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MINI-REVIEW

Biocatalytic ketone reduction—a powerful tool for the production of chiral alcohols—part II: whole-cell reductions

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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. Due to 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 with whole cells. Microbial transformations feature different characteristics in comparison to isolated enzymes. Enzymes that are used in whole-cell biotransformations are often more stable due to the presence of their natural environment inside the cell. 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. Many whole-cell biocatalysts offer their internal cofactor regeneration that can be used by adding cosubstrates, glucose or, in the case of cyanobacteria, simply light. In this paper, various processes carried out on laboratory and industrial scales are presented. Thereby, attention is turned to process parameters, e.g., conversion, yield, enantiomeric excess, and process strategies, e.g., the application of biphasic systems. The biocatalytic production of chiral alcohols utilizing isolated enzymes is presented in part I of this review (Goldberg et al., *Appl Microbiol Biotechnol*, 2007).

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Introduction

Microbial transformations using bacteria or fungi as catalyst are known since many decades. Especially organisms from the group of the yeasts, e.g., *Saccharomyces cerevisiae* were applied in biocatalytic processes (Csuk 1991; Breuer et al. 2004). In comparison to isolated enzymes, whole-cell applications have distinct different characteristics. Enzymes utilized as whole cells are usually more stable due to the surrounding of their natural environment. Furthermore, especially in fermentative processes, the cells have internal cofactor regeneration, so that the addition of cheap glucose is sufficient to drive the reaction (Nakamura and Matsuda 2002; Faber 2004).

In part I (Goldberg et al. 2007), the production of chiral alcohols with isolated enzymes was discussed in detail. This part deals with the utilization of whole rested or fermented cells as heterogeneous catalysts for the reduction of prochiral ketones.

Reduction of prochiral ketones using whole cells

In most processes for ketone reduction catalyzed by whole cells, glucose is used for the internal cofactor regeneration in the metabolism of the cells or just the cultivated cells without any additives.

Without cofactor regeneration

β-Hydroxy acids and esters are versatile building blocks in the organic chemistry, especially in the preparation of

natural products and drugs like cholesterol antagonists or antidepressants or anti-cancer agents (Shaw et al. 2003). For the microbial reduction of α- and β-keto esters, many examples are described in literature (Csuk 1991).

For the reduction of keto acids or esters, 95 micoorganisms were tested, the best results were found with *Candida parapsilosis* and *Rodococcus erythropolis* (Peters et al. 1992). Other studies deal with the screening of several yeast genes overexpressed in *Escherichia coli* cells for the reduction of the same kind of substrates (Kaluzna et al. 2005).

In subsequent studies of the company Bristol–Myers Squibb, USA, *Pichia methanolica* was found to be the best microbe for reduction of ethyl-5-oxo-hexanoate and 5-oxo-hexanenitrile to the corresponding (*S*)-alcohols (Nanduri et al. 2001). Glucose was added to the reaction medium for the internal cofactor regeneration. The alcohols **1a** and **1b** (Fig. 1) could be produced with conversions between 80 and 90% and enantiomeric excesses (*ee*) of >95%. Compound **1a** was also synthesized on gram scale with a yield of 90%.

Various microorganisms were tested for their ability to perform an enantioselective reduction of (*S*)-4-chloro-3-hydroxy-butanoates (**2**, *R*=Me; Fig. 1). The best results gave, among many others, *Geotrichum candidum* SC 5469 with a conversion of 98% and an *ee* of up to 97% (Patel et al. 1992). After heat treatment, this value could be improved to 99%. The active dehydrogenase was β-1,4-nicotinamide adenindinucleotide phosphate (NADPH) dependent; for the biotransformation, buffer solution without glucose addition was used. Preparative productions of **2** were carried out in a 750-l fermenter with 50 kg of frozen cells and 5 kg substrate. After extraction and purification by distillation, a reaction yield of 89% with an optical purity of 98% was achieved.

Retinoids like vitamin A are essential compounds for growth, vision, tissue homeostasis, and reproduction. Synthetic retinoids can be used as pharmaceuticals; few examples are already in clinical use.

Recently, biocatalytic reductions were used for the synthesis of retinoids (Patel et al. 2002). An alcohol dehydrogenase (ADH) from *Aureobasidium pullulans* SC 13849, used as cell suspension, showed the best results in the production of **4** (Fig. 1). The cells were grown in a 25-l bioreactor, harvested by centrifugation and stored at −60°C.

A conversion of 98% and an *ee* of 98% were reached after 16 h reaction in buffer containing additional NADPH and glucose for the internal cofactor regeneration. The same results were obtained, performing a fermentation–bioreduction process, but due to low cell concentrations, the reaction time increases dramatically to 106 h.

Product separation with XAD-16 resin and subsequent extraction with acetonitrile gave a preparative yield of 94%; this was done up to an amount of 12 g alcohol. Besides the described biocatalyst, five other strains were tested, but they showed significant lower conversions and *ee*.

A key intermediate in the synthesis of longer-acting and more-potent antihypertensive agents, like the benzothiazepinone calcium channel blocker, is the secondary alcohol **9** (Fig. 2; Floyd et al. 1990).

Diketone **8** exists predominantly in the achiral enol form, so there is a rapid equilibrium between the two ketone enantiomers; the reduction gives four possible stereoisomers. Remarkably, the microbial reduction with suspended whole cells of *Nocardia salmonicolor* SC 6310 catalyzed that bioconversion in 96% reaction yield with 99.8% *ee* at 2 g l^{-1} substrate concentration after 24 h (Patel et al. 1991). The product was isolated by ethyl acetate extraction. In this process, no additional substances for cofactor regeneration were added.

Cofactor regeneration by glucose addition

Carbonic anhydrase inhibitors (CAIs) are topically active agents for the treatment of intraocular pressure associated with glaucoma. Many orally administrated CAIs show severe side effects, so intense and ongoing research in this area has been mounted (Blacklock et al. 1993). The com-

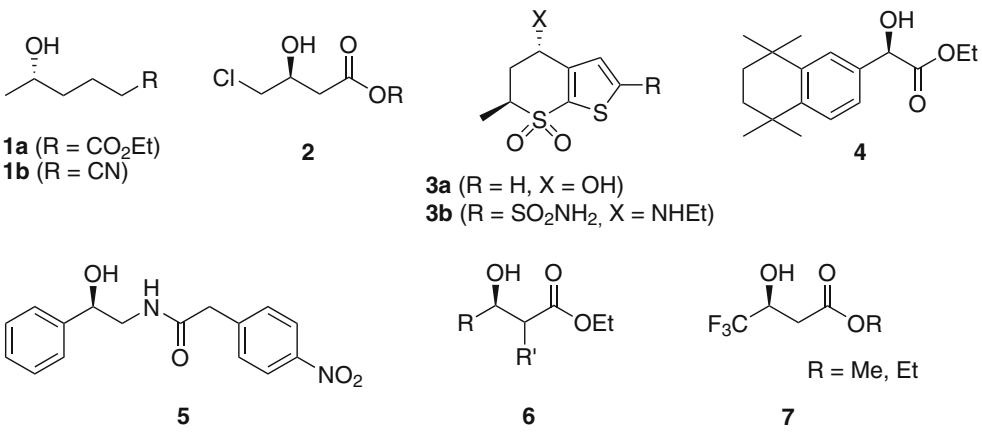

Fig. 1 Exemplary products from whole-cell biotransformations

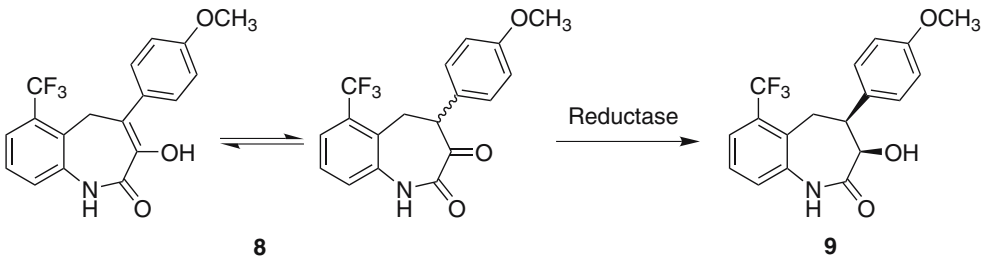

Fig. 2 Microbial production of benzothiazepinone **9**

pany Merck & Co invented and marked the CAI Trusopt® (Dorzolamide, **3b**; Fig. 1). A feature in the current synthesis of this drug is an asymmetric reduction to form the amino-stereo center (Zaks and Dodds 1997). The intermediate alcohol (**3a**) can be produced from its corresponding ketone by applying a whole-cell biotransformation.

The biocatalytic production of similar compounds was already published (Jones et al. 1991), but the major group of tested yeasts and other microorganisms showed a preferred production of the undesired *cis*-isomer. The screening of about 39 micro-organisms showed that the NADPH-dependent alcohol dehydrogenases from *Neurospora crassa* IMI 19419 or *Pichia haplophila* CBS 2008 are appropriate for the synthesis of the *trans*-isomer with selectivities of 95% and conversions of 100% in both cases. The reactions were performed with suspended cells in a glucose buffer solution (Holt 1996).

Approximately 30% of the adult population suffers from obesity, which is closely associated with type II diabetes, coronary artery diseases, and hypertension. Compound **5** is proposed to elevate the metabolic rate through thermogenesis; this will lead to weight loss (Shih et al. 1999).

Among 14 different yeasts, *Candida sorbophila* MY 1833 was identified as the most active catalyst for the synthesis of alcohol **5** (Fig. 1; Chartrain et al. 1999). An *ee* of 95% and a yield of 85% could be reached. The alcohol has a very low solubility in water; it was added as ethanol slurry. During the reaction, a two-phase system is formed, consisting of the aqueous phase and small beads containing substrate and product.

An optimization of the medium and the reaction conditions gave optimum glucose and yeast nitrogen-base concentrations of 7 g l^{-1} and 15 g l^{-1}, respectively. The glucose feed rate of 1.5 g l^{-1} was found to support the highest initial bioreduction rates. A scale up of the reaction was performed in 280 ml bioreactors. After 6 days bioreduction, a conversion of 98% at an alcohol production rate up to 250 mg l^{-1} h^{-1} was achieved. A total of 4.9 kg of alcohol with an *ee* of 98.2% was produced. An overall yield of 62% (final isolated product) was achieved during the large-scale bioreduction. Remarkable is the high volumetric product concentration of 60 g l^{-1} that was achieved in the process.

Enantiopure diols are valuable compounds in the synthesis of pharmaceuticals or ligands for the chemical asymmetric catalysis.

Resting cells of *Lactobacillus kefir* DSM 20587 catalyzed the enantio- and diastereoselective reduction of 2,5-hexanedione (**10**) to (2*R*,5*R*)-hexanediol (**12**; Fig. 3; Haberland et al. 2002a, b).

In batch experiments, *ee* and diastereomeric excesses (*de*) of >99% were achieved. Glucose was added to drive the internal cofactor regeneration; the utilized alcohol dehydrogenase is NADPH dependent. *L. kefir* is known to be heterofermentative, so that 1 mol lactic acid is formed per mol glucose. The resulting pH shift was regulated by addition of sodium hydroxide solution (4 M) by an autotitrator. The selectivity toward the hexanediol was 100%.

In subsequent investigations, a continuous process was developed (Haberland et al. 2002b). The reactor setup consists of a continuously operated stirred tank reactor (CSTR) on a 2-l scale with cell retention by an ultrafiltration module with a molecular weight cutoff (MWCO) of 400 kD (Fig. 4). A buffered glucose solution was the main feed to the reactor. The substrate and the sodium hydroxide solutions were added independently. The process was kept under anaerobic conditions by applying a flow of nitrogen gas. The optical purities of the product were again very

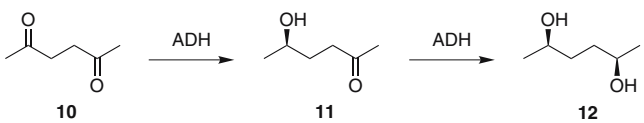

Fig. 3 Diastereoselective production of (2*R*, 5*R*)-hexanediol **12**

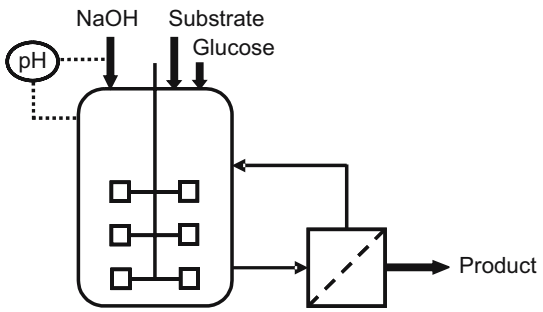

Fig. 4 Reactor set-up of the CSTR with cell retention

high with *de* and *ee* >99%. By changing from batch to a continuously operated process, the ratio $g_{\text{product}}\, g_{\text{cell dry weight}}^{-1}$, and therefore, the productivity was increased by factor 30 to 15 g g^{-1}. A typical space-time yield for the continuous process over 5 days was 64 g l^{-1} day^{-1}.

The biocatalytic synthesis of (2*S*,5*S*)-hexanediol (*ent*-**12**) was achieved with cells of *S. cerevisiae* and glucose for the internal cofactor regeneration. On a preparative scale with 25 mmol hexanedione as substrate complete conversion, an *ee* of >99% and a *de* of 96% could be achieved. After purification, the yield was 75% (Bertau and Burli 2000).

The intermediate in the synthesis of optical active hexanediol (Goldberg et al. 2007) is the 5-hydroxyhexane-2-one (**11**; Fig. 3). These γ-hydroxyketones are used in the preparation of optically active tetrahydrofuranes for biodegradable biopolymers, perfumes, and drugs (Watanabe et al. 1998).

The enantioselective reduction of (2,5)-hexanedione (**10**) was achieved using *L. kefir* DSM 20587 (Haberland et al. 2002a). Data from a typical batch reactor suggest the use of a plug flow reactor (PFR) as the most suitable reactor set up to synthesize the intermediate (5*R*)-hydroxyhexane-2-one (**11**) as the main product (Tan et al. 2006). The cells were immobilized into a sodium cellulose sulfate (NaCS) matrix with an immobilization yield of 40%. Due to the production of lactic acid in the metabolism of the cells, a pH gradient is present in the plug flow reactor. To avoid titration, the productivity and selectivity of the reaction was studied over a pH range in a typical batch reactor. The maximum conversion in the continuous process was 100% with a selectivity of 95% hydroxyketone. The production of **11** was extended to an operation time of 6 days. During this time (91 residence times), a space-time yield of 87 g l^{-1} day^{-1} and a productivity of 0.7 g $g_{\text{wet cell weight}}^{-1}$ were obtained. Despite the long operating time, the NaCS-immobilized *L. kefir* had a residual activity of 68%.

Whole cells of *L. kefir* could also be used for the two-step reduction of dioxohexanoates in a fed-batch approach (Amidjojo et al. 2005). High regio- and stereoselectivies of >99.5% were achieved. In batch experiments, the influence of buffer concentration, temperature, pH, and oxygen were investigated.

Yeast cells from, e.g., *S. cerevisiae* or *Pichia pastoris* are widely used for the asymmetric reduction of prochiral ketones because the reductions are easy to perform and the cells are inexpensive and readily available (Engelking et al. 2006). In some cases, multiple reductase enzymes from one cell are involved in the reduction of particular ketones. Due to the different stereospecificies of these enzymes, the desired alcohol lacks high optical purity (Rodriguez et al. 1999). By genetic engineering, three major reductases from yeast were either knocked out by replacement of the reductase gene with a selectable marker or overexpressed to increase the amount of a certain reductase. The combination of these approaches gives modified "second-generation" yeast cells that overexpress a single reductase and lacks a competing reductase (Rodriguez et al. 2001). The overexpressed or knocked out enzyme were fatty acid synthase, aldo-keto reductase, and α-acetoxy ketone reductase. Biotransformation with the genetically modified strains, glucose, or galactose as carbon source and various β-keto esters as substrates showed that the *ee* of the products could be increased significantly in most cases. Also, the stereoselectivity could be inverted with some substrates. Similar results could be obtained with different α-substituted ketones as substrates (Stewart 2001).

Cyanobacteria

A different approach to supply the internal metabolism of biocatalyst with energy is the utilization of cyanobacteria (Havel and Weuster-Botz 2006). An example is the reduction of halide substituted acetophenones by *Synechococcus* sp. PCC 7942 (Nakamura et al. 2000). The algae absorb light and carbon dioxide and uses this energy to produce the (*S*)-alcohol with a yield of >90% and an *ee* of >99%.

Enzyme-coupled cofactor regeneration

The application of recombinant biocatalysts increases the amount of active enzyme in the clone cell. Due to the high enzyme concentration in the cell, the internal cofactor regeneration is usually not fast or efficient enough. To manage this problem, the desired enzyme can be overexpressed in host cells together with glucose dehydrogenases for the cofactor regeneration. Some examples for this approach are presented:

The enantioselective reductions of alkyl-3-oxobutanoates to the corresponding hydroxyl esters (**6**) were investigated by Kizaki et al. (2001). As biocatalyst, recombinant *E. coli* cells overexpressing a CR S1 from *Candida magnoliae* for the reduction and a glucose dehydrogenase (GDH) from *Bacillus megaterium* for the regeneration of the cofactor NADPH were applied.

A two-phase aqueous/organic solvent system with *n*-butyl acetate was used to avoid substrate degradation and enzyme deactivation; both enzymes are tolerant to this solvent. The cofactor regeneration process produces gluconic acid as byproduct. The resulting pH shift was regulated by titration with sodium hydroxide addition.

Various substrates were tested to investigate the substrate specificity with *R* being halide, hydroxy or azide substituted alkyl groups (*R*′=*H*). In nearly all cases, enantiomeric excesses of 99% are described. The relative reactivity is best for $–CH_2Cl$ (100%) and $–CH_2OH$ (80%) substituents,

while azides and longer alkyl chains cause a drastic decrease in activity.

Alkyl 2-substituted 3-oxobutanoates (**6**, $R' \neq H$) are interesting substrates because the product contains two stereogenic centers, so the reduction of substrates with $R = CH_3$ and $R' = CH_3$, Cl was investigated. The syn/anti ratios and *ee* of the isomers were moderate.

An aldehyde dehydrogenase from *Sporobolomyces salmonicolor* catalyzes the production of trifluoro-3-hydroxybutanoates (**7**). This enzyme and a GDH from *B. megaterium* for NADPH regeneration were overexpressed simultaneously in *E. coli* JM109 cells and first used for the reduction of 4-chloro-3-oxo-butanoate with productivities of 300 g l^{-1} and *ee* of 92% (Shaw et al. 2003).

The synthesis of **7** was performed in a two-phase system with water and ethyl acetate to avoid inhibitory effects by substrate and product. The reduction proceeded with an *ee* of 99% and a yield of 50% and is carried out at Lonza AG, Switzerland.

The Degussa AG (Germany) reported the development of a method using designed biocatalysts, designer bugs, for the asymmetric reduction of ketones that tolerate high substrate concentrations and do not require an external addition of cofactor. High conversions of >90% and *ee* of >99% could be achieved (Gröger et al. 2006). As enzymes, the (*R*)-specific ADH from *L. kefir* and the (*S*)-specific ADH from *R. erythropolis*, both characterized by NADPH dependence and activities of more than 1,000 U mg^{-1} were used. These enzymes were combined with GDH from *Thermoplasma acidophilum* or *Bacillus subtilis*, respectively, for cofactor regeneration with high activity. The host for the enzymes was in both cases *E. coli* DSM 1445. With the model substrate 4-chloro-acetophenone conversions of 95% at substrate concentrations of 500 mmol l^{-1} (78 g l^{-1}) could be reached. In this case, the substrate and product are forming a second phase due to the low solubility in water. Several other substrates with concentrations of up to 1.0 M were reduced with the same result.

Besides the GDH, the related glucose-6-phosphate dehydrogenase (G6PDH) can be applied for the regeneration of NADPH. Whole cells of *Bacillus pumillus* Phe-C3 containing a NADPH-dependent keto reductase and G6PDH were used for the reduction of trifluoro β-keto esters to form the corresponding (*R*)-alcohol (*ent*-**11**, *R*=Et; Zhang et al. 2006), which is an important intermediate in the synthesis of befloxatone. The biocatalyst was permeabilized by toluene treatment to enable migration of external NADPH/nicotinamide adenine dinucleotide phosphate (oxidized form, $NADP^+$) into the cell. The transformations were performed with 20–60 mmol l^{-1} substrate, up to 100 mmol l^{-1} glucose-6-phosphate for the cofactor regeneration, and 0.005–1.0 mmol l^{-1} of additional NADPH or $NADP^+$. Optical purities of 96% and a conversion of 98% could be reached in the optimization process.

In addition to the whole cell biotransformations with enzyme-coupled cofactor regeneration with GDH, some examples utilizing formate dehydrogenase (FDH) were reported.

An NADPH-dependent ADH from *L. brevis* and a β-1,4-nicotinamide adenindinucleotide (NAD^+) dependent FDH from *Mycobacterium vaccae* N10 were coexpressed in *E. coli* cells and used for the asymmetric reduction of methyl acetoacetate (Ernst et al. 2005). The in vitro activity of the ADH was 6.5 U mg^{-1} with NADPH and 0.7 U mg^{-1} with NADH. The activity of the FDH was 1.3 U mg^{-1}. Thus, the functional overexpression of an ADH in the presence of a FDH was sufficient to enable and sustain the desired reduction reaction with NADH, instead of NADPH, as a cofactor. In the presence of formate, the intracellular concentration ratio of NADH to NAD^+ was increased sevenfold. The application of 40 mmol l^{-1} substrate gave a yield of 100% with a productivity of 200 μmol $g_{cell\ dry\ weight}^{-1}$ min^{-1}. The use of FDHs is associated with a pH shift due to formate consumption. In this case, a pH shift of only 0.1 after a reaction time of 2 h could be achieved by using a high buffer concentration of 500 mmol l^{-1}.

In the same group, a similar approach with the NADH-dependent MDH from *Leuconostoc pseudomesenteroides* ATCC 12291 with the same FDH was applied (Kaup et al. 2004). The reduction of 15 mmol l^{-1} D-fructose to D-mannitol was catalyzed by resting cells of the recombinant *E. coli* cells (3 $g_{cell\ dry\ weight}$ l^{-1}), which produced 216 mmol l^{-1} D-mannitol in 17 h.

A different approach for the combination of NADH- and NADPH-dependent reductases was published by Weckbecker and Hummel (2004). A NADPH dependent ADH from *L. kefir* and a NAD^+-dependent FDH from *C. boidinii* were coexpressed together with a pyridine nucleotide transhydrogenase from *E. coli* JM83. Reactions were carried out with 1 mmol l^{-1} additional NAD^+ and $NADP^+$, and 10 mmol l^{-1} of the substrate acetophenone. The conversions of the reduction after 12 h with or without integrated trans-

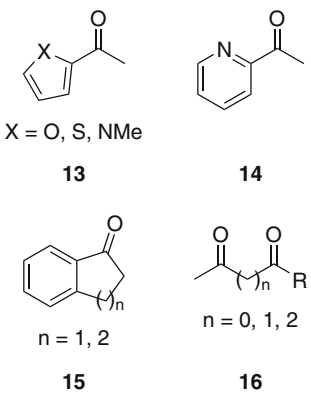

Fig. 5 Substrates for ADH-'A' from *R. ruber* DSM 44541

hydrogenase were 66 or 19%, respectively. In the case of 66%, the equilibrium was not reached.

The substrate-coupled approach for cofactor recycling is only rarely used in whole-cell bioreductions. Some examples are presented here:

Substrate-coupled cofactor regeneration

After intensive screening investigations, Kroutil et al. found an effective ketone reduction system based on the NADH-dependent ADH-′A′ of *Rhodococcus ruber* DSM 44541 (Stampfer et al. 2002). Beside applications of the isolated enzyme (Goldberg et al. 2007), whole lyophilized cells can be used for the asymmetric reduction of various substrates like alkyl-, aryl-, and heteroaryl-substituted 2-ethanones and different diketones (Fig. 5). In many cases, *ee* of 99% could be reached (Stampfer et al. 2004; Edegger et al. 2006a). Due to its high stability toward elevated concentrations of organic solvents, a substrate-coupled approach with 2-propanol was applied for cofactor regeneration. Further development gave a recombinant *E. coli* with a noticeable higher activity with a small loss on stability (Edegger et al. 2006b).

The ADH in this *E. coli*/ADH-′A′ cells was also applied in combination with an in situ coproduct removal process (Goldberg et al. 2006). By leading a water and 2-propanol saturated air stream through the reaction mixture, the coproduct acetone could be removed completely. With this method, the conversion of acetophenone, raspberry ketone, and 2,5-hexanedione were increased to completeness.

Whole cells of *L. kefir* were used for the NADPH-dependent asymmetric reduction of 4-chloro acetoacetate, applying a substrate-coupled cofactor regeneration with 2-propanol as cosubstrate (Amidjojo and Weuster-Botz 2005). The results of the biotransformations were compared to experiments with glucose addition. The *ee* could be improved from 88 to >99% with 2-propanol addition. The yield could even be enhanced from 20 to 100%. The process was scaled up to a 200-ml stirred tank reactor with 5% *v*/*v* 2-propanol. A final (*S*)-alcohol concentration of 1.2 mol l^{-1} and an *ee* of 99.5% were achieved within 14 h. The space-time yield and specific product capacity were 85.7 mmol l^{-1} h^{-1} and 24 mmol $g^{-1}_{\text{dry cell weight}}$, respectively.

Conclusions and outlook

The fact that biocatalytical methods have successfully replaced well-established chemical methods for the synthesis of chiral compounds in industry points out the advantage of biological catalysts. Oxidoreductases, namely alcohol dehydrogenases or carbonyl reductases, have become a powerful tool in the synthesis of chiral alcohols.

When wild-type microorganisms are used as biocatalysts, insufficient values for selectivity and, in particular, enantioselectivity may occur due to the presence of a vast variety of active enzymes within the cell. By applying isolated enzymes as biocatalysts, undesired side reactions can be avoided. But in most cases, whole-cell biocatalysts are more stable because they are able to offer a natural environment to the enzyme. Furthermore, the use of whole-cell biocatalysts is sometimes desirable, as there is no need for downstream processing and purification of enzymes. By developing recombinant microorganisms, it became possible to create whole-cell biocatalysts that can compete against isolated enzymes. The overexpression of oxidoreductases and cofactor regenerating enzymes leads to sufficient specific activities of these enzymes, so that side reactions are negligible. To the author's opinion, the number of biotechnological processes catalyzed by such tailor-made microorganisms will increase within the next years, especially on industrial scale. The high selectivity and specific activity of biocatalysts, as well as the easy handling and availability of microorganisms, are major reasons for that trend. Furthermore, new and improved enzymes will become available from metagenomic screenings and directed evolution techniques. Beside the reduction of ketones, the direct hydroxylation and oxidation of methylene groups will also become a significant method in the biocatalytic synthesis of chiral alcohols if suitable catalysts are available.

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