3 h with 92 % yield and greater than 99 % e.e. for L-6-hydroxynorleucine in the bovine liver GDH catalysed step.

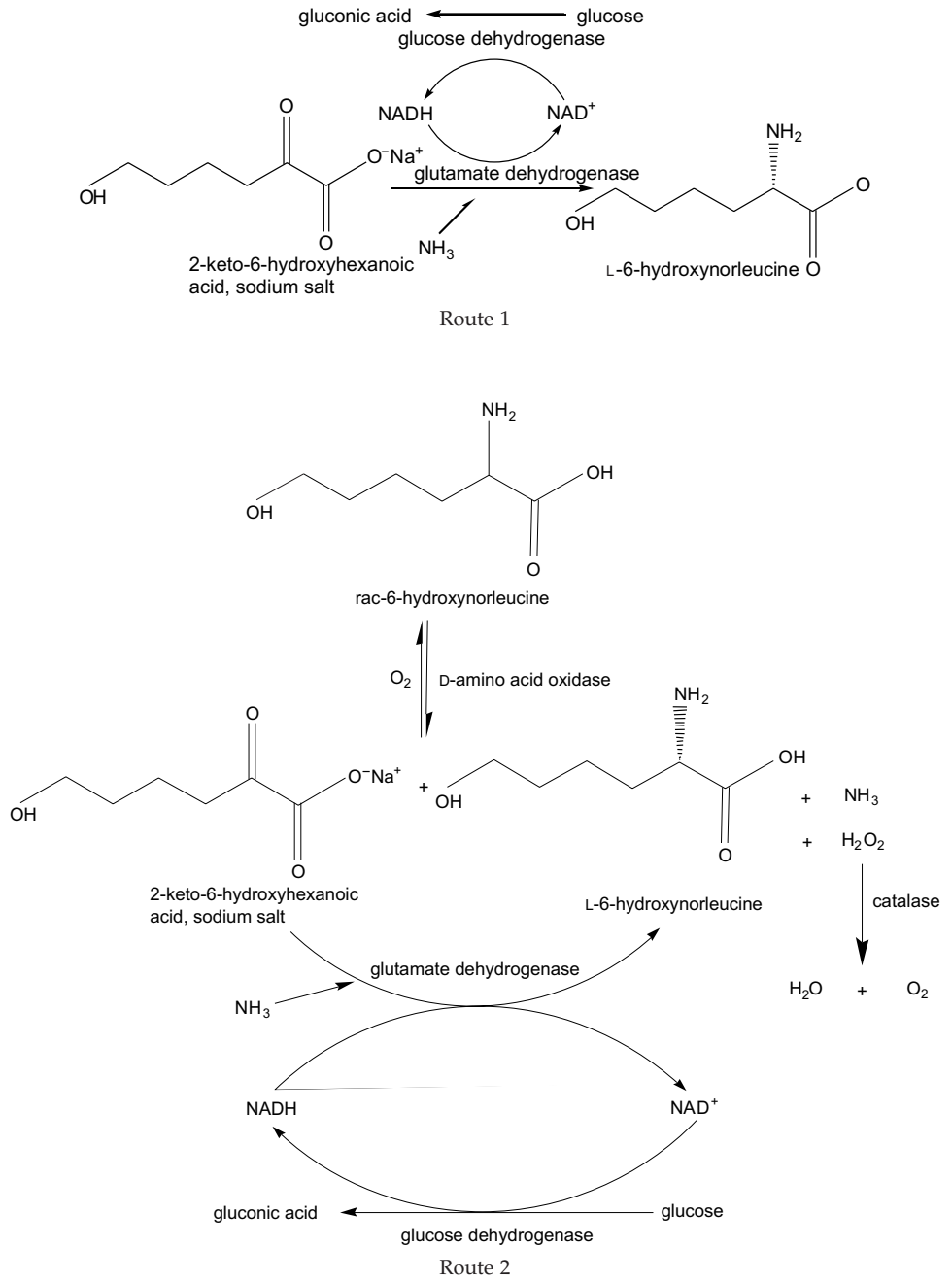

Fig. 4. Enzymatic synthesis of L-6-hydroxynorleucine

The second method (Route 2) (Fig. 4) also gave good results: after the oxidation step, the mixture of 2-keto-6-hydroxy hexanoic acid and L-6-hydroxynorleucine was entirely converted into L-6-hydroxynorleucine with 97 % yields from racemic 6-hydroxynorleucine and >98 % e.e. for L-6-hydroxynorleucine (*53*).

Production of Fine Chemicals with E.C. 3 – Hydrolases

Esterase and lipase

Lipases, esterases and proteases are just some of the major representatives of the vast class of hydrolases. Due to their broad substrate specificity, they are mainly used for the resolution of different racemic compounds to obtain chiral products. However, in the best case only 50 % of the substrate can be converted to the desired enantiomer by hydrolytic cleavage unless a de-racemization process is applied. Hydrolases are widely distributed in microorganisms, plants and mammals. From animals a variety of tissue-specific lipases and esterases have been isolated (*56*). During the last few years much attention has been paid to the use of enzymes from microbial sources due to their easily available gene expression technologies and scale-up means (*57*). Also, high productivity of microbial hosts for native secreted enzymes and high stability of many of these enzymes favoured the selec-

tion of microbial hydrolases as candidates for industrial application. However, there are many reactions for commercial products carried out by hydrolases (mainly lipases, esterases and proteases) from higher eukaryotes. In particular, porcine pancreatic lipase (PPL) and pig liver esterase (PLE) have been widely used for production of chiral compounds.

Porcine pancreatic lipase is the cheapest and one of the most widely used lipases for kinetic resolution of non-natural substrates (*58*). In particular, it was applied for the resolution of glycidyl esters, which are converted to central chiral building blocks for the production of enantiopure pharmaceuticals (*59*). A process based on hydrolysis of racemic glycidylbutyrate with porcine pancreatic lipase was developed by Ladner and Whitesides (*60*). The 89 % theoretical yield and 92 % e.e. for the products make its industrial use feasible (Fig. 5). It was finally developed by Andeno-DSM and used for the production of (*R*)-glycidol on a multi-ton scale (*61*).

A further well-known industrial application of PPL is the kinetic resolution of 2,3-epoxy-1-tridecanol (Fig. 6) by selective acylation. The unconverted (2*R*,3*S*)-2,3-epoxy-1-tridecanol was isolated with 27 % yield and 99 % e.e. and used to synthesize the moth pheromone Disparlur® (*62*).

There are also lab-scale investigations of PPL-mediated resolution that show interesting perspectives (*63–65*). The enzymatic resolution of N-Boc-piperidine-2-ethanol can be achieved by the use of *Pseudomonas cepacia* lipase (Lipase PS) and porcine pancreatic lipase, respectively, exploiting their opposite enantioselectivities. Thus, both enantiomers of N-Boc-piperidine-2-ethanol can be obtained by lipase technology. A gram scale availability of (*R*)-enantiomer by lipase PS (90 % e.e.) and (*S*)-enantiomer by PPL (95 % e.e.) extends their synthetic application to provide general access to enantiopure piperidine alkaloids (*66*).

Pig liver esterase (PLE) plays a crucial role in the enantioselective hydrolysis of prochiral or racemic esters. Many applications have been reported for asymmetric synthesis employing this enzyme (*67–70*).

Unlike pig pancreatic lipase, which is highly active in organic media, PLE shows very low activity in organic solvents. Many factors, including water activity, the nature of the organic solvents and the formulation of the enzyme, affect the activity and enzyme stability in organic solvents. Immobilization on solid supports, covalent binding of methoxypoly(ethylene glycol) (MPEG) residues, lyophilisation by organic polymers, cross-linking of enzyme crystals, sol-gel entrapment, and entrapment in reverse micelles are the most important formulations that usually improve the performance of this enzyme in organic media (*71–74*).

A procedure has been developed for the highly enantioselective enzyme-catalyzed resolution of the antiviral agent 2′,3′-dideoxy-5-fluoro-3′-thiacytidine (FTC, Emtriva®) and related compounds, based on PLE mediated hydrolysis of their butyrate ester derivatives (*75*). PLE preferentially hydrolyzed the (+)-enantiomers and the use of butyrate esters facilitated the separation of the optically enriched, unreacted substrate from the medium by an extraction with $CHCl_3$. This process was successfully applied for the preparation of multi-gram quantities of enantiomerically pure (–)-FTC, which had an excellent activity/toxicity against HIV-1 (*75*) (Fig. 7). Emtriva® was approved as an anti-HIV drug in 2003 (*76*).

Using commercially available pig liver esterase, enantioselective hydrolysis on a series of substituted α-amino acid phenolic esters was performed (*77*). (*R*)-amino acid esters are novel compounds possessing hypnotic activity. Attempting an asymmetric synthesis of (*R*)-enantiomers, the reactions of bis(2-methoxyethyl)amine with α-bromo intermediates were first carried out to produce α-amino acid phenolic esters followed by de-

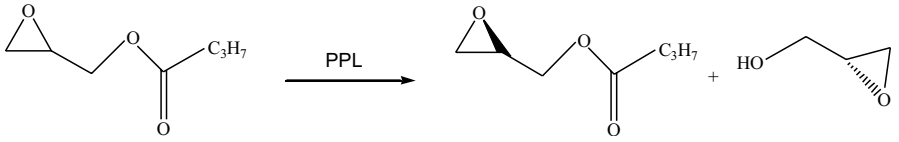

Fig. 5. (*R*)-glycidol synthesis based on porcine pancreatic lipase (PPL) hydrolysis

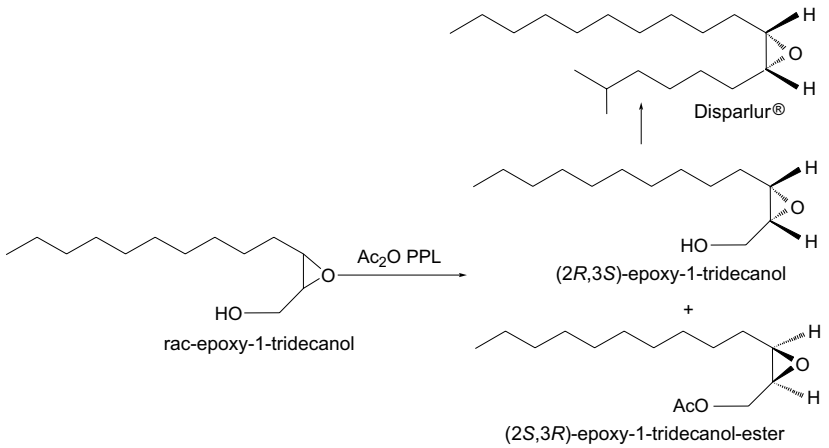

Fig. 6. Synthesis of Disparlur® precursor based on kinetic resolution

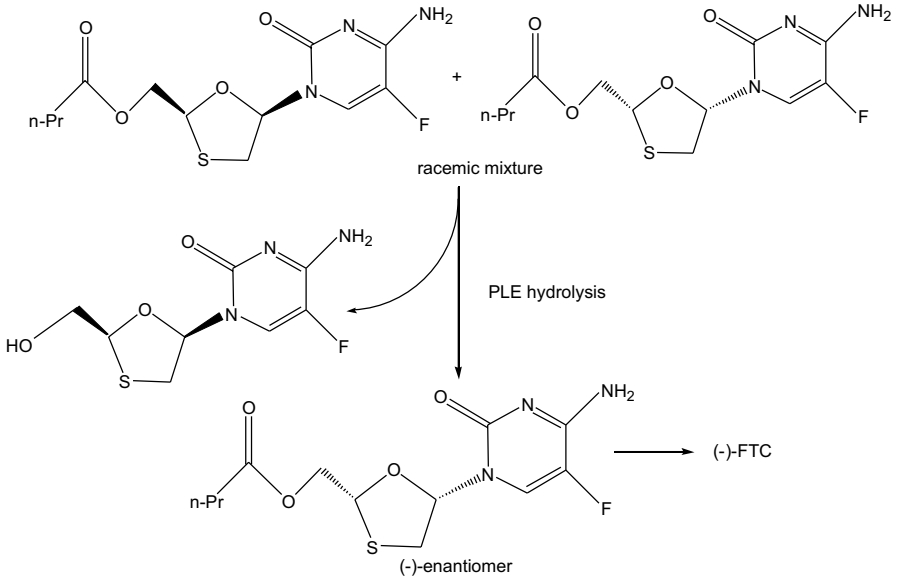

Fig. 7. PLE catalyzed resolution of 2′,3′-dideoxy-5-fluoro-3′-thiacytidine

-racemization. The active (*R*)-enantiomers were recovered in 25–30 % yield (max. 50 %) and in greater than 99.5 % e.e. These reactions have been scaled up and have been used to obtain multi-gram quantities of target compounds (see Fig. 8) (*77*).

The PLE-catalyzed hydrolysis of racemic hydroxytramadol derivatives (racemic δ-hydroxytramadol: potential analgesics) in aqueous buffer at pH=7.0 in the presence of 16 % acetone proceeded readily. It gave the alcohol with 93 % e.e. and 96 % yield and the ester with 72 % e.e. and 99 % yield, respectively (the quoted yields are based on one enantiomer) (*67*). An economical multi-mol scale PLE-catalyzed resolution of δ-hydroxytramadol butyrate ester can be achieved since the enzyme can be stabilized by the addition of bovine serum albumin and recovered by membrane filtration with only a minor loss of activity after large-scale hydrolysis in water (*67*).

For the repetitive two phase hydrolysis of meso-diester (dimethyl *cis*-cyclohex-4-ene-1,2-dicarboxylate) to the corresponding monomethyl ester (monomethyl *cis*-cyclohex-4-ene-1,2-dicarboxylate) by PLE on a multi-gram scale (Fig. 9), the enzyme was stabilized against organic solvents by immobilization in a polysulphone hollow fibre ultrafiltration membrane reactor. The e.e. value of monomethyl ester was still high (>97 %) and the enzyme retained 62 % activity (*68*). A further PLE-catalyzed centrosymmetric meso compound hydrolysis was reported with 100 % conversion and an e.e. for the products higher than 99.5 % (*78*).

Pig liver esterase was primarily used as a crude enzyme in powdered pig liver. Its application was limited due to the variable fractions in the extracts, which differed from batch to batch, and the relatively expensive enzyme source. However, it was demonstrated that PLE is a versatile biocatalyst that converts a broad range of compounds with a high stereoselectivity. A PLE gene has been cloned recently (*79,80*) and highly over-expressed in the methylotrophic yeast *Pichia pastoris* (*81*).

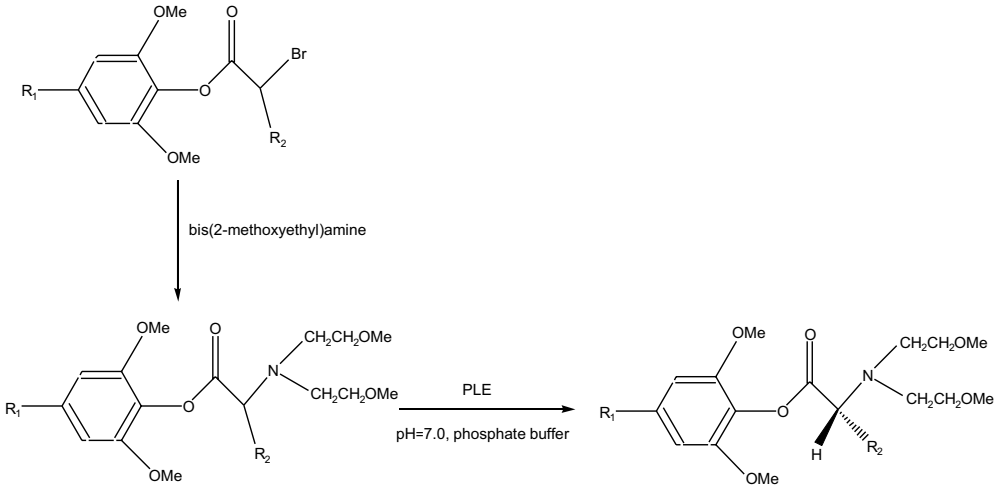

Fig. 8. PLE catalyzed hydrolysis of substituted α-amino acid phenolic esters (compound 1: R_1=H, R_2=Et; compound 2: R_1=Me, R_2=Et; compound 3: R_1=H, R_2=^{n}Pr)

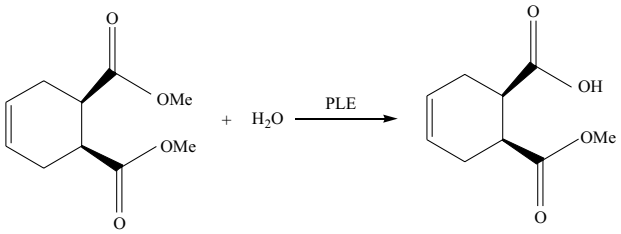

Fig. 9. PLE resolution of dimethyl *cis*-cyclohex-4-ene-1,2-dicarboxylate

A recombinant PLE isoenzyme (rPLE) could be produced with reliable product quality without interference of other isoenzymes and hydrolases, but it showed striking differences in substrate specificity and enantioselectivity compared to commercial enzymes from Fluka, Sigma or Roche Diagnostics. This was especially seen for the activity on naphthyl-butyrate (*81*) and the enantioselectivity for the kinetic resolution of (*R,S*)-1-phenyl-2-propyl acetate (*82*) and (*R,S*)-1-phenyl-2-butyl acetate (*83*) (Fig. 10).

Thus it must be emphasized that the expressed enzyme showed interesting features, but the name recombinant pig liver esterase (rPLE) is misleading since many bioconversions employing this enzyme result in products other than those obtained with the commercial native enzyme preparations. Variants of the cloned esterase gene were obtained by site-directed mutagenesis and the enantioselectivity was further increased. A six-fold increase in enantioselectivity compared to rPLE wild type was observed in the hydrolysis of racemic phenylethyl acetate using a variant containing a single amino acid change (E77G) (*84*).

Carboxypeptidase B and trypsin

Carboxypeptidase B preferentially hydrolyzes the basic amino acids lysine, arginine and ornithine from the C-terminal position of polypeptides. Trypsin is a pancreatic serine protease with a substrate specificity based on positively charged lysine and arginine side chains. The key source for these enzymes is pig pancreas. They are mainly used in the process for the production of human insulin, which is a polypeptide hormone and used as the therapeutic for the wide-spread disease diabetes mellitus (*85*).

There are four main routes to produce insulin: extraction from human pancreas, chemical synthesis, conversion of porcine insulin to human insulin and fermentative production by genetically engineered microorganisms. The last of these was used by Eli Lilly, Hoechst Marion Roussel and Novo Nordisk. Pig pancreas carboxypeptidase B and trypsin were involved in these processes (*59*). At first, the proinsulin gene with an N-terminally attached methionine was over-expressed intracellularly as a fusion protein with the *trpE* gene in *Escherichia coli*. Methionine is needed for the subsequent cleavage of the fusion partner *trpE*. The proinsulin was formed after purification, cyanogen bromide (CNBr) cleavage of the fusion protein and an oxidative sulfitolysis process. Then proinsulin was cleaved by carboxypeptidase B from pig pancreas to yield human insulin. The reaction was carried out in aqueous medium at 30–35 °C with more than 95 % yield. For the trypsin method, the precursor proinsulin was directly produced in a fermentation process employing recombinant *Escherichia coli*, this production being subsequently followed by the enzymatic reaction. The reaction conditions were different in this case (temperature: 6 °C) and the yield was only >70 % (*59,85*). Similar processes were also performed in Hoechst Marion Roussel: their carboxypeptidase process also started with expression of a proinsulin fusion protein in *Escherichia coli* and the conversion and yield were >99.9 % and >90 %, respectively, after 4–6 h of reaction in aqueous medium at 30–35 °C (reactor volume: 7500 L; capacity: >0.5 t/year). In a different approach, the pre-proinsulin gene was produced directly by cultivation of recombinant *E. coli*, using the trypsin method. More than 99.9 % conversion and >65 % yield were achieved after 6 h of reaction time at 6 °C (reactor volume: 10 000 L; capacity: >0.5 t/year). Novo Nordisk combined recombinant expression in yeast with trypsin conversion: proinsulin was over-expressed in *Saccharomyces cerevisiae* and directly converted to the threonin ester of insulin by transpeptidation with trypsin (batch reaction). To prevent the possible cleavage by trypsin at position 22 of chain B, low water concentrations were maintained through the addition of organic solvents. A surplus of threonin ester, low temperature (6 °C) and low pH (<7.0) were applied. More than 99.9 % conversion and a yield greater than 97 % were obtained in this process and the threonin ester formed could be converted into human insulin by simple hydrolysis (*86*).

Production of Fine Chemicals with E.C. 4 – Lyases

Hydroxynitrile lyase (HNL)

In many plants, the release of HCN from damaged plant tissues is believed to serve as a defensive strategy against herbivoral attack or as a nitrogen source for the biosynthesis of L-asparagine. Hydroxynitrile lyases (HNLs, MDLs) are responsible for these reactions and their preferred natural substrates, cyanohydrins, are separated from the catalysts by compartmentalization on tissue or subcellular level. In synthetic chemistry, HNLs are mainly employed for the reverse reactions. Syntheses of optically

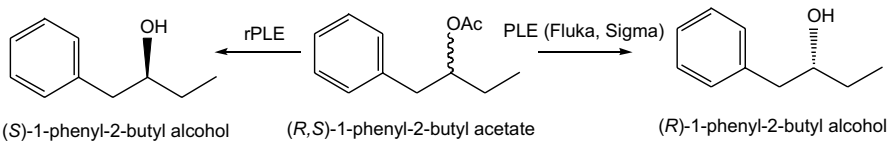

Fig. 10. Different enantioselectivity of recombinant PLE and commercially available PLE for the hydrolysis of (*R,S*)-1-phenyl-2-butyl acetate

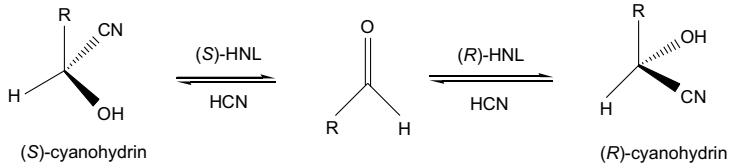

Fig. 11. Reversible addition of HCN to aldehydes and ketones by HNLs

active cyanohydrins are carried out by selective enzymatic addition of (hydro)cyanide to carbonyl groups (Fig. 11).

Cyanohydrins are important building blocks for syntheses of pharmaceuticals and agrochemicals such as α-hydroxy acids, α-hydroxy ketones and β-amino alcohols. HNLs are known from more than 2000 different living sources and almost a dozen enzymes have been purified and characterized from cyanogenic plants. The main sources were Rosaceae (*Prunus amygdalus*, *Pa*HNL), Gramineae (*Sorghum bicolor*, *Sb*HNL), Euphorbiaceae (*Hevea brasiliensis*, *Hb*HNL; *Manihot esculenta*, *Me*HNL) and Linaceae (*Linum usitatissimum*, *Lu*HNL) (*87*). Since 1996, several HNL genes have been cloned and over-expressed in *Escherichia coli* (*88*), *Saccharomyces cerevisiae* (*89*) and *Pichia pastoris* (*89,90*), thereby making the large scale production of (*S*)- and (*R*)-cyanohydrins by HNLs feasible.

*Sb*HNL is (*S*)-selective and was the first (*S*)-HNL used in organic solvents (where predominantly enzymatic reaction takes place) for the preparation of (*S*)-cyanohydrins such as (*S*)-*p*-hydroxymandelonitrile. Its major drawbacks include limited availability and limited substrate specificity: only aromatic and heteroaromatic aldehydes are accepted, while aliphatic aldehydes or ketones are not converted (*91,92*). In order to improve accessibility and expand the substrate range for these types of reactions, *Me*HNL from cassava and *Hb*HNL from *Hevea brasiliensis* were investigated in detail. These two HNLs are nowadays available in sufficient amounts for synthetic processes due to successful over-expression in *Escherichia coli* and *Pichia pastoris*, respectively. They are mainly used for the production of (*S*)-*m*-phenoxybenzaldehyde cyanohydrin in a biphasic process (98 % yield, 99 % e.e.) by DSM and Nippon Shokubai (*93–95*). This cyanohydrin is an intermediate for the production of pyrethroids and is produced in ton-scale at DSM (*96*) (Fig. 12).

The 3D-structures of *Hb*HNL and *Me*HNL have been determined (*97,98*) and provided the basis for substrate specificity improvements by rational enzyme design. Docking simulations between *Hb*HNL and chiral cyanohydrins showed the presence of a catalytic triad composed of a serine, a histidine and an aspartate residue and indicated a general acid-base catalysis mechanism (*99*). The active site seems to be accessible from the surface only through a short channel. This narrow channel is capped by a bulky tryptophan residue (W128), which limits the substrate transport efficiency to the enzyme's active site. Thus, although *Hb*HNL and *Me*HNL accept a wide range of carbonyl compounds, they showed low catalytic efficiency for the conversion of aldehydes with bulky substituents. By replacing the tryptophan at position 128 with smaller amino acids such as alanine or glycine, large substrates can enter and bind to the active center efficiently. This resulted in higher production and shorter reaction time (*100*).

(*R*)-hydroxynitrile lyases are mainly used for synthesis of (*R*)-mandelonitrile and its derivatives, which can subsequently be hydrolyzed to their corresponding carboxylic acids. The resulting α-hydroxy acids are used as chiral building blocks for synthesis of active pharmaceutical compounds and as racemate-resolving agents. There are two main representative recombinant (*R*)-hydroxynitrile lyases: *Lu*HNL and *Pa*HNL. *Lu*HNL has been cloned and over-expressed in *Pichia pastoris*, but with aromatic aldehydes the conversion to cyanohydrins could not be done to completion with this recombinant HNL and the enantioselectivity was low (*101*). *Pa*HNL from almonds is an excellent tool for the preparation of (*R*)-cyanohydrins and it is also the first discovered (*R*)-HNL (*87*). *Pa*HNL can be isolated and purified from almond meal. Aliphatic, aromatic and heterocyclic aldehydes as well as ketones are good substrates for this enzyme (*102,103*). For high enantioselectivity, especially for substrates that are accepted less efficiently than mandelonitrile, cyanohydrin synthesis has to be performed at low pH-values where the competitive chemical reaction is suppressed and the enantiomerically pure cyanohydrins that are produced are stable. However, the enzyme from natural sources is relatively expensive and lacks sufficient stability at low pH. In addition, enzyme preparations from different batches and suppliers contain different proportions of isoenzymes. This might be the main reason for varying enantioselectivity and conversion rates observed for the same substrate in different processes (*104*).

Recently at the Research Centre for Applied Biocatalysis, Graz, the *Pa*HNL isoenzyme 5 gene has been cloned, expressed and optimized successfully for industrial ap-

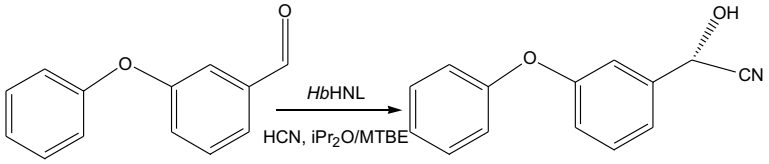

Fig. 12. (*S*)-*m*-phenoxybenzaldehyde cyanohydrin synthesis by *Hevea brasiliensis* hydroxynitrile lyase (*Hb*HNL) in a biphasic process (iPr_2O: isopropanol; MTBE: methyl tertiary butyl ether)

plications through a comprehensive molecular engineering process employing *Pichia pastoris* (*8,90*). It is noteworthy that this recombinant *Pa*HNL isoenzyme 5 shows high stability even at pH-values lower than 3. When secretion of over-expressed enzyme was driven by its native secretory signal sequence, 250 mg of active secreted enzyme per litre of culture supernatant were obtained. The recombinant enzyme showed extensive glycosylation and twice higher specific activity than the isolated *Pa*HNL from almond seeds. In a further step, the first amino acid of the mature protein, leucine, was changed into glutamine and the native plant signal sequence was replaced by the alpha mating factor leading sequence from *Saccharomyces cerevisiae*. In this manner, the enzyme expression level was improved approximately 4-fold. A production level of 1 g/L provides almost unlimited access to this biocatalyst, which in nature is expressed in floral tissues of almond trees (*8,90*). The recombinant enzyme is used industrially on a ton-scale for several processes at DSM.

Enzymatic synthesis of (*R*)-2-chlorobenzaldehyde cyanohydrin is the key step in the synthesis of (*R*)-2-chloromandelic acid, which is the chiral building block for the synthesis of the antidepressant and platelet aggregation inhibitor Clopidogrel® (*105*). This reaction was carried out in microaqueous or biphasic systems. Native *Pa*HNL is employed for this process of Nippon Shokubai and Clariant, resulting in an e.e. of greater than 83 % (Fig. 13) (*106–108*).

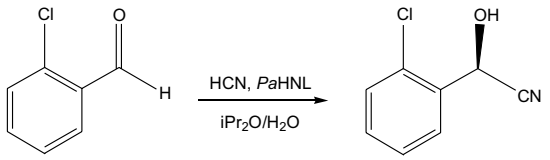

Fig. 13. Synthesis of (*R*)-*o*-chloromandelonitrile via *Pa*HNL (e.e.= 83 %) (iPr_2O: isopropanol)

The produced (*R*)-*o*-chloromandelonitrile can then be converted into (*R*)-*o*-chloromandelic acid without racemization (*1*). However, *ortho*-substituted substrates were converted with relatively low yield, low turnover frequencies (TOF) and low e.e. values (*109*).

At DSM, a mutant of *Pa*HNL5 is employed for the production of (*R*)-2-chloromandelic acid. Based on the structural information of the isoenzyme *Pa*HNL1 (*110*), as well as on docking experiments of chloro-substituted benzaldehydes with a model of *Pa*HNL5, the amino acid residues which are in close contact with the substrate were identified. Alanine 111 appeared to undergo steric clashes with the chloro-substituent in the *ortho*-position. Changing alanine at position 111 to glycine resulted in improved enzymatic activity with 2-Cl-benzaldehyde and can be used for the production of (*R*)-*o*-chloromandelic acid with >95 % yield and >95 % e.e. (*8*).

The other lyases or C-C bond-forming enzymes (*i.e.* other than HNLs) that are used in industrial applications currently originate exclusively from bacteria and fungi (*111*).

Production of Fine Chemicals with E.C. 5 – Isomerases

Epimerase

Epimerases are applied in industry to produce N-acetylneuraminic acid (Neu5Ac) from N-acetyl-D-glucosamine (GlcNAc) and pyruvate with porcine kidney N-acyl-D-glucosamine 2-epimerase (GlcNAc 2-epimerase) and *Escherichia coli* N-acetylneuraminate lyase (Neu5Ac lyase) (*59*). Neu5Ac has many biological functions as a receptor, by masking receptors and by regulation of the immune system (*112*). Due to these versatile functions, it has been used for the production of different therapeutics (*113*).

An integrated process was developed with porcine kidney GlcNAc 2-epimerase and *Escherichia coli* Neu5Ac lyase in a membrane reactor operated continuously at pH=7.5. The shortcomings of this process are that the epimerase was not available in large quantities and that it requires ATP as an allosteric activator. Additionally, both enzymes were inhibited by high concentrations of Neu5Ac (*114*).

The porcine GlcNAc 2-epimerase (*115*) and *Escherichia coli* Neu5Ac lyase (*116*) have been cloned and over-expressed in *Escherichia coli* to provide sufficient enzyme for the large-scale production of Neu5Ac (*117*) and this also promises to be applicable to the mass production of sialic acid at low cost (*118*).

For the production of Neu5Ac, the enzymatic epimerization and aldol condensation proceeded in a reversible process (Fig. 14). An excess molar amount of pyruvate was used to shift the equilibrium towards synthesis of Neu5Ac. The original reaction mixture consisted of 27

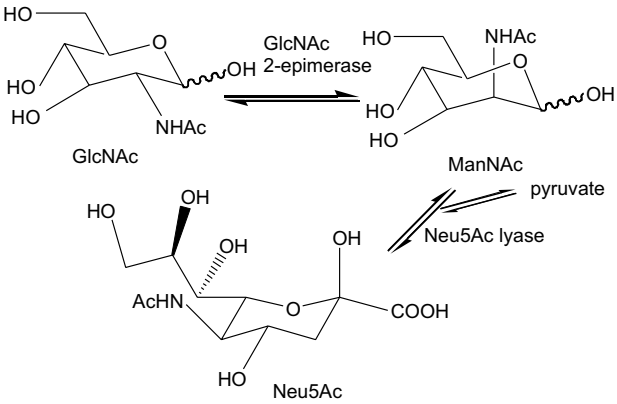

Fig. 14. Enzymatic synthesis of Neu5Ac from GlcNAc and pyruvate

kg of GlcNAc and 8 kg of pyruvate in 150 L of water before both enzymes were added. At the beginning, the molar ratio of GlcNAc to pyruvate was only 1:0.6 to avoid inhibition of GlcNAc 2-epimerase. The reaction was started by the addition of the two enzymes and continued by the addition of pyruvate step-by-step. After 240 h, the conversion of GlcNAc reached 77 % and 23 kg of Neu5Ac were obtained. This process was applied for multi kg production of Neu5Ac (Marukin Shoyu, Japan) (*59*).

Conclusions and Perspectives

For a long time biocatalysis has been taken as a prospective area for chemical synthesis, but the industrial applications were moderate, especially for enzymes from higher eukaryotes. Despite the fact that only a few industrial applications of eukaryotic enzymes have been reported, this review summarizes a significantly increasing use of these catalysts in organic syntheses.

Simple and quick access to large enzyme quantities was one of the major bottlenecks hampering industrial applications. Due to recent progress in heterologous enzyme production, this problem is now largely solved. Also the problem of heterogeneities in enzyme preparations has been solved by heterologous expression of single isoenzymes. In addition, a variety of processes employing hydrolases and other enzymes from animal sources have been reported, some of which were aimed to be used for the production of active pharmaceutical intermediates. However, there is still considerable skepticism about the employment of enzymes from animal resources due to the high risk of infectious contaminations (especially prions). Therefore, the efficient cloning and heterologous over-expression of these enzymes in suitable (microbial) hosts can be regarded as seminal progress in this field of biocatalysis.

The future will show if the discovery and enzyme engineering processes can also be accelerated to make higher eukaryotes ever more competitive as a source for robust and efficient industrial enzymes.

Acknowledgements

The authors thank B. Hauer and K. Faber for helpful suggestions and also W. Skranc for critically reviewing the manuscript. We are also grateful to the Technologie Impulse Gesellschaft (TIG), the province of Styria, the Steirische Wirtschaftsfoerderungsgesellschaft (SFG) and the city of Graz for financial support.

References

1. M. Breuer, K. Ditrich, T. Habicher, B. Hauer, M. Kesseler, R. Sturmer, T. Zelinski, *Angew. Chem.-Int. Edit. 43* (2004) 788–824.
2. A. J. Straathof, S. Panke, A. Schmid, *Curr. Opin. Biotechnol. 13* (2002) 548–556.
3. A. Schmid, J. S. Dordick, B. Hauer, A. Kiener, M. G. Wubbolts, B. Witholt, *Nature, 409* (2001) 258–268.
4. B. Schulze, M. G. Wubbolts, *Curr. Opin. Biotechnol. 10* (1999) 609–615.
5. J. B. van Beilen, Z. Li, *Curr. Opin. Biotechnol. 13* (2002) 338–344.
6. N. Pras, H. J. Woerdenbag, W. vanUden, *Agric. Biotechnol. News Info. 7* (1995) 231–234.
7. N. Pras, H. J. Woerdenbag, W. vanUden, *Plant Cell Tissue Organ Cult. 43* (1995) 117–121.
8. A. Glieder, R. Weis, W. Skranc, P. Poechlauer, I. Dreveny, S. Majer, M. G. Wubbolts, H. Schwab, K. Gruber, *Angew. Chem.-Int. Edit. 42* (2003) 4815–4818.
9. J. L. Cereghino, J. M. Cregg, *FEMS Microbiol. Rev. 24* (2000) 45–66.
10. V. Guallar, M. Jacobson, A. McDermott, R. A. Friesner, *J. Mol. Biol. 337* (2004) 227–239.
11. L. Snajdrova, P. Kulhanek, A. Imberty, J. Koca, *Carbohydr. Res. 339* (2004) 995–1006.
12. R. Weis, R. Luiten, W. Skranc, H. Schwab, M. G. Wubbolts, A. Glieder, *FEMS Yeast Res. 5* (2004) 179–189.
13. E. T. Farinas, T. Bulter, F. H. Arnold, *Curr. Opin. Biotechnol. 12* (2001) 545–551.
14. M. Leisola, J. Jokela, O. Pastinen, O. Turunen, H. Schoemaker: *Industrial Use of Enzymes*, Eolss Publishers, Oxford (2001).
15. R. Scott: *Cheesemaking Practice*, Applied Science Publishers Ltd., London (1981).
16. G. S. Kelly, *Altern. Med. Rev. 1* (1996) 243–257.
17. B. Wuethrich, *Hautarzt, 36* (1985) 123–125.
18. F. P. Guengerich, T. Shimada, *Chem. Res. Toxicol. 4* (1991) 391–407.
19. F. J. Gonzalez, *Trends Pharmacol. Sci. 13* (1992) 346–352.
20. A. D. Rodrigues, T. H. Rushmore, *Curr. Drug Metab. 3* (2002) 289–309.
21. D. S. Streetman, *America's Pharmacist, 121* (1999) 57–62.
22. J. Lill, L. A. Bauer, J. R. Horn, P. D. Hansten, *Am. J. Health-Syst. Pharm. 57* (2000) 1579–1584.
23. S. H. Preskorn: *Clinical Pharmacology of Selective Serotonin Reuptake Inhibitors*, Professional Communications, Inc., Caddo (1996).
24. T. Aoyama, S. Yamano, P. S. Guzelian, H. V. Gelboin, F. J. Gonzalez, *Proc. Natl. Acad. Sci. USA, 87* (1990) 4790–4793.
25. C. L. Crespi, B. W. Penman, D. T. Steimel, H. V. Gelboin, F. J. Gonzalez, *Carcinogenesis, 12* (1991) 355–359.
26. J. T. Buters, K. R. Korzekwa, K. L. Kunze, Y. Omata, J. P. Hardwick, F. J. Gonzalez, *Drug Metab. Dispos. 22* (1994) 688–692.
27. H. F. Bligh, C. R. Wolf, G. Smith, J. D. Beggs, *Gene, 110* (1992) 33–39.
28. J. P. Renaud, M. A. Peyronneau, P. Urban, G. Truan, C. Cullin, D. Pompon, P. Beaune, D. Mansuy, *Toxicology, 82* (1993) 39–52.
29. M. Bureik, B. Schiffler, Y. Hiraoka, F. Vogel, R. Bernhardt, *Biochemistry, 41* (2002) 2311–2321.
30. E. M. Gillam, T. Baba, B. R. Kim, S. Ohmori, F. P. Guengerich, *Arch. Biochem. Biophys. 305* (1993) 123–131.
31. T. Shimada, E. M. Gillam, P. Sandhu, Z. Guo, R. H. Tukey, F. P. Guengerich, *Carcinogenesis, 15* (1994) 2523–2529.
32. J. A. Blake, M. Pritchard, S. Ding, G. C. Smith, B. Burchell, C. R. Wolf, T. Friedberg, *FEBS Lett. 397* (1996) 210–214.
33. P. M. Patrick, W. C. Roland, F. T. Herbert, *Cytochrome P450 Expression in Enterobacteria. WO9802554* (1996).
34. T. H. Rushmore, P. J. Reider, D. Slaughter, C. Assang, M. G. Shou, *Metab. Eng. 2* (2000) 115–125.
35. P. M. Shaw, N. A. Hosea, D. V. Thompson, J. M. Lenius, F. P. Guengerich, *Arch. Biochem. Biophys. 348* (1997) 107–115.
36. J. E. Seip, S. K. Fager, J. E. Gavagan, L. W. Gosser, D. L. Anton, R. DiCosimo, *J. Org. Chem. 58* (1993) 2253–2259.
37. Y. Lindqvist, *J. Mol. Biol. 209* (1989) 151–166.
38. D. L. Anton, R. DiCosimo, J. E. Gavagan, *Production of Glyoxylic Acid with Glycolate Oxidase and Catalase Immobilized on Oxirane Acrylic Beads. US Patent 5439813* (1995).
39. P. Macheroux, S. B. Mulrooney, C. H. Williams Jr., V. Massey, *Biochim. Biophys. Acta, 1132* (1992) 11–16.
40. K. Stenberg, Y. Lindqvist, *Protein. Expr. Purif. 8* (1996) 295–298.
41. P. Macheroux, V. Massey, D. J. Thiele, M. Volokita, *Biochemistry, 30* (1991) 4612–4619.
42. M. S. Payne, K. L. Petrillo, J. E. Gavagan, L. W. Wagner, R. DiCosimo, D. L. Anton, *Gene, 167* (1995) 215–219.

43. M. S. Payne, K. L. Petrillo, J. E. Gavagan, R. DiCosimo, L. W. Wagner, D. L. Anton, *Gene*, *194* (1997) 179–182.

44. W. Adam, M. Lazarus, B. Boss, C. R. Saha-Moeller, H. U. Humpf, P. Schreier, *J. Org. Chem. 62* (1997) 7841–7843.

45. W. Adama, M. Lazarus, C. R. Saha-Moeller, P. Schreier, *Tetrahedron: Asymmetry*, *9* (1998) 351–355.

46. W. Kroutil, H. Mang, K. Edegger, K. Faber, *Adv. Synth. Catal. 346* (2004) 125–142.

47. J. D. Stewart, *Curr. Opin. Chem. Biol. 5* (2001) 120–129.

48. C. Barbieri, E. Caruso, P. D'Arrigo, G. P. Fantoni, S. Servi, *Tetrahedron: Asymmetry*, *10* (1999) 3931–3937.

49. K. I. Fuhshuku, N. Funa, T. Akeboshi, H. Ohta, H. Hosomi, S. Ohba, T. Sugai, *J. Org. Chem. 65* (2000) 129–135.

50. T. Matsuda, T. Harada, N. Nakajima, K. Nakamura, *Tetrahedron Lett. 41* (2000) 4135–4138.

51. T. Matsuda, T. Harada, N. Nakajima, T. Itoh, K. Nakamura, *J. Org. Chem. 65* (2000) 157–163.

52. P. E. Peterson, T. J. Smith, *Struct. Fold. Des. 7* (1999) 769–782.

53. R. L. Hanson, M. D. Schwinden, A. Banerjee, D. B. Brzozowski, B. C. Chen, B. P. Patel, C. G. McNamee, G. A. Kodersha, D. R. Kronenthal, R. N. Patel, L. J. Szarka, *Bioorg. Med. Chem. 7* (1999) 2247–2252.

54. R. N. Patel, *Biomol. Eng. 17* (2001) 167–182.

55. J. A. Robl, M. P. Cimarusti, *Tetrahedron Lett. 35* (1994) 1393–1396.

56. J. Yourno, W. Mastropaolo, *Blood*, *58* (1981) 939–946.

57. A. Pandey, S. Benjamin, C. R. Soccol, P. Nigam, N. Krieger, V. T. Soccol, *Biotechnol. Appl. Biochem. 29 (Pt 2)* (1999) 119–131.

58. K. Faber: *Biotransformations in Organic Chemistry*, Springer, Heidelberg (2000).

59. A. Liese, K. Seelbach, C. Wandrey: *Industrial Biotransformations*, Wiley-VCH, Weinheim (2000).

60. W. E. Ladner, G. M. Whitesides, *J. Am. Chem. Soc. 106* (1984) 7250–7251.

61. R. A. Sheldon: *Lipase-Mediated Reactions with Nitrogen Nucleophiles*, Elsevier, London (1991).

62. E. I. Fukusaki, S. Satoda, S. Senda, T. Omata, *J. Biosci. Bioeng. 87* (1999) 103–104.

63. V. S. Parmar, H. N. Pati, A. Azim, R. Kumar, Himanshu, K. S. Bisht, A. K. Prasad, W. Errington, *Bioorg. Med. Chem. 6* (1998) 109–118.

64. S. Rodríguez, P. Clapés, F. Camps, G. Fabriàs, *J. Org. Chem. 67* (2002) 2228–2233.

65. Y. Nagao, S. Miyamoto, K. Hayashi, A. Mihira, S. Sano, *Chem. Pharm. Bull. 50* (2002) 558–562.

66. M. Angoli, A. Barilli, G. Lesma, D. Passarella, S. Riva, A. Silvani, B. Danieli, *J. Org. Chem. 68* (2003) 9525–9527.

67. H. J. Gais, C. Griebela, H. Buschmann, *Tetrahedron: Asymmetry*, *11* (2000) 917–918.

68. H. A. Sousa, J. G. Crespo, C. A. M. Afonso, *Tetrahedron: Asymmetry*, *11* (2000) 929–934.

69. X. Liang, A. Lohse, M. Bols, *J. Org. Chem. 65* (2000) 7432–7437.

70. M. S. Yu, I. Lantos, Z. Q. Peng, J. Yu, T. Cacchio, *Tetrahedron Lett. 41* (2000) 5647–5651.

71. F. Theil, *Tetrahedron*, *56* (2000) 2905–2919.

72. A. M. P. Koskinen, A. M. Klibanov: *Enzymatic Reactions in Organic Media*, Kluwer Academic Publishers, Dordrecht (1995).

73. G. Carrea, S. Riva, *Angew. Chem.-Int. Edit. 39* (2000) 2226–2254.

74. J. M. Harris, S. Zalpinsky: *Poly (Ethylene Glycol) Chemistry and Biological Applications*, American Chemical Society, Washington (1997).

75. L. K. Hoong, L. E. Strange, D. C. Liotta, *J. Org. Chem. 57* (1992) 5563–5565.

76. E. De Clercq, *Int. J. Biochem. Cell Biol. 36* (2004) 1800–1822.

77. D. J. Bennett, K. I. Buchanan, A. Cooke, O. Epemolu, N. M. Hamilton, E. J. Hutchinson, A. Mitchell, *J. Chem. Soc. Perkin Trans. I*, *4* (2001) 362–365.

78. C. Böhm, W. F. Austin, D. Trauner, *Tetrahedron: Asymmetry*, *14* (2003) 71–74.

79. M. Matsushima, H. Inoue, M. Ichinose, S. Tsukada, K. Miki, K. Kurokawa, T. Takahashi, K. Takahashi, *FEBS Lett. 293* (1991) 37–41.

80. E. Heymann, K. Peter, *Biol. Chem. Hoppe Seyler*, *374* (1993) 1033–1036.

81. S. Lange, A. Musidlowska, C. Schmidt-Dannert, J. Schmitt, U. T. Bornscheuer, *Chembiochem*, *2* (2001) 576–582.

82. A. Musidlowska, S. Lange, U. T. Bornscheuer, *Angew. Chem.-Int. Edit. 40* (2001) 2851–2853.

83. A. Musidlowska-Persson, U. T. Bornscheuer, *J. Mol. Catal. B-Enzym. 19–20* (2002) 129–133.

84. A. Musidlowska-Persson, U. T. Bornscheuer, *Tetrahedron: Asymmetry*, *14* (2003) 1341–1344.

85. M. R. Ladisch, K. L. Kohlmann, *Biotechnol. Progr. 8* (1992) 469–478.

86. Novo Nordisk, *Process for Preparing Insulin Esters. GB2069502* (1981).

87. F. Effenberger, S. Foerster, H. Wajant, *Curr. Opin. Biotechnol. 11* (2000) 532–539.

88. H. Wajant, S. Foerster, A. Sprauer, F. Effenberger, K. Pfizenmaier, *Ann. NY Acad. Sci. 799* (1996) 771–776.

89. M. Hasslacher, M. Schall, M. Hayn, H. Griengl, S. D. Kohlwein, H. Schwab, *J. Biol. Chem. 271* (1996) 5884–5891.

90. R. Weis, P. Poechlauer, R. Bona, W. Skranc, R. Luiten, M. G. Wubbolts, H. Schwab, A. Glieder, *J. Mol. Catal. B-Enzym. 29* (2004) 211–218.

91. F. Effenberger, B. Hoersch, S. Foerster, T. Ziegler, *Tetrahedron Lett. 31* (1990) 1249–1252.

92. U. Niedermeyer, M. R. Kula, *Angew. Chem.-Int. Edit. 29* (1990) 386–387.

93. M. Hasslacher, M. Schall, H. Schwab, E. Hayn, S. Kohlwein, H. Griengl, *DSM, (S)-Hydroxynitrile Lyase from Hevea brasiliensis. WO9703204* (1997).

94. H. Semba, *Method for the Production of Optically Active Cyanohydrins. EP1026256*, Nippon Shokubai (2000).

95. C. van Den Broek, H. J. Wories, R. Reintjens, R. Neuhofer, M. Schmidt, I. Wirth, H. Griengl, P. Poechlauer, A. Zabelinskaja-Machova, *Enzymatic Process for the Preparation of (S)-Cyanhydrines. EP0927766* (1999).

96. H. U. Blaser, E. Schmidt: *Asymmetric Catalysis on Industrial Scale*, WILY-VCH Verlag GmbH & Co. KGaA, Weinheim (2003).

97. J. Zuegg, K. Gruber, M. Gugganig, U. G. Wagner, C. Kratky, *Protein Sci. 8* (1999) 1990–2000.

98. H. Lauble, K. Decanniere, H. Wajant, S. Foerster, F. Effenberger, *Acta. Crystallogr. Sect. D-Biol. Crystallogr. 55* (1999) 904–906.

99. K. Gruber, *Proteins*, *44* (2001) 26–31.

100. F. Effenberger, H. Lauble, H. Bühler, H. Wajant, S. Foerster, H. Schwab, C. Kratky, U. Wagner, E. Steiner, *(S)-Hydroxynitrile Lyases with Improved Substrate Binding Arising from Modifications Affecting the Substrate Binding Site and Their Uses. EP0969095* (2000).

101. K. Trummler, J. Roos, U. Schwaneberg, F. Effenberger, S. Foerster, K. Pfizenmaier, H. Wajant, *Plant Sci. 139* (1998) 19–27.

102. F. Effenberger, *Angew. Chem.-Int. Edit. 33* (1994) 1555–1564.

103. F. Effenberger, B. Hoersch, F. Weingart, T. Ziegler, S. Kühner, *Tetrahedron Lett. 32* (1991) 2605–2608.

104. G. de Gonzalo, R. Brieva, V. Gotor, *J. Mol. Catal. B-Enzym. 19–20* (2002) 223–230.

105. A. Bousquet, A. Musolino, *Hydroxyacetic Ester Derivatives, Preparation Method and Use as Synthesis Intermediates. EP1021449, Sanofi-Synthelabo* (1998).

106. P. Poechlauer, H. Mayrhofer, *Method for Producing Optically and Chemically Pure (R)- and (S)-Hydroxycarboxylic Acids. EP1148042, DSM Fine Chemicals Austria* (2001).

107. N. Okuda, H. Semba, Y. Dobashi, *A Method for Producing Alpha-Hydroxycarboxylic Acid. EP1160235, Nippon Shokubai* (2001).

108. H. Semba, Y. Dobashi, *An Enzyme Reaction Method and a Method for Enzymatically Producing an Optically Active Cyanohydrin. EP1160329, Nippon Shokubai* (2001).

109. B. Kirschbaum, G. Wilbert, F. Effenberger, *Process for Preparing Optically Active Cyanohydrins and Secondary Products. US2002052523, Clariant GmbH* (2002).

110. I. Dreveny, C. Kratky, K. Gruber, *Protein Sci. 11* (2002) 292–300.

111. M. Breuer, B. Hauer, *Curr. Opin. Biotechnol. 14* (2003) 570–576.

112. R. Schauer: *Sialic Acids, Chemistry, Metabolism, and Function*, Springer-Verlag, Wien (1982).

113. M. von Itzstein, W. Y. Wu, G. B. Kok, M. S. Pegg, J. C. Dyason, B. Jin, T. Van Phan, M. L. Smythe, H. F. White, S. W. Oliver, P. M. Colman, J. N. Varghese, D. M. Ryan, J. M. Woods, R. C. Bethell, V. J. Hotham, J. M. Cameron, C. R. Penn, *Nature, 363* (1993) 418–423.

114. U. Kragl, D. Gygax, O. Ghisalba, C. Wandrey, *Angew. Chem.-Int. Edit. 30* (1991) 827–828.

115. I. Maru, Y. Ohta, K. Murata, Y. Tsukada, *J. Biol. Chem. 271* (1996) 16294–16299.

116. Y. Ohta, Y. Tsukada, T. Sugimori, K. Murata, A. Kimura, *Agric. Biol. Chem. 53* (1989) 477–481.

117. I. Maru, J. Ohnishi, Y. Ohta, Y. Tsukada, *Carbohydr. Res. 306* (1998) 575–578.

118. I. Maru, J. Ohnishi, Y. Ohta, Y. Tsukada, *J. Biosci. Bioeng. 93* (2002) 258–265.

Enzimi viših eukariota za industrijsku biokatalizu

Sažetak

Industrijska proizvodnja finih kemikalija, sastojaka hrane, farmaceutskih proizvoda, agrokemikalija i njihovih međuprodukata temelji se na sve većoj primjeni biokatalize, tj. na enzimima ili cijelim stanicama što kataliziraju pretvorbu molekula. Jednostavni postupci otkrivanja, kloniranja i povećane ekspresije, te brzi rast daju prednost fungima i kvascima, a osobito bakterijama kao izvorima biokatalizatora. Viši eukarioti sadrže skoro neograničeni broj potencijalnih biokatalizatora. Danas je opskrba tim enzimima u industrijskim procesima ograničena zbog velike heterogenosti enzimskih pripravaka i opasnosti od infekcijskih primjesa. U prošlosti se samo nekoliko životinjskih te biljnih enzima iz poljoprivrednih otpadaka koristilo u proizvodnji hrane. Uporaba ekspresiranih bakterijskih sojeva ili nekonvencionalnih kvasaca za heterološku proizvodnju djelotvornih eukariotskih enzima može nadomjestiti njihov nedostatak i omogućiti dovoljnu količinu homogenih enzimskih pripravaka za pouzdanu i ekonomski opravdanu primjenu u industrijskom mjerilu. Idealni enzimski procesi nisu opasni za okoliš te provode skoro 100 %-tnu pretvorbu najčešće neprirodnih supstrata u čiste proizvode. Najnovija dostignuća pokazuju da su isplativi biokatalitički procesi u industrijskom mjerilu, koristeći enzime iz viših eukariota (biljaka ili životinja), kao i njihova primjena u pilot-postrojenjima.