

Biocatalytic ketone reduction—a powerful tool for the production of chiral alcohols—part I: processes with isolated enzymes

Katja Goldberg · Kirsten Schroer · Stephan Lütz ·
Andreas Liese

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Abstract Enzymes are able to perform reactions under mild conditions, e.g., pH and temperature, with remarkable chemo-, regio-, and stereoselectivity. Because of this feature, the number of biocatalysts used in organic synthesis has rapidly increased during the last decades, especially for the production of chiral compounds. The present review highlights biotechnological processes for the production of chiral alcohols by reducing prochiral ketones. These reactions can be catalyzed by either isolated enzymes or whole cells that exhibit ketone-reducing activity. The use of isolated enzymes is often preferred because of a higher volumetric productivity and the absence of side reactions. Both types of catalysts have also deficiencies limiting their use in synthesis of chiral alcohols. Because reductase-catalyzed reactions are dependent on cofactors, one major task in process development is to provide an effective method for regeneration of the consumed cofactors. In this paper, strategies for cofactor regeneration in biocatalytic ketone reduction are reviewed. Furthermore, different processes carried out on laboratory and industrial scales using isolated enzymes are presented. Attention is turned to process parameters, e.g., conversion, yield, enantiomeric excess, and process strategies, e.g., the application of biphasic systems or methods of in situ (co)product recovery. The biocatalytic production of chiral alcohols utilizing whole

cells is presented in part II of this review (Goldberg et al., *Appl Microbiol Biotechnol*, 2007).

Keywords Ketone reduction · Cofactor regeneration · Chiral alcohol

Introduction

Today, chiral compounds are the most important building blocks in the chemical and pharmaceutical industry for the production of, for example, chemical catalysts, liquid crystals, flavors, agrochemicals, or drugs (Daubmann et al. 2006a). Optical active secondary alcohols are especially widely used as intermediates for the introduction of chiral information into the product. In the industry, usually well-established chemical methods are used (Blaser et al. 2003), but in the last decades, the interest for creating stereogenic centers by applying biocatalytic methods has risen (Honda et al. 2006). In particular, the number of industrial processes using alcohol dehydrogenases (ADHs) is increasing (Breuer et al. 2004; Buchholz and Gröger 2006; Liese et al. 2006). These biocatalysts, used as isolated enzymes or whole cells, catalyze the stereoselective reduction of prochiral ketones with remarkable chemo-, regio-, and stereoselectivity (Fig. 1; Wandrey 2004).

Biocatalytic production of chiral alcohols

The biocatalytic production of chiral alcohols is possible following several pathways. Examples with enzymes from the classes oxidoreductases (EC 1), hydrolases (EC 3), and lyases (EC 4) are described in literature (Fig. 2).

Oxidoreductases catalyze redox reactions; thus, the transfer of electrons from or to the substrate. The reduction

Katja Goldberg and Kirsten Schroer did equally contribute to this review.

K. Goldberg · A. Liese (✉)
Institute of Technical Biocatalysis,
Hamburg University of Technology,
21073 Hamburg, Germany
e-mail: liese@tuhh.de

K. Schroer · S. Lütz
Institute of Biotechnology 2, Forschungszentrum Jülich GmbH,
52425 Jülich, Germany

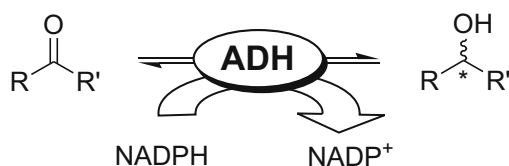


Fig. 1 Asymmetric biocatalytic reduction of ketones (nicotine amide dinucleotide cofactor: NADP^+ oxidized form, NADPH reduced form)

of ketones by, for example, ADHs (Peters 1998; also referred to as carbonyl reductases [CR] or ketone reductases) is the issue of this review and will be explained in detail later. Moreover, alcohols can also be obtained by oxidation of CH bonds by monooxygenases (van Beilen et al. 2003).

Hydrolases play the most important role in industrial enzymatic processes (Liese et al. 2006). Between 1987 and 2003, around 60% of the published articles were dealing with enzymes of this class (Faber 2004). The lipases as a subgroup of the hydrolases catalyze the hydrolytic cleavage of the carbon–oxygen single bonds in esters or analogous carboxylic derivatives to produce acids and alcohols (Bornscheuer et al. 1994; Jaeger et al. 1997; Reetz et al. 1997; Jaeger and Eggert 2002; Detry et al. 2006; Elend et al. 2006). In most cases, chiral products are obtained by the (dynamic) kinetic resolution of racemic mixtures.

Alcohols can also be obtained by using phosphate or sulphate esters as a substrate for phosphatases or sulphatases (Wallner et al. 2005), respectively. However, these

enzymes are only rarely used for the production of alcohols (Faber 2004).

Lyases catalyze the formation of carbon–carbon bonds. By setting ketones or aldehydes as a substrate, alcohols are available (Adam et al. 1999; Pohl and Liese 2006). A widespread catalyst for this reaction is the benzaldehyde lyase (BAL), which produces hydroxyketones from two aldehydes (Demir et al. 2002; Kihumbu et al. 2002; Kurlemann and Liese 2004; Domínguez de María et al. 2006; Hildebrand et al. 2006; Stillger et al. 2006).

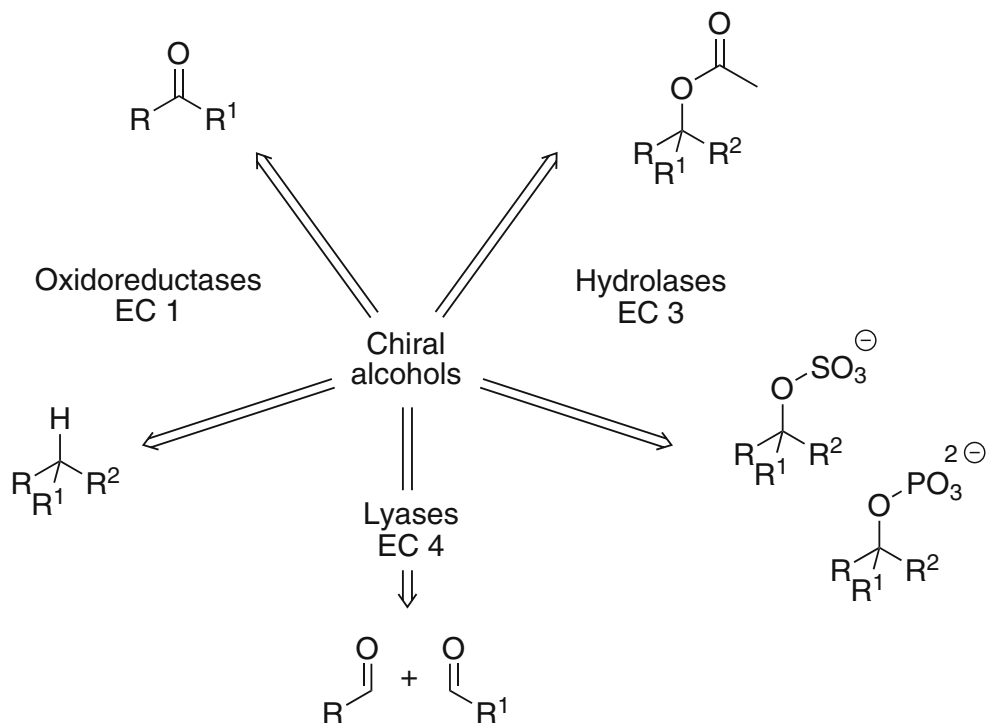
Other examples are the pyruvate decarboxylase, which couples, for example, acetaldehyde and benzaldehyde to phenylacetylcarbinol (Rosche et al. 2002; Leksawadi et al. 2004) or the deoxyriboaldolase that is able to catalyze the aldol condensation with chloroacetaldehyde with two molecules of acetaldehyde in the industrial production of statines (Liu et al. 2004; Müller 2005).

Another group of enzymes to be mentioned are the hydroxynitrile lyases. The coupling of ketones with hydrogen cyanide leads to nitrile substituted alcohols (Griengl et al. 1997; Avi et al. 2004; Fechter and Griengl 2004; Gaisberger et al. 2004).

Cofactor regeneration

The majority of ADHs are dependent on the nicotinamide cofactors β -1,4-nicotinamide adenindinucleotide (NADH) or β -1,4-nicotinamide adenindinucleotide phosphate (NADPH). Rarely, enzyme-bound cofactors from the group

Fig. 2 Retrosynthesis: examples for the biocatalytic production of alcohols



of the flavines (FAD; Walsh 1980) or methoxatines (pyrroloquinoline quinone, PQQ; McWhirter and Klapper 1990) are found. Because all these cofactors are too expensive to be used stoichiometrically—with prices ranging from €1,300 per mol for NAD^+ to more than €2,700,000 per mol for PQQ (Leonida 2001)—there has been significant interest in developing efficient cofactor regeneration processes (Wichmann and Vasic-Racki 2005; Lütz 2006). FAD- and PQQ-dependent enzymes are seldomly used in preparative synthesis (Liese et al. 2006) and thus not considered in this review.

Cofactor regeneration is carried out parallel to the conversion of substrate to product (Fig. 3). In the case of the ADHs, the production of NADPH can be carried out by means of chemical, electrochemical, photochemical, and enzymatic methods. A further approach to cofactor regeneration in whole-cell biotransformation processes is the usage of the metabolism of cultivated or resting cells utilizing glucose or other compounds as nutrient (Haberland et al. 2002).

Electrochemical regeneration

Cofactors only switch between the oxidized and reduced state, so electrochemical methods are appealing. Direct cathodic reduction (Simon et al. 1985; Biade et al. 1992) of NADP^+ suffer from low regioselectivity and side reactions because of high overpotentials (Hollmann and Schmid 2004). Therefore, organic and metal containing electron shuttles for the transfer of electrons between electrode and NADP^+ or NADPH, respectively, were developed (Steckhan et al. 1990, 1991).

Typical organic electron shuttles (Fig. 4) are based on large conjugated systems. In the case of methyl viologen (MV, **1**), enzymes like ferredoxin NADP^+ reductase for NADPH or diaphorase for NADH are necessary to transfer the reduction equivalents from the viologen to NADP^+ (Yuan et al. 1997).

In the glycerol dehydrogenase-catalyzed oxidative resolution of racemic 1,2-diols 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate (**2**) is applied to regenerate NAD^+ (Schröder et al. 2003; Degenring et al. 2004). Further examples are 1,10-phenanthroline-5,6-dione (**3**) or its N-monomethylated derivatives (Hilt et al. 1997).

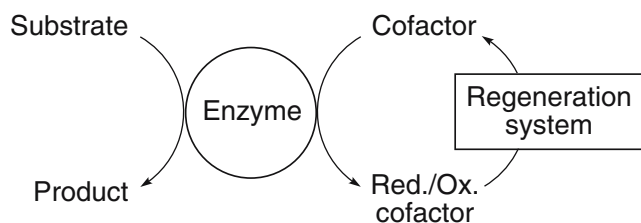


Fig. 3 Cofactor regeneration in general

Besides the application of organic reduction equivalents, metal-containing electron shuttles were described: for example, $[\text{Cp}^*\text{Rh}(\text{bpy})(\text{H}_2\text{O})]^{2+}$ ($\text{Cp}^*=\text{C}_5\text{Me}_5$, $\text{bpy}=2,2'$ -bipyridine) performs fast and quantitative reductions of NADP^+ to NADPH (Steckhan et al. 1990; Vuorilehto et al. 2004; Hollmann et al. 2006).

Chemical regeneration

Inorganic salts like sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$; Jones et al. 1972) offer easy handling but suffer at the same time from enzyme deactivation processes at higher salt concentrations (Raunio and Lilius 1971).

Since the 1980s, the first examples utilizing hydrogen and metal complexes (Abril and Whitesides 1982; Wagenknecht et al. 2003) were described. More often than not, late transition metals like rhodium, ruthenium, and platinum and their complexes are applied.

A similar approach is the combination of a platinum carbonyl cluster with the dye safranin (**4**, Fig. 5) in a two-phase system (Bhaduri et al. 1998; Lütz 2006).

Photochemical regeneration

Homogeneous photosensitizers such as ruthenium or zinc complexes, dyes like methylene blue, and heterogeneous semiconductor powders and colloids like cadmium sulfide or titanium dioxide have been used for the light-induced production of methyl viologen and subsequent regeneration of NADPH in the presence of dihydrolipoamide dehydrogenase or ferredoxin reductase, respectively (Julliard 2004; Willner et al. 1990; Rickus et al. 2002).

Enzymatic regeneration

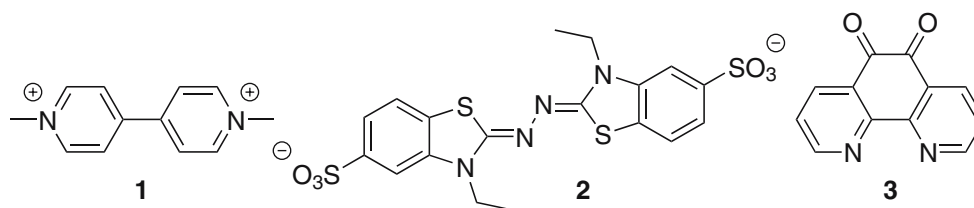
For this method, there are two different approaches: the enzyme-coupled and the substrate-coupled process (Hummel and Kula 1989).

The enzyme-coupled approach uses a sacrificial cosubstrate that is converted by a second enzyme in the opposite redox direction. Several methods are established in literature, some examples are explained in detail:

- Formate oxidation by formate dehydrogenase (FDH)

A widespread NADPH regeneration system is the oxidation of formate to carbon dioxide by FDH (Hummel and Kula 1989; Seelbach et al. 1996; Tishkov et al. 1999). A process for the production and purification of FDH from *Candida boidinii* in large scale has been developed (Weuster-Botz et al. 1994), and the enzyme is used in the industrial production of *L-tert-leucine* (Bommarius et al. 1995). Advantages are inertness, readily removal of the coproduct carbon

Fig. 4 Exemplary organic electron shuttles for cofactor regeneration



dioxide and thereby a favorable thermodynamic equilibrium, good availability, low cost of the FDH, and the cheap cosubstrate formate. In some cases, the change of pH during the cofactor regeneration causes inhibitory effects (Nidetzky et al. 1996); this can be overcome by applying a fed-batch process (Neuhauser et al. 1998). Most of the FDHs are NADH dependent, while many ADHs are NADPH dependent. In most cases, the lower activity of the FDH towards NADPH is tolerated (Ernst et al. 2005). An approach to overcome this drawback is the use of transhydrogenases that can move reduction equivalents between NADH and NADPH (Weckbecker and Hummel 2004).

- Glucose dehydrogenase (GDH) or glucose-6-phosphate dehydrogenase (G6PDH)

The GDH or G6PDH oxidizes the cosubstrates glucose or glucose-6-phosphate, respectively, and is suited for the regeneration of NADPH (Kizaki et al. 2001; Weckbecker and Hummel 2005). Both enzymes are rather inexpensive, highly active, and stable. Because of the hydrolysis of the coproducts gluconolactone or 6-phosphogluconolactone to the corresponding acids, the reaction is nearly irreversible. The natural preference for NADP^+ is a benefit for the use of these enzymes as cofactor-regenerating enzymes. A drawback is the high cost of glucose-6-phosphate and the demand for permeabilization when whole cells are applied as biocatalysts (Makino et al. 1989; Kataoka et al. 1998).

- Alcohol oxidation by ADH

For the enzyme-coupled cofactor regeneration applying a second ADH, only few examples are known (Leonida 2001). The reduction of pyruvate to L-lactate can also be used for the cofactor regeneration in enzyme-coupled processes (Kim and Whitesides 1988; Liese et al. 1996).

- Direct reduction of NADP^+ with hydrogen/hydrogenase

Hydrogenases are bidirectional enzymes that catalyze the production and oxidation of molecular hydrogen. The direct regeneration of NADPH using this enzyme is probably the most elegant solution, as the molecule is consumed completely and no coproduct is formed (Klibanov and Puglisi 1980; Wong et al. 1981; Greiner et al. 2003; Mertens et al. 2003; Mertens and Liese 2004).

- Phosphite dehydrogenase (PTDH)

The oxidation of phosphite to phosphate with the concomitant reduction of NAD^+ to NADH is another approach to cofactor recycling (Vrtis et al. 2002). This enzyme can also use NADP^+ as a cofactor but with a lower activity (Costas et al. 2001). PTDH has been combined with several dehydrogenases such as lactate dehydrogenase, horse liver alcohol dehydrogenase (HLADH), malate dehydrogenase (Vrtis et al. 2002), xylose reductase, and ADH (Johannes et al. 2007).

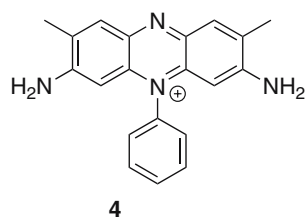
The enzyme-coupled process requires the application of two enzymes at the same time. The substrate-coupled approach, thus applying only one enzyme for the production of the desired compound and the cofactor regeneration, is a powerful alternative in comparison to the enzyme-coupled approach (Tishkov et al. 1999; Stillger et al. 2002).

The auxiliary cosubstrate in the substrate-coupled process for the production of chiral alcohols is in most cases 2-propanol, which is oxidized to the coproduct acetone. Because there is a competition between substrate, product, cosubstrate, and coproduct, a thermodynamic equilibrium is present; hence, the maximum conversion is limited by the thermodynamics of the system. In situ (co)product removal (ISPR) processes like organophilic pervaporation, gassing out, reduced pressure, distillation, or crystallization can be applied to shift the equilibrium (Lye and Woodley 1999; Stark and von Stockar 2003; Takors 2004; von Scala et al. 2005; Buque-Taboada et al. 2006; Goldberg et al. 2006).

Reduction of prochiral ketones catalyzed by isolated enzymes

The use of isolated enzymes as biocatalysts offers some advantages in comparison to whole cells. Because of the presence of only one or two enzymes (in case of enzyme-coupled cofactor regeneration approaches), side reactions can be avoided and thus the reduction of enantioselectivity.

Fig. 5 Molecular structure of safranin 4



Therefore, downstream processing can be simplified. In comparison to processes catalyzed by whole cells, diffusion limitations do not occur. On the other hand, there is a need for an addition of cofactors to the process when isolated ADHs are applied. Furthermore, isolated enzymes may show high sensibility towards high concentrations of substrates and organic solvents (Liese et al. 1998; Schmid et al. 2001; Villela et al. 2003). Advantages of isolated dehydrogenases over their natural whole-cell biocatalysts have been reviewed several times (Kula and Kragl 2000; Hummel et al. 2003; Faber 2004). The decision on the application of isolated enzymes or whole cells depends on the specific requirements of the industrial reaction system.

The following section shows processes carried out in industrial and laboratory scales using isolated ADHs in a substrate-coupled approach or in combination with a second enzyme for cofactor regeneration. Different strategies for overcoming limitations and improving process parameters like conversion and space–time yield as well as for increasing the stability of biocatalysts should be pointed out.

Enzyme-coupled cofactor regeneration

Bristol-Myers Squibb uses an ADH from *Acinetobacter calcoaceticus* as catalyst for the production of 6-benzyloxy-(3*R*,5*S*)-dihydroxy-hexanoic acid ethyl ester, which is a key chiral intermediate for anticholesterol drugs. Cofactor NAD⁺ is added to the reaction medium together with GDH that catalyzes the regeneration of NADH. The producing ADH is applied as crude cell extracts. The process is carried out in a batch process with a yield of 92% and an enantiomeric excess (*ee*) of 99% (Patel et al. 1993). Furthermore, Codexis published the use of ADH and GDH, for example, for the production of ethyl-(*S*)-4-chloro-3-hydroxybutyrate. The process is carried out with almost complete conversion (>99.5%) and an *ee* greater than 99.9%. In this case, the enzymes have been optimized by directed evolution to achieve the process goals (Davis et al. 2005).

There are several examples for the use of FDH as a cofactor-regenerating enzyme in industrial biocatalysis. Ciba Spezialitätenchemie AG uses (*R*)-lactate-NAD oxidoreductase from *Staphylococcus epidermis* together with FDH from *C. boidinii* for the synthesis of (*R*)-2-hydroxy-4-phenyl-butyric acid. The process is carried out in a continuously operated stirred tank reactor equipped with an ultrafiltration membrane to retain the enzymes. To avoid degassing of the side product CO₂, which causes mechanical force, a pressure of 3 bar is applied. The reaction is carried out in a scale of 0.2 L with a space–time yield of 410 g L⁻¹ day⁻¹ and an *ee* of 99.9% (Schmidt et al. 1992). The company Pfizer makes use of the same combination of enzymes to produce (*R*)-3-(4-fluorophenyl)-2-hydroxypro-

panoate in an industrial scale. This reaction is also carried out in a continuously operated enzyme membrane reactor (EMR) with a scale of 2.2 L. This process is also characterized by a good space–time yield of 560 g L⁻¹ day⁻¹ and an *ee* of 99.9% (Tao and McGee 2002).

The two described industrial processes with enzyme-coupled regeneration of cofactors via FDH are both characterized by a simple reactor setup and an easy reaction strategy. Some interesting processes dealing with enzyme-coupled cofactor regeneration via FDH and two phase systems had been published, which are described in detail below. The main goal of applying biphasic systems is the feasibility of increasing the amounts of hydrophobic substrates to reach higher productivities. Beside biphasic systems, other methods like the usage of cyclodextrin-containing buffers as reaction medium have been applied to achieve higher concentrations of poorly soluble substrates (Zelinski et al. 1999).

A simple way to create an aqueous/organic biphasic system is the formation of microemulsions. Depending on the mixture and temperature, small droplets of the non-aqueous phase are dispersed in water, or in case of an oil-rich emulsion, reverse micelles of water can be dispersed in oil. Orlich et al. (2000) reported the application of reverse micelles for ADH-catalyzed reduction of ketones in an enzyme-coupled approach (Fig. 6). The microemulsion contained water, cyclohexane, and Marlipal 013-16 as the surfactant. The stability of the investigated ADHs and FDH where both increased in comparison to former aqueous/organic biphasic systems known from literature so far. Furthermore, it was possible to perform successful semi-batch experiments reducing a prochiral ketone (2-butanone) in full conversion and enantioselectivity.

Liese et al. (1998) reported the enantioselective reduction of 2-octanone catalyzed by *Candida parapsilosis* CR (CPCR). To increase the substrate solubility, an emulsion membrane reactor was developed, which consists of two reactor units. The first one was a stirred emulsion vessel in which the aqueous phase was separated from the organic phase by a hydrophilic ultrafiltration membrane. The organic phase was pure substrate. The substrate saturated

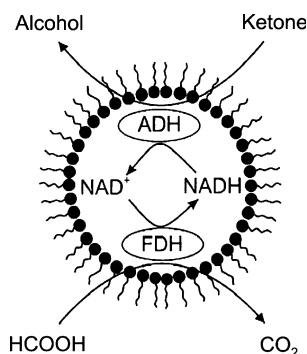


Fig. 6 Reaction scheme in a reverse micelle

the aqueous phase then entered an EMR where the substrate 2-octanone was reduced to (*S*)-2-octanol by the *CPCR* (Fig. 7). By this reactor design, the product can be extracted into the organic phase, which is beneficial for the driving force of the reaction equilibrium and for downstream processing. Furthermore, there is no direct contact between the enzyme and an organic interphase. By applying this reactor design, poorly soluble ketones can be supplied in higher concentrations compared to reactor systems with only one aqueous phase. In the emulsion membrane reactor, conversion of 91%, a space–time yield of $11 \text{ g L}^{-1} \text{ day}^{-1}$, and an *ee* greater than 99.5% were obtained. In contrast to the latter two-phase reactor system, a continuously operated EMR could be operated over a period of more than 4 months at a space–time yield of $21.1 \text{ g L}^{-1} \text{ day}^{-1}$ (97% conversion, total turnover number [*ttn*]=13.6) in the range of solubility of the reactants. In the case of the emulsion reactor, the *ttn* could be increased to 124, because of the higher concentration of substrate that can be applied. Furthermore, downstream processing was simplified because the product is concentrated in the organic phase.

To avoid direct contact between the aqueous and the organic phase, biphasic systems can be operated with membrane modules. Kruse et al. (1996) developed a continuously operated reactor system of three main cycles. The EMR cycle represents a continuously operated loop reactor supplied with a hollow-fiber ultrafiltration membrane. The hydrophobic product of the enzyme-catalyzed process is extracted by means of a hydrophobic membrane into the hexane cycle, making the recycling of the hydrophilic cofactor possible in the NAD^+ cycle. Thus, the residence time of the cofactor can be decoupled from the residence time of the substrate (Fig. 8). Chiral hydrophobic alcohols like (*S*)-1-phenylpropan-2-ol, (*S*)-4-phenylbutan-2-ol, and (*S*)-6-methylhept-5-en-2-ol have

been produced in this reactor setup by using ADH from *Rhodococcus erythropolis* together with FDH from *C. boidinii*. For the three processes, conversions of 72, 80, and 65% could be achieved. The *ee* was higher than 99% in all cases. The described reactor setup led to *ttns* for NAD^+ up to $1,350 \text{ mol}_P \text{ mol}_{\text{NAD}}^{-1}$, which means a 25-fold increase in comparison to standard techniques without cofactor retention (Kruse et al. 1996).

In a similar reactor performance the reduction of acetophenone was carried out. The reaction was catalyzed by *CPCR* in a enzyme-coupled approach with FDH. The process has been compared to chemical borane reduction using a homogeneously soluble polymer-bound oxazaborolidine catalyst. The biological method yielded (*S*)-phenylethanol in *ee* >99% with a space–time yield of $88 \text{ g L}^{-1} \text{ d}^{-1}$. Although the chemical methods provided higher space–time yield ($1,400 \text{ g L}^{-1} \text{ d}^{-1}$), it became obvious, that the chemical method offers some disadvantages in terms of *ee*, which was only 94%, and stability of the catalyst (Rissom et al. 1999).

Mertens et al. (2003) reported direct regeneration of NADPH with a hydrogenase from *Pyrococcus furiosus*. The enzyme is capable of generating the NADPH directly from the oxidized NADP^+ without producing any by-products other than protons. Thus, the enzyme not only offers the feasibility to catalyze the enzyme-coupled in situ regeneration of NADPH but also the synthesis of NADPH. By applying an ADH from *Thermoanaerobium* sp. together with the hydrogenase from *P. furiosus*, acetophenone and (2*S*)-hydroxy-1-phenyl-propanone have been reduced enantioselectively to the corresponding alcohol with complete conversion and an *ee* greater than 99.5%.

Substrate-coupled cofactor regeneration

For the use of isolated enzymes in biotransformation processes with substrate-coupled cofactor regeneration, it

Fig. 7 Flow scheme of the emulsion reactor (EMR)

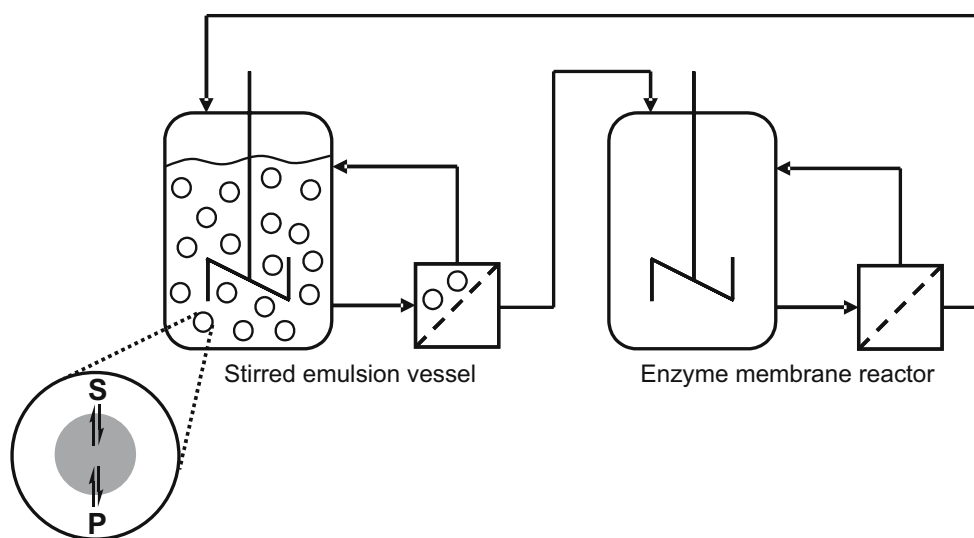
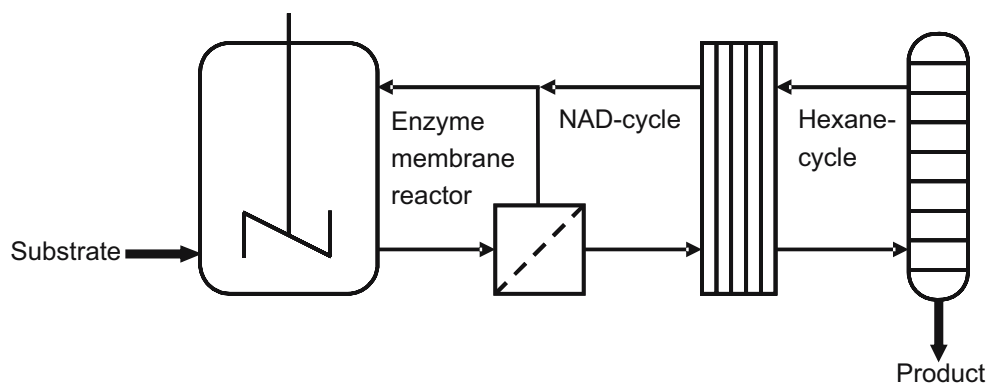


Fig. 8 Conceptual view of the three-cycle reactor

is essential that the enzyme can withstand elevated concentrations of cosubstrate. HLADH and ADH from *Lactobacillus brevis* (*LbADH*; Hummel 1997) are known to be able to operate at rather high alcohol concentrations. For that reason, these enzymes were used as catalysts for the enantioselective preparation of propargylic alcohols in a substrate-coupled approach (Schubert et al. 2002). Recently, Kosjek et al. (2004) reported the purification and characterization of a new chemotolerant ADH-‘A’ from *Rhodococcus ruber* DSM 44541. This enzyme can withstand acetone concentrations up to 50% v/v and 2-propanol concentrations even up to 80% v/v. Because of this feature, this enzyme is a very interesting biocatalyst for synthesis in a substrate-coupled process.

Successful efforts have been done to make biocatalysts more stable against organic solvents. HLADH was crystallized in the presence of NADH. The resulting crystals were then treated with glutardialdehyde to yield cross-linked enzyme crystals. These crystals exhibited high stability towards organic solvents like 2-propanol in comparison to solubilized HLADH (St. Clair et al. 2000).

A good operating stability can also be achieved by immobilizing the ADHs. Hildebrand and Lütz (2006) reported the reduction of acetophenone by immobilized *LbADH* in a plug flow reactor. The enzyme has been immobilized on Sepabeads®. By optimizing the immobilization procedure, a 60-fold increase in stability in comparison to soluble enzyme could be achieved. With the immobilized *LbADH*, a plug-flow-reactor could be operated for more than 10 weeks. During that time, conversion could be held constant at 60% with an *ee* greater than 99.5% and a space–time yield of 30 g L⁻¹ day⁻¹.

Isolated *LbADH* was also applied for the reduction of more complex ketones in a substrate-coupled cofactor regeneration approach. 5-Hydroxy-3-oxocarboxylates are valuable intermediates in the synthesis of chiral building blocks such as 3,5-dihydroxycarboxylates and β -keto δ -lactones. A preparative-scale reduction of *tert*-butyl 3,5-dioxohexanoate resulted in 99.4% *ee*, complete regioselectivity, and 77% isolated yield of the corresponding alcohol. Furthermore, a chloroderivate of the same com-

pound was reduced by *LbADH*. The product *tert*-butyl-(*S*)-6-chloro-5-hydroxy-3-oxohexanoate could be produced with greater than 99.5% *ee* and 72% isolated yield (Wolberg et al. 2000).

Nevertheless, some biotransformation processes with substrate-coupled cofactor regeneration demand removal of the coproduct acetone because acetone not only affects the activity of the enzyme but also causes a thermodynamic limitation. Stillger et al. (2002) used two different strategies for in situ acetone removal in the biocatalytic reduction of ethyl 5-oxohexanoate to (*S*)-ethyl 5-hydroxyhexanoate catalyzed by *CPCR*. In a first reactor setup, acetone has been stripped out from the reactor system by gassing the aqueous solution with humidified compressed air. Because of its low volatility, acetone can be removed effectively from a reaction system by this method. Compared to batch reactions without acetone removal, the conversion could be increased from 75% to more than 97%. In both cases, an *ee* of 99.5% could be achieved. In another approach, acetone was removed by a pervaporation step (Fig. 9) resulting in a conversion of 95% with an *ee* of 99.5% (Stillger et al. 2002).

Acetone removal is not restricted to biotransformation processes on laboratory scale. It is also realized as an important element in industrial processes. Wacker Fine Chemicals produces several β -keto esters on ton scale (Daußmann et al. 2006b). Isolated *LbADH* is used as biocatalyst for the synthesis of (*R*)-ethyl-3-hydroxybutyrate in a process with substrate-coupled cofactor regeneration

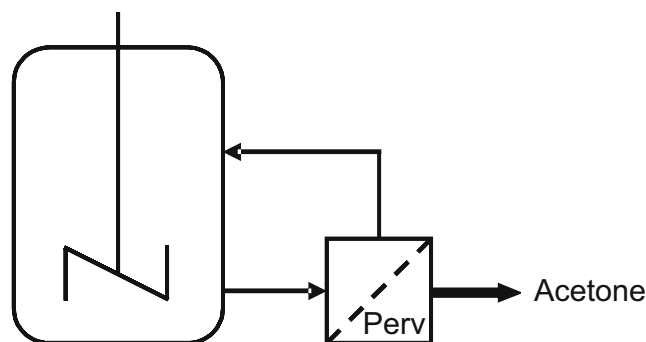
**Fig. 9** Flow scheme of the pervaporation reactor

Table 1 Partition coefficients for 2-propanol and acetone (Eckstein et al. 2004)

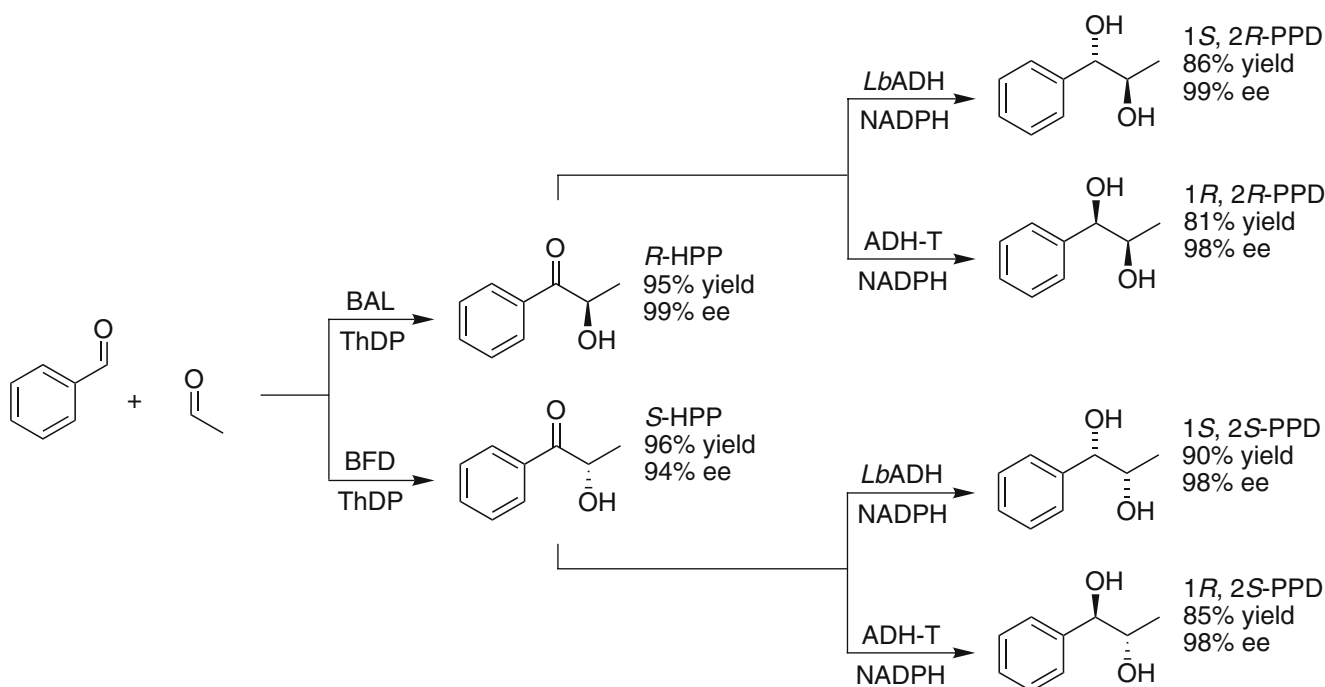
Substance	MTBE/buffer	[BMIM][CF ₃ SO ₂] ₂ N/buffer
2-Propanol	1.0	0.4
Acetone	1.1	2.0

via oxidation of 2-propanol. The coproduct acetone is removed by continuous stripping. This strategy shifts the equilibrium reaction towards complete conversion and simplifies downstream processing. Furthermore, it allows a continuous reuse of the aqueous phase in standard reactor vessels. The process is carried out with a yield of 96%, an *ee* of 99.8%, and a space–time yield of 92 g L⁻¹ day⁻¹ (Rosen et al. 2004). The synthesis of (*R*)-methyl-3-hydroxybutyrate is also catalyzed by *LbADH* in a substrate-coupled approach. The process is performed in a stirred batch reactor with a yield of 94% and an *ee* of 99.8%. Acetone is evaporated continuously under reduced pressure (Daubmann et al. 2006c).

The main problem of using enzymes as biocatalysts for the reduction of prochiral ketones is the low solubility of many ketones in aqueous solutions. For that reason, the creation of a biphasic system is preferable for the reduction of some hydrophobic substrates. As the occurrence of liquid–liquid interphases and presence of residual amounts of organic solvent in water can lead to deactivation of the biocatalyst, the low enzyme stability is a major drawback in biphasic enzymatic reactions. Villela et al. (2003) investi-

gated the stability of three different ADHs in biphasic systems. Several solvents with different properties have been used as organic phase, e.g., nonane, cyclohexane, toluene, dichloromethane, *tert*-butyl methyl ether (MTBE) and ethyl acetate. It has been investigated if the log*P* value, which characterizes the polarity of an organic solvent, can be used as parameter to guide the solvent choice. It could be shown that ADHs show good stability in biphasic systems with MTBE as the organic solvent; in systems containing dichloromethane as the organic solvent, the stabilities of the enzymes were rather low. There was no correlation found between the polarity of the organic solvent and the influence on the stability of an enzyme. In addition to the hydrophobicity of a solvent, its functionality should be considered as well when screening for a solvent that should be applied in a biphasic system.

When a biphasic system is applied for enzymatic reactions, the nonaqueous phase cannot only be used as a substrate reservoir but also for the continuous extraction of products and the coproduct acetone. A novel approach is the use of ionic liquids as the nonaqueous phase in enzymatic biphasic systems (Eckstein et al. 2004). The partition coefficients of 2-propanol and acetone in a biphasic system containing buffer and the ionic liquid [BMIM][CF₃SO₂]₂N (1-butyl-3-methylimidazolium *bis* ((trifluoromethyl)sulfonyl)amide) significantly differ from their partitioning behavior in buffer/MTBE (see Table 1). *LbADH* has been used as a biocatalyst in biphasic systems with buffer/[BMIM][CF₃SO₂]₂N and buffer/MTBE,

**Fig. 10** Enzymatic synthesis of 1-phenylpropane-1,2-diol (PPD) stereoisomers via hydroxyl-1-phenylpropanone (HPP)

respectively. The enzyme catalyzed the conversion of 2-octanone to (*R*)-2-octanol with an *ee* greater than 99%. Cofactor regeneration was carried out in the substrate-coupled approach using 2-propanol as cosubstrate. In the biphasic system with buffer/[BMIM][$(\text{CF}_3\text{SO}_2)_2\text{N}$], the produced acetone is continuously removed from the buffer phase leading to lower acetone concentrations in the buffer phase. Because of faster cofactor regeneration, a higher reaction rate could be observed. Within the first 180 min, the reduction is much faster in the biphasic system containing the ionic liquid, leading to a conversion of 88%, while the reduction reaches a conversion of only 61% in the presence of MTBE. Other applications of ionic liquids in biocatalysis, which mainly deals with isolated lipases, have recently been reviewed (Kragl et al. 2002; van Rantwijk et al. 2003).

Enzymatic gas-phase reactions involve the conversion of a gaseous substrate to a gaseous product using a dry enzyme as the catalyst. This method offers some advantages in comparison to biocatalysis in aqueous media. Poorly water-soluble substrates can be applied as volatile compounds. The dilute product then can easily be recovered using fractionated condensation (Lamare et al. 2004). *LbADH* and *ADH-T* from *Thermoanaerobacter* sp. (Findrik et al. 2005) were investigated regarding their ability to catalyze the reduction of acetophenone in a gas-phase reaction with substrate-coupled cofactor regeneration via oxidation of 2-propanol. By optimization of the reaction conditions water activity, cofactor-to-protein molar ratio, and reaction temperature, which all affected the initial reaction rate and also the stability of the biocatalyst, productivities of $1,000 \text{ g L}^{-1} \text{ day}^{-1}$ for *LbADH* and $600 \text{ g L}^{-1} \text{ day}^{-1}$ for *ADH-T* could be achieved (Trivedi et al. 2006). Other works are dealing with the effects of immobilization and drying the biocatalysts. It could be shown that the addition of sucrose to the cell extract before immobilization of the enzyme led to a longer half-life time of the biocatalyst (Ferloni et al. 2004). Furthermore, the method for drying the enzyme has an impact to the enzyme stability (Trivedi et al. 2005).

Optically active diols are interesting building blocks in asymmetric synthesis. They can be obtained via chemical routes, but only one of the diol stereoisomers is accessible in good stereoisomeric purity from a given starting material (Koike et al. 2000; Choudary et al. 2001). Furthermore, the enzymatic and microbial reduction of 1,2-diketones and α -hydroxyketones does not afford the synthesis of all four possible stereoisomers (Mochizuki et al. 1995; Bortolini 1997). By employing a combination of enantioselective lyases, e.g., thiamine diphosphate-dependent benzoylformate decarboxylase or BAL, and diastereoselective ADHs, it has become possible to synthesize all four 1-phenylpropane-1,2-diol stereoisomers separately in a

reaction cascade (Fig. 10). As substrates, simple molecules like benzaldehyde and acetaldehyde were used (Kihumbu et al. 2002).

Conclusion and outlook

The given examples point out that oxidoreductases namely, ADHs or CRs, have become a powerful tool in the synthesis of chiral alcohols. It could be shown that by developing appropriate process engineering strategies, it became possible to remarkably optimize a couple of biocatalytic processes catalyzed by isolated enzymes, e.g., in terms of space–time yield and catalyst usage. Successful attempts have been reported for the use of ADHs in nonconventional media like biphasic systems or gas phases. Such methods offer the possibility to apply remarkable high concentrations of substrate to the biocatalyst and thus increase volumetric productivities. Furthermore, thermodynamic limitations can be overcome by applying adequate ISPR techniques. These examples clearly demonstrate that by applying reaction engineering, ADHs can be successfully used as catalysts in preparative organic chemistry and on an industrial scale for the synthesis of chiral alcohols.

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