Biocatalytic Reductions: From Lab Curiosity to "First Choice"

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ABSTRACT

Enzyme-catalyzed reductions have been studied for decades and have been introduced in more than 10 industrial processes for production of various chiral alcohols, α-hydroxy acids and α-amino acids. The earlier hurdle of expensive cofactors was taken by the development of highly efficient cofactor regeneration methods. In addition, the accessible number of suitable dehydrogenases and therefore the versatility of this technology is constantly increasing and currently expanding beyond asymmetric production of alcohols and amino acids. Access to a large set of enzymes for highly selective C=C reductions and reductive amination of ketones for production of chiral secondary amines and the development of improved D-selective amino acid dehydrogenases will fuel the next wave of industrial bioreduction processes.

1. Introduction

Nature's toolbox for catalyzing reduction reactions is vast but until recently was not considered as competitive and applicable in industry. Indeed early reports on bioreduction processes were often only impressive because of mild reaction conditions without the need for hydrogen and unmatched regio- and enantioselectivities of the enzymes. Other industrially important parameters such as catalyst costs, productivities ($g \cdot L^{-1} \cdot h^{-1}$), final product concentrations, and/or complex downstream processing were, however, often not attractive for industrial applications.

Many of these limitations were caused by the fact that the initial processes used living or resting cells as biocatalysts. These wild-type strains (often yeasts) typically grew slowly, suffered from high product or substrate concentrations, and contained a significant level of undesired enzymes causing side reactions. In addition, required special know-how (e.g., in microbiology) and equipment (e.g., fermentors and microfiltration units) were not standard in the chemical industry. Therefore it is not surprising that bioreductions were far from being a standard synthesis tool for quite some time.

With the introduction of recombinant DNA technology and the development of efficient cofactor regeneration concepts in the early 1980s, many of these hurdles were lowered. Indeed, the first industrial enzymatic reductive amination process with *in situ* cofactor regeneration was enabled by these developments and introduced in the 1990s for production of L-*tert*-leucine based on a reductive amination of the corresponding keto acid.[1] Since then, the number of industrial bioreduction processes for manufacturing of primary and secondary alcohols and α-amino as well as α-hydroxy acids has grown to more than 10 industrial processes today.[2] We expect this to rapidly grow during the next years because the chance of finding a suitable biocatalyst is very high. The availability of continuously expanding off-the-shelf enzyme libraries (e.g., more than 100 recombinant keto reductases are available today) and the application of modern enzyme development and screening tools such as directed evolution[3] and metagenome screening[4] will fuel this growth.

Indeed, enzymatic carbonyl reduction is moving from a niche application to a first choice approach, although the general perception lags behind the convincing status of the actual technology. Moreover, new applications for bioreductions are on the horizon. For example, the development of efficient enone and enoate reductases for C=C reductions, amine dehydrogenases, and D-selective amino acid dehydrogenases will find new applications for production of chiral amines and other valuable products. The aim of this Account is to present the current status
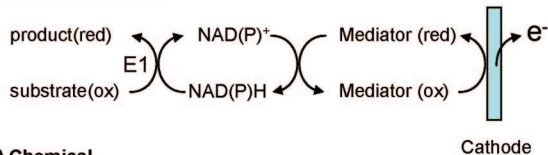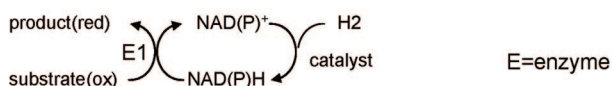
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a) Coupled Enzyme

b) Coupled Substrate
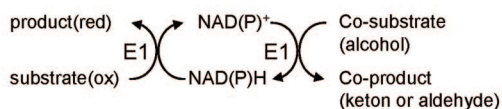
c) In-vivo
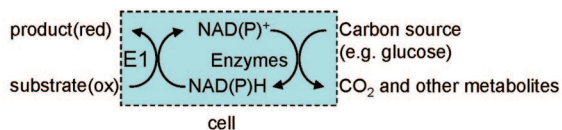
d) Photo Chemical

e) Electro Chemical

f) Chemical

FIGURE 1. Overview of cofactor regeneration concepts.

Table 1. Comparison of Different Enzyme Systems for Cofactor Regeneration

enzyme	activity	stability	cosubstrate cost	workup
formate DH[a]	−	±	±	+
glucose-6-phospate DH[a]	+	±	−	−
phosphite DH[a]	−	±	+	+
glucose DH[a]	+	+	±	±
hydrogenase	−	−	+	+
aldehyde DH[a]	±	±	±	−

[a] DH = dehydrogenase.

of enzymatic reductions and to discuss emerging areas with a strong focus on industrial applicability.

2. Cofactor Regeneration

The majority of enzymes applied in industrial bioreduction processes use nicotine amide cofactors (NADH and NADPH). These cofactors transfer a hydride ion with the help of an enzyme, usually in a highly regio- and stereoselective way, to a substrate and are therefore oxidized and stoichiometrically consumed during the reaction. The high price of the cofactor makes an efficient regeneration system a prerequisite for industrial bioreduction processes.

Many different regeneration systems have been developed since the 1980s. They can be classified as coupled enzyme, coupled substrate, *in vivo* (i.e., relying on the natural cofactor regeneration system of living organisms), electro-enzymatic, photochemical, and chemical approaches. An overview of the different concepts is shown in Figure 1. The last three approaches (Figure 1,d–f) seem not yet competitive for commercial applications due to typically lower total turnover numbers (moles of cofactor consumed per mole of produced product), side reactions, or the requirement for expensive mediators. Coupled enzyme and coupled substrate approaches look currently most attractive, but *in vivo* approaches are also of

industrial relevance.[5] In the following, we will focus on coupled enzyme and coupled substrate methods.

2.1. Coupled Enzyme Approach. Cofactor regeneration systems based on the coupled enzyme approach have been explored since the beginning of the 1980s. For example, Whitesides, Wong, and co-workers described the use of hydrogenases, formate dehydrogenases, aldehyde dehydrogenases, and glucose-6-phosphate dehydrogenases for regeneration of NADH as well as NADPH.[6–8] Additional enzyme systems have been developed since then based on malic enzymes,[9] phosphite dehydrogenases,[10] and glucose dehydrogenases.[11]

Table 1 attempts to sketch a rough picture about advantages and disadvantages of the different enzyme systems, which indicates a trade-off between enzyme and cosubstrate related properties of the different systems. Systems with lower enzyme cost contributions are compromised by higher cosubstrate costs and vice versa. Thus there is no single best or ideal cofactor regeneration system at the moment that, however, does not limit industrialization and therefore the competitiveness of bioreduction processes based on the coupled enzyme approach.

In the mid-1990s, the first enzyme coupled cofactor regeneration process was implemented at a ton scale based on the formate dehydrogenase from *Candida boidinii*.[1] Yields of >70% and productivities of >600 $g \cdot L^{-1} \cdot d^{-1}$ are reported based on a repetitive batch process for production of L-*tert*-leucine.[2]

Glucose dehydrogenase based methods are also commercialized despite seemingly more complex workup procedures. For example, Kaneka reported the production of a statin intermediate (4-chloro-3-hydroxybutanoate) using an alcohol dehydrogenase (ADH) from *Candida magnoliae* and a glucose dehydrogenase from *Bacillus megaterium*, which are coexpressed in *Escherichia coli*. A total turnover number of 21.600 mol product/mol NADPH, high product concentrations (>20% w/v), reasonable yields (85%), and high optical purities (>99.5% ee) were reported, which clearly demonstrates that modern bioreduction processes including recent developments of recombinant whole-cell processes[12–14] are highly competitive with other alternative reduction methods.

2.2. Coupled Substrate Approach. The seemingly simplest approach for cofactor regeneration makes use of a single enzyme that simultaneously transforms substrate plus a cheap cosubstrate according to the scheme in Figure 1b. Due to the reversibility of dehydrogenase reactions, the cosubstrate must be applied in large excess
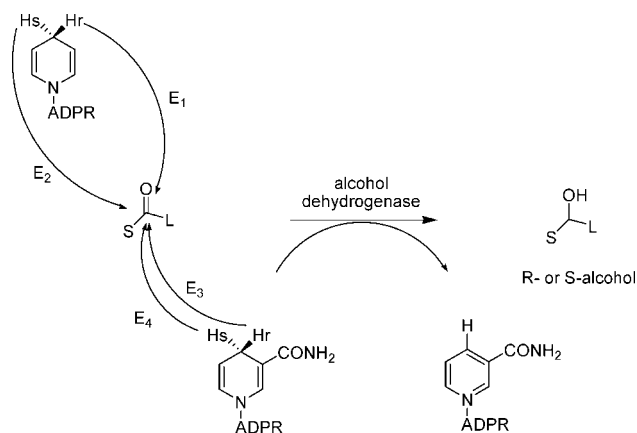
FIGURE 2. Stereochemical mechanism of hydride transfer from NAD(P)H to carbonyl carbon of a substrate bearing small (S) and large (L) substitution groups (E_3/E_4 follow Prelog's rule[25] via front attack (*re* face), in contrast to back attacking E_1/E_2 (*si* face)).

to drive the equilibrium in the desired direction and allow sufficient yields. Consequently, the coupled substrate system is often compromised by enzyme inhibition and enzyme inactivation caused by the high cosubstrate or coproduct concentrations. Furthermore product isolation can be hampered if the desired product has similar physico-chemical properties as the cosubstrate or coproduct.

Several remarkably stable dehydrogenases are known that tolerate high cosubstrate concentrations. For example, the alcohol dehydrogenase from *Thermoanaerobium brockii* tolerates 2-propanol concentrations of up to 20% (v/v)[15] and the alcohol dehydrogenase from *Rhodococcus ruber* DSM44541 is reported to accept even up to 50% (v/v) 2-propanol.[16]

Industrially relevant processes based on the coupled substrate approach are, for example, described by Industrial Enzyme Products (IEP) for production of 2-butanol and ethyl-4-(*S*)-chloro-3-hydroxybutyrate.[17,18]

3. Alcohol Production by Reduction of Ketones

Carbonyl reductions catalyzed by alcohol dehydrogenases (E.C.1.1.) have been described in detail during the last decades.[19,20] Most reports describe the reduction of ketones to the corresponding chiral (*R*)- or (*S*)-alcohols, but alcohol dehydrogenases can also be used to catalyze selective oxidations.[20]

Dehydrogenase reductions proceed according to the following steps: (1) enzyme–reduced coenzyme complex binds ketone substrate; (2) hydride is transferred from reduced cofactor (NAD(P)H) to ketone; (3) formed (chiral) alcohol product is released from enzyme–oxidized cofactor complex; (4) oxidized cofactor uncouples from enzyme and is reduced again by a reductive cofactor regeneration system (see cofactor regeneration above).

Step 2 can follow four stereochemical patterns (E_1–E_4) depending on (i) hydride attack via the *si* or *re* face of the sp²-hybridized carbon atom in the C=O moiety and depending on (ii) attack by pro-*R*- or pro-*S*-hydride of NAD(P)H, which is illustrated in Figure 2. Alcohol dehy-

drogenases (ADHs) can be classified according to these four reduction patterns. *Pseudomonas* sp. ADH[21] and *Lactobacillus kefir* ADH[22] belong to E_1, whereas *Geotrichum candidum* glycerol DH[23] and *Mucor javanicus* dihydroxyacetone reductase[24] belong to E_2. The yeast ADH,[25] horse liver ADH,[26,27] and *Moraxella* sp. ADH[28] show an E_3 pattern, while E_4 is not common within the ADH enzyme family.

A large variety of different ADHs are known and an increasing number (>100) are available off-the-shelf at small scale. Some of the best known industrially relevant enzymes are described as follows:

1 *Rhodococcus* sp. ADH:[29] broad substrate range (methyl ketones are very well accepted), obeys Prelog's rule (mostly (*S*)-alcohols are produced), high enantioselectivity, moderate cosolvent tolerance, NADH-dependent.

2 *Lactobacillus* sp. and *Pseudomonas* sp. ADH's:[21,22] NADPH- and NADH-dependent, respectively, broad substrate range, accepting high concentrations of 2-propanol for substrate-coupled cofactor regeneration, obey anti-Prelog's rule (mostly (*R*)-alcohols are formed).

3 *Thermoanaerobium brockii* ADH:[30] broad substrate range (well-suited for aliphatic ketone reductions), small substrates are converted to (*R*)-alcohols, while sterically more demanding ketones yield mainly (*S*)-alcohols, NADPH-dependent, very high enantioselectivity.

4 *Candida* yeast ADHs: (*S*)-selective ADH from *Candida macedoniensis* has been coexpressed by Shimizu et al. for the commercial production of ethyl (*S*)-4-chloro-3-hydroxybutanoate.[13]

5 Baker's yeast whole cells: broad substrate range (but often compromised stereoselectivity), low barrier usage for chemists, applied on large scale, key ADHs have been sequenced and overexpressed in *E. coli*.[31]

Although homogeneous catalysis is a highly competitive method for production of chiral alcohols, an increasing number of examples show that ADHs can outperform chemical alternatives based on (i) regioselectivity, (ii) enantioselectivity, (iii) acting in mild aqueous conditions, (iv) low catalyst costs, or (v) versatility.

The substrate spectrum of all alcohol dehydrogenases reported in the literature is broad enough to claim that almost every target ketone or aldehyde substrate can be converted to the corresponding alcohol. However, substrates of which both side groups flanking the carbonyl are very bulky, such as phenyl isopropyl ketone, tend to be poor substrates of most known ADHs resulting in low productivities and high biocatalyst cost contributions. This gap has to be filled by the discovery of new ADHs that can convert sterically hindered ketones with high activity.

4. Production of α-Amino Acids by Reductive Amination of α-Keto Acids

Amino acid dehydrogenases (AADHs; EC 1.4.1.X) can catalyze the reductive amination of α-keto acids to α-amino acids, with the concomitant oxidation of the cofactor NAD(P)H. Due to thermodynamic reasons, the equilibrium of the AADH-catalyzed reaction is usually far

on the side of the aminated products. The K_{eq} for the leucine/ketoleucine reaction, for instance, equals 9×10^{12} M^{-2},[32] whereas for the phenylalanine/phenylpyruvate reaction, a calculated K_{eq} of 2.2×10^{13} M^{-2} has been reported.[33] Consequently, the maximum conversion in the AADH-catalyzed amination reaction is very close to 100%, which implies an almost quantitative use of the α-keto acid starting compound.

Of the large number of AADHs identified in nature, only a few are industrially relevant for the synthesis of enantiomerically pure α-H-α-amino acids.[34,35] Besides alanine dehydrogenases (AlaDH, EC 1.4.1.1) and glutamate dehydrogenases (GluDH, EC 1.4.1.2–4), these are particularly phenylalanine dehydrogenases (PheDH, EC 1.4.1.20) and even more leucine dehydrogenases (LeuDH, EC 1.4.1.9). All of these dehydrogenases catalyze the reductive conversion of α-keto acids to the corresponding L-amino acids [(S)-configuration] only. Most of the synthetic work with LeuDHs has been performed with the enzymes from several *Bacillus* species and from *Thermoactinomyces intermedius*,[36] which appeared to be NADH-dependent. An extensive study to address the synthetic scope and limitations of the LeuDHs from *B. stearothermophilus*, *B. cereus*, and *B. sphaericus* showed that these enzymes have remarkably similar substrate spectra and accept α-keto acids with hydrophobic, aliphatic, branched, and unbranched carbon chains from four up to and including six C-atoms in a straight chain. Furthermore, they can convert some alicyclic α-keto acids but do not have any activity for aromatic substrates, like L-phenylalanine.[33] The specific activities of these LeuDHs for the reference substrate 2-oxo-4-methyl-pentanoic acid (ketoleucine) range from 3.3 U/mg (*B. sphaericus*) to 120 U/mg (*B. stearothermophilus*) of protein at 30 °C.[34]

The aromatic amino acid gap in the substrate spectrum of LeuDH was closed by the identification of an enzyme catalyzing the oxidative deamination of L-phenylalanine, first in a *Brevibacterium* strain[38] and later among others in a *Rhodococcus* sp.[39] and in *T. intermedius*.[40] Studies showed that the substrate specificity of these PheDHs is very broad, not only accepting similar types of substrates as the LeuDHs but also α-keto acids with an aromatic side chain. Consequently, the substrate ranges of LeuDH and PheDH are complementary. Especially the *Rhodococcus* PheDH is an attractive biocatalyst due to its high stability and intrinsic high specific activity (935 U/mg for the deamination of L-phenylalanine).[33] Also all PheDHs are NADH-dependent.

A number of different process concepts have been explored to efficiently operate the reductive amination process depicted in Figure 3. Wandrey and co-workers developed processes with isolated enzymes in enzyme–membrane reactors (EMR),[37] whereas others engaged in the development of whole cell processes.[2]

At Bristol-Myers Squibb, for instance, a recombinant *Pichia pastoris* coexpressing a *T. intermedius* PheDH gene and the endogenous formate dehydrogenase (FDH) was applied for the production of 15 kg of (S)-2-amino-5-(1,3-
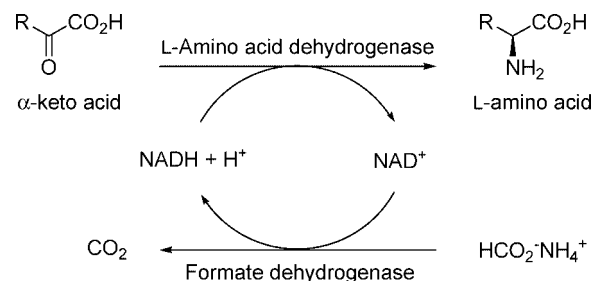

FIGURE 3. Reductive amination process to enantiomerically pure L-amino acids with FDH-based cofactor regeneration.

dioxolan-2-yl)-pentanoic acid (allysine ethylene acetal), with 97% yield and >98% ee.[41]

Galkin et al. constructed among others two different recombinant *E. coli* strains both expressing the FDH from *Mycobacterium vaccae*[42] in combination with either the LeuDH[36] or the PheDH[43] from *T. intermedius*. The whole-cell biocatalysts produced L-leucine, L-valine, L-norvaline, and L-methionine (combination LeuDH–FDH) and L-phenylalanine and L-tyrosine (combination PheDH–FDH) in high chemical yields (>88%) and excellent enantioselectivity (>99.9%). Advantageously, the intracellular NAD$^+$ pool in the resting cells was sufficiently high to produce these amino acids up to 0.3 M. Higher product concentrations, however, could not be obtained, probably due to intracellular degradation of the cofactor.[44]

Recently, also Degussa reported on the construction of a whole-cell biocatalyst for the asymmetric reductive amination of α-keto acids as a cost-attractive alternative to their current route, which is based on the application of (expensive) isolated and purified enzymes.[45,46] Application of this new whole-cell biocatalyst for the synthesis of L-*tert*-leucine showed that this reaction proceeded even without the addition of external cofactor. However, substrate concentrations of 0.5 M and higher led to incomplete conversion, which was in line with the earlier observations by Galkin et al.[44] This problem could be solved by addition of a low amount of additional cofactor (1–10 μM) or, more attractively, by fed-batch operation. Continuous addition of a 1.25 M solution of the substrate trimethylpyruvic acid over a period of 12 h (equaling a final substrate concentration of 1 M) resulted in an overall conversion of >95% after 24 h without the need for an external cofactor. The ee of the L-*tert*-leucine obtained after workup (yield 84%) was >99%.[45] More recently the same whole-cell biocatalyst has succesfully been applied for the synthesis of L-neopentylglycine with >95% conversion and >99% ee at substrate concentrations of up to 88 g/L, causing the resulting amino acid to precipitate from the reaction mixture. Nevertheless, the resting cells stayed intact, and the reaction proceeded without addition of external cofactor.[46]

Until recently, synthesis of D-amino acids via enzymatic reductive amination had not been reported because a suitable D-AADH was not availble. In 2006, however, the first known highly stereoselective D-AADH by engineering of the enzyme *meso*-2,6-diaminopimelic acid D-hydrogenase from *Corynebacterium glutamicum* was reported by *Biocatalytics*.[47]
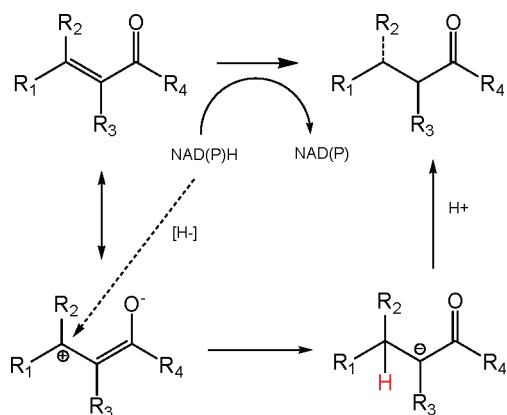
FIGURE 4. Hypothetical reaction mechanism of reductases active on enones and enals (R_4 = H).

The examples discussed above clearly indicate that enzymatic reductive amination has matured to an attractive technology for the commercial synthesis of enantiomerically pure L-α-H-α-amino acids, whereas the first steps have been taken toward the development of a similar technology for the production of D-α-H-α-amino acids.

5. Reduction of Carbon–Carbon Double Bonds

In homogeneous catalysis, the (stereoselective) reduction of carbon–carbon double bonds has been studied in depth with mainly (chiral) rhodium or ruthenium phosphines, resulting in a high number of reductive (enantioselective) transformations documented since the mid-1980s.[48] In the field of biocatalysis, this type of reduction has first been explored with baker's yeast cells on various substrates[49,50] under mild and aqueous conditions. Optically active 1-nitroalkanes were produced from 1-nitro-1-alkenes in studies conducted by Ohta et al.[51] using baker's yeast as catalyst. Later on, it became clear that one of the NADH-(P)H-linked flavin-containing "old yellow enzyme" (OYE, EC 1.6.99.1) analogues was catalyzing this reaction, which is one of the best explored families of enzymes for the reduction of C=C bonds. OYE analogues are also active on substituted α,β-unsaturated ketones and aldehydes to produce the corresponding enantiopure reduction products. Examples are found in the work of Fuganti et al.[52] The reduction of these families of olefins most likely proceeds in two hypothetical steps (see Figure 4),[53] involving (i) hydride transfer from NAD(P)H via the enzyme reduced flavin to the β-carbon of the olefin forming a free-rotating intermediate that is negatively charged on the α-carbon, followed by (ii) a protonation at this α-carbon finalizing the reaction. In members of this olefin reductase family conducting the reduction reaction in a stereoselective way, often specific tyrosine residues are responsible for the chirality of the end product.

Meanwhile, the variety of olefin reductases has been expanded far beyond the borders of baker's yeast cells. A summary of known enzymes and their origins is given in Table 2. The gene sequence of most of the mentioned enzymes is known (not for *Astasia* sp. and *Nicotiana* sp.) and (bio)chemists are just starting to explore these

Table 2. Summary of Known C=C Bond Reducing Enzymes and Their Origins

origin		enzyme name	ref
Candida macedoniensis	homologue	OYE	58
Candida albicans	homologue	OYE	60
Kluyveromyces lactis	homologue	OYE	61
Bacillus subtilis	YqjM	reductase	54
Escherichia coli	NemA	reductase	62
Pseudomonas putida	xenobiotic	reductaseAandB	63
Lycopersicon esculentum	LeOYE		64
Arabidopsis thaliana	enone	reductases	65
Astasia longa	enone	reductase	66
Nicotiana tabacum	enone	reductase	67

enzymes as organic synthesis tools for research purposes. Extensive substrate spectra of these reductases have not been published so far.

OYE and its homologues bind flavin mononucleotide (FMN) tightly but noncovalently.[54,55] During reductions with members of the OYE family, NAD(P)H is the physiological reductant, and several compounds can act as oxidants (electron acceptors) such as quinines and many α,β-unsaturated aldehydes and ketones.[55] During reduction, the electron-carrier FMN mostly accepts two high-potential electrons in its isoalloxazine ring with NAD(P)H as the electron donor.[56] The initial step is the binding of NAD(P)H and the transfer of its two electrons to FMN, forming the reduced complex $FMNH_2$. FMN can also accept one single electron instead of two by forming a semiquinone radical intermediate. By either method, FMN serves as a temporary electron sink during the reduction reaction. The next step is the transfer of electrons from $FMNH_2$ to an electron-accepting substrate, often the β-carbon of enones, enals, and 1-nitro-1-alkenes.[55,56] Non-flavin-containing reductases carry out C=C reductions by the conjugate addition of hydride directly from NAD(P)H in the absence of flavin.

Studies with whole yeast cells have shown that OYE reductases can catalyze several C=C reductions in a stereoselective manner.[57] As an example, α-Me-cinnamaldehyde could be reduced by whole baker's yeast cells toward the chiral aldehyde, which was further reduced to the corresponding alcohol with high ee and conversion (>99%) due to excess reducing ADH activity in the presence of glucose. Also other α-substituted cinnamaldehydes showed the same reduction profile when reduced with whole yeast cells. End products were mostly (*S*)-enantiomers resulting from (*E*)-isomer cinnamaldehyde derivative substrates. Most reductases prefer (*E*)-isomers as substrates, although conjugated cyclic substrates such as cyclohexenone derivatives are well reduced by many representatives. The substrate spectrum of a random combined set of prokaryotic, yeast, and plant reductases ranges from very small enones and enals to larger or complex substrates such as chalcone, (9*R*,13*R*)-*cis*-oxo-phytodienoic acid, nonenal derivatives, and (hydro)jasmone. Most reductases however do not reduce the corresponding esters, acids, or amides, although exceptions have been observed for these substrates in *Clostridia* sp. Activities that are found with C=C reductases are within the range of 0.5–50 U mg^{-1} of reductase, which indicates

that space time yield values exceeding 50 g·L^{-1}·d^{-1} could be obtained in developed processes with wild-type enzymes.

Coexpression of the enone reductase from *C. macedonensis* with a glucose dehydrogenase (GDH) from *Bacillus megaterium* has been documented for the reduction of ketoisopherone by Kataoka et al.[58] In this process, a high substrate concentration of up to 10 wt % was accepted by the C=C reductase.

6. Outlook

The knowledge about redox enzymes and their applications described in this Account have their origin in the 1980s, when biocatalysis with whole yeast cells was explored and sometimes applied as an efficient black box system. Since 1990, several enzymes responsible for the reduction of ketones, keto acids, and double bonds have been investigated and costs for cofactor regeneration have been reduced to low levels allowing the industrial use of NADH- as well as NADPH-dependent enzymes. As a result, more than 10 industrial bioreduction processes have been introduced for the production of α-amino acids, α-hydroxy acids, and alcohols.

It is therefore likely that other NADH- and NADPH-dependent dehydrogenases and reductases will find new applications in bioredox processes. Although never reported to be implemented at industrial scale, lab-scale processes with C=C reductases have shown considerable potential and might be the next enzymes to enter the arena of highly relevant hydrogenation tools.[68] Furthermore, first reports on recombinant amine dehydrogenases whose substrate spectrum goes beyond keto acids exist, which will certainly be rapidly integrated for industrial production of chiral amines by reductive amination of the corresponding ketones if they become commercially available.[69]

Although current cofactor regeneration systems are cost efficient and technically proven, clean and cheap hydrogen-driven hydrogenases are clearly the next hurdle to take in the years to come. Currently known hydrogenases have been reported from thermophilic organisms such as *Pyrococcus furiosis*, but activity levels and stabilities are still inferior.[59] It is foreseen that implementation of hydrogenase technology will only be stimulated and paid off by introducing bioredox processes for bulk chemicals, which will most probably not be the case before 2015. Long before that time, however, C=C reductases will have reached the point of industrial implementation and bioredox technology in general will increasingly impact the way the chemical industry is tackling its most demanding challenges, precisely balancing between sustainability and profitability.

References

(1) Bommarius, A. S.; Drauz, K.; Hummel, W.; Kula, M.-R.; Wandrey, C. Some new developments in reductive amination with cofactor regeneration. *Biocatalysis* **1994**, *10*, 37–47.

(2) Liese, A.; Seelbach, K.; Wandrey, C. *Industrial Biotransformations*; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2006.

(3) May, O.; Voigt, C. A.; Arnold, F. H. Enzyme engineering by directed evolution. In *Enzyme Catalysis in Organic Synthesis*; Drauz, K., Waldman, W., Eds.; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2002; pp 95–138.

(4) Short, J. M. Recombinant approaches for accessing biodiversity. *Nat. Biotechnol.* **1997**, *15*, 1322–1323.

(5) Holt, R. A. Microbial asymmetric reduction in the synthesis of a drug intermediate. *Chim. Oggi* **1996**, *14*, 17–20.

(6) Wong, C.-H.; Daniels, L.; Orme-Johnson, W. H.; Whitesides, G. M. Enzyme-catalyzed organic synthesis: NAD(P)H regeneration using dihydrogen and the hydrogenase from Methanobacterium thermoautotrophicum. *J. Am. Chem. Soc.* **1981**, *103*, 6227–6228.

(7) Wong, C.-H.; Whitesides, G. M. Enzyme-catalyzed organic synthesis: NAD(P)H cofactor regeneration by using glucose-6-phosphate and the glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides. *J. Am. Chem. Soc.* **1981**, *103*, 4890–4899.

(8) Wong, C.-H.; Whitesides, G. M. Enzyme-catalyzed organic synthesis: NAD(P)H cofactor regeneration using ethanol/alcohol dehydrogenase/aldehyde dehydrogenase and methanol/alcohol dehydrogenase/aldehyde dehydrogenase/formate dehydrogenase. *J. Org. Chem.* **1982**, *47*, 2816–2818.

(9) Suye, S.-I. Coenzyme regeneration with malic enzyme reaction system. *Recent Res. Dev. Ferment. Bioeng.* **1998**, *1*, 55–64.

(10) Johannes, T. W.; Woodyer, R. D.; Zhao, H. Efficient regeneration of NADPH using an engineered phosphite dehydrogenase. *Biotechnol. Bioeng.* **2006**, *96*, 18–26.

(11) Weckbecker, A.; Hummel, W. Glucose dehydrogenase for the regeneration of NADPH and NADH. *Methods Biotechnol.* **2005**, *17*, 225–237.

(12) Kataoka, M.; Rohani, L. P. S.; Wada, M.; Kita, K.; Yanase, H.; Urabe, I.; Shimizu, S. *Escherichia coli* transformant expressing the glucose dehydrogenase gene from *Bacillus megaterium* as a cofactor regenerator in a chiral alcohol production system. *Biosci., Biotechnol., Biochem.* **1998**, *62*, 167–169.

(13) Kizaki, N.; Yasohara, Y.; Hasegawa, J.; Wada, M.; Kataoka, M.; Shimizu, S. Synthesis of optically pure ethyl (*S*)-4-chloro-3-hydroxybutanoate by *Escherichia coli* transformant cells coexpressing the carbonyl reductase and glucose dehydrogenase genes. *Appl. Micorbiol. Biotechnol.* **2001**, *55*, 590–595.

(14) Gröger, H.; Chamouleau, F.; Orologas, N.; Rollmann, C.; Drauz, K.; Hummel, W.; Weckbecker, A.; May, O. Enantioselective reduction of ketones with "designer cells" at high substrate concentrations: highly efficient access to functionalized optically active alcohols. *Angew. Chem., Int. Ed.* **2006**, *45*, 5677–5681.

(15) Villela, F. M.; Stillger, T.; Mueller, M.; Liese, A.; Wandrey, C. Is log P a convenient criterion to guide the choice of solvents for biphasic enzymatic reactions? *Angew. Chem., Int. Ed.* **2003**, *42*, 2993–2996.

(16) Edegger, K.; Gruber, C. C.; Poessl, T. M.; Wallner, S. R.; Lavandera, I.; Faber, K.; Niehaus, F.; Eck, J.; Oehrlein, R.; Hafner, A.; Kroutil, W. Biocatalytic deuterium- and hydrogen-transfer using overexpressed ADH-'A': enhanced stereoselectivity and 2H-labeled chiral alcohols. *Chem. Commun.* **2006**, 2402–2404.

(17) Gupta, A.; Zimmer, A.; Bobkova, M. Enzymic biosynthesis of 2-butanol from 2-butanone in an aqueous two-phase system. World Patent WO 2005108593, 2005.

(18) Gupta, A.; Breese, K.; Bange, G.; Neubauer, P. Use of alcohol dehydrogenase in two-phase system for enantioselective reduction of keto compounds. World Patent WO 2002086126 2002.

(19) Whitesides, G. M.; Chenault, H. K.; Bertschy, H.; Simon, H.; Bommarius, A. S. In *Enzyme Catalysis in Organic Synthesis A Comprehensive Handbook*; Drauz, K., Waldmann, H. Eds.; Wiley-VCH: Weinheim, Germany, 1995; pp 595–665.

(20) Faber, K. *Biotransformations in Organic Chemistry*, 2nd ed., Springer-Verlag: Berlin, 1995; pp 145–180.

(21) Bradshaw, C. W.; Fu, H.; Shen, G.-J.; Wong, C.-H. A *Pseudomonas* sp. alcohol dehydrogenase with broad substrate specificity and unusual stereospecificity for organic synthesis. *J. Org. Chem.* **1992**, *57*, 1526–1532.

(22) Bradshaw, C. W.; Hummel, W.; Wong, C.-H. *Lactobacillus kefir* alcohol dehydrogenase: A useful catalyst for synthesis. *J. Org. Chem.* **1992**, *57*, 1532–1536.

(23) Nakamura, K.; Shiraga, T.; Miyai, T.; Ohno, A. Stereochemistry of NAD(P)-coenzyme in the reaction catalyzed by glycerol dehydrogenase. *Bull. Chem. Soc. Jpn.* **1990**, *63*, 1735–1737.

(24) Dutler, H.; Van Der Baan, J. L.; Hochuli, E.; Kis, Z.; Taylor, K. E.; Prelog, V. Dihydroxyacetone reductase from *Mucor javanicus*. 1. Isolation and properties. *Eur. J. Biochem.* **1977**, *75*, 423–432.

(25) Prelog, V. Specification of the stereospecificity of some oxidoreductases by diamond lattice sections. *Pure Appl. Chem.* **1964**, *9*, 119–130.

(26) Jones, J. B. Enzymes in organic synthesis. *Tetrahedron* **1986**, *42*, 3351–3403.

(27) Jones, J. B.; Beck, J. F. In *Applications of Biochemical Systems in Organic Chemistry*; Jones, J. B., Sih, C. J., Perslman, D. Eds.; John Wiley and Sons: New York, 1976; pp 107–401.

(28) Velonia, K.; Tsigos, I.; Bouriotis, V.; Smonou, I. Stereospecificity of hydrogen transfer by the NAD(+)-linked alcohol dehydrogenase from the Antarctic psychrophile *Moraxella* sp. TAE123. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 65–68.

(29) Zelinski, T.; Kula, M.-R. A kinetic study and application of a novel carbonyl reductase isolated from Rhodococcus erythropolis. *Bioorg. Med. Chem.* **1994**, *2*, 421–428.

(30) Keinan, E.; Hafeli, E. K.; Seth, K. K.; Lamed, R. Thermostable enzymes in organic synthesis. 2. Asymmetric reduction of ketones with alcohol dehydrogenase from *Thermoanaerobium brockii*. *J. Am. Chem. Soc.* **1986**, *108*, 162–169.

(31) Kaluzna, I. A.; Matsuda, T.; Sewell, A. K.; Stewart, J. D. Systematic investigation of *Saccharomyces cerevisiae* enzymes catalyzing carbonyl reductions. *J. Am. Chem. Soc.* **2004**, *126*, 12827–12832.

(32) Sanwal, B. D.; Zink, M. W. L-Leucine dehydrogenase of *Bacillus cereus*. *Arch. Biochem. Biophys.* **1961**, *94*, 430–435.

(33) Brunhuber, N. M. W.; Thoden, J. B.; Blanchard, J. S.; Vanhooke, J. L. *Rhodococcus* L-phenylalanine dehydrogenase: Kinetics, mechanism, and structural basis for catalytic specifity. *Biochemistry* **2000**, *39*, 9174–9187.

(34) Bommarius, A. S. Reduction of C=N bonds. In *Enzyme catalysis in organic synthesis*; Drauz, K., Waldman, H. Eds.; Wiley-VCH Verlag GmbH: Weinheim, Germany, 2002; pp 1047–1063.

(35) Ohshima, T.; Soda, K. Stereoselective biocatalysis: amino acid dehydrogenases and their applications. In *Stereoselective Biocatalysis*; Patel, R. N. Ed.; Marcel Dekker, Inc.: New York, 2000; pp 877–902.

(36) Ohshima, T.; Nishida, N.; Bakthavatsalam, S.; Kataoka, K.; Takada, H.; Yoshimura, T.; Esaki, N.; Soda, K. The purification, characterization, cloning and sequencing of the gene for a halostable and thermostable leucine dehydrogenase from Thermoactinomyces intermedius. *Eur. J. Biochem.* **1994**, *222*, 305–312.

(37) Lütz, S.; Rao, N.; Wandrey, C. Membranes in biotechnology. *Chem. Eng. Technol.* **2006**, *29*, 1404–1415.

(38) Hummel, W.; Weiss, N.; Kula, M.-R. Isolation and characterization of a bacterium possessing L-phenylalanine dehydrogenase activity. *Arch. Microbiol.* **1984**, *137*, 47–52.

(39) Hummel, W.; Schütte, H.; Schmidt, E.; Wandrey, C.; Kula, M.-R. Isolation of L-phenylalanine dehydrogenase from *Rhodococcus* sp. M4 and its application for the production of L-phenylalanine. *Appl. Microbiol. Biotechnol.* **1987**, *26*, 409–416.

(40) Ohshima, T.; Takada, H.; Yoshimura, T.; Esaki, N.; Soda, K. Distribution, purification, and characterization of thermostable phenylalanine dehydrogenase from thermophilic actinomycetes. *J. Bacteriol.* **1991**, *173*, 3943–3948.

(41) Hanson, R. L.; Howell, J. M.; LaPorte, T. L.; Donovan, M. J.; Cazzulino, D. L.; Zannella, V.; Montana, M. A.; Nanduri, V. B.; Schwarz, S. R.; Eiring, R. F.; Durand, S. C.; Wasylyk, J. M.; Parker, W. L.; Lui, M. S.; Okuniewicz, F. J.; Chen, B.-C.; Harris, J. C.; Natalie, K. J., Jr.; Ramig, K.; Swaminathan, S.; Rosso, V. W.; Pack, S. K.; Lotz, B. T.; Bernot, P. J.; Rusowicz, A.; Lust, D. A.; Tse, K. S.; Venit, J. J.; Szarka, L. J.; Patel, R. N. Synthesis of allysine ethylene acetal using phenylalanine dehydrogenase from *Thermoactinomyces intermedius*. *Enzyme Microb. Technol.* **2000**, *26*, 348–358.

(42) Galkin, A.; Kulakova, L.; Tishkov, V.; Esaki, N.; Soda, K. Cloning of formate dehydrogenase gene from a methanol-utilizing bacterium *Mycobacterium vaccae* N10. *Appl. Microbiol. Biotechnol.* **1995**, *44*, 479–483.

(43) Takada, H.; Yoshimura, T.; Ohshima, T.; Esaki, N.; Soda, K. Thermostable phenylalanine dehydrogenase of *Thermoactinomyces intermedius*: cloning, expression, and sequencing of its gene. *J. Biochem. (Tokyo)* **1991**, *109*, 371–376.

(44) Galkin, A.; Kulakova, L.; Yoshimura, T.; Soda, K.; Esaki, N. Synthesis of optically active amino acids from α-keto acids with *Escherichia coli* cells expressing heterologous genes. *Appl. Environ. Microbiol.* **1997**, *63*, 4651–4656.

(45) Menzel, A.; Werner, H.; Altenbuchner, J.; Gröger, H. From enzymes to "Designer Bugs" in reductive amination: a new process for the synthesis of L-tert-leucine using a whole cell-catalyst. *Eng. Life Sci.* **2004**, *4*, 573–576.

(46) Gröger, H.; May, O.; Werner, H.; Menzel, A.; Altenbuchner, J. "Second-Generation Process" for the synthesis of L-neopentylglycine: Asymmetric reductive amination using a recombinant whole cell catalyst. *Org. Process Res. Dev.* **2006**, *10*, 666–669.

(47) Vedha-Peters, K.; Gunawardana, M.; Rozzell, J. D.; Novick, S. J. Creation of a broad-range and highly stereoselective D-amino acid dehydrogenase for the one-step synthesis of D-amino acids. *J. Am. Chem. Soc.* **2006**, *128*, 10923–10929.

(48) Blaser, H.-U.; Malan, C.; Pugin, B.; Spindler, F.; Steiner, H.; Studer, M. Selective hydrogenation for fine chemicals: Recent trends and new developments. *Adv. Synth. Catal.* **2003**, *345*, 103–151.

(49) Gramatica, P.; Manitto, P.; Poli, L. Chiral synthetic intermediates via asymmetric hydrogenation of α-methyl-α,β-unsaturated aldehydes by baker's yeast. *J. Org. Chem.* **1985**, *50*, 4625–4628.

(50) Kawai, Y.; Saitou, K.; Hida, K.; Ohno, A. Asymmetric reduction of α,β-unsaturated ketones with baker's yeast. *Tetrahedron: Asymmetry* **1995**, *6*, 2143–2144.

(51) Ohta, H.; Kobayashi, N.; Ozaki, K. Asymmetric reduction of nitro olefins by fermenting baker's yeast. *J. Org. Chem.* **1989**, *54*, 1802–1804.

(52) Fuganti, C.; Pedrocchi-Fantoni, G.; Sarra, A.; Servi, S. Stereochemistry of baker's yeast mediated reduction of α,β-unsaturated δ-lactones in the goniothalamin series. *Tetrahedron:Asymmetry* **1994**, *5*, 1135–1138.

(53) Brown, B. J.; Hyun, J. w.; Duvvuri, S.; Karplus, P. A.; Massey, V. The role of glutamine 114 in old yellow enzyme. *J. Biol. Chem.* **2002**, *277*, 2138–2145.

(54) Fitzpatrick, T. B.; Amrhein, N.; Macheroux, P. Characterization of YqjM, an old yellow enzyme homolog from *Bacillus subtilis* involved in oxidative stress response. *J. Biol. Chem.* **2003**, *278*, 19891–19897.

(55) Niino, Y. S.; Chakraborty, S.; Brown, B. J.; Massey, V. A new old yellow enzyme of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **1995**, *270*, 1983–1991.

(56) Berg, J. M.; Tymoczko, J. L.; Stryer, L. *Biochemistry*, 5th ed.; W.H. Freeman and Company: New York, 2002, ISBN 0-7167-4684-0

(57) Sapra, R.; Verhagen, M. F.; Adams, M. W. Purification and characterization of a membrane-bound hydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J. Bacteriol.* **2000**, *182* (12), 3423–3428.

(58) Kataoka, M.; Kotaka, A.; Hasegawa, A.; Wada, M.; Yoshizumi, A.; Nakamori, S.; Shimizu, S. Old yellow enzyme from *Candida macedoniensis* catalyzes the stereospecific reduction of the C=C bond of ketoisophorone. *Biosci., Biotechnol., Biochem.* **2002**, *66*, 2651–2657.

(59) Sapra, R.; Verhagen, M. F.; Adams, M. W. Purification and characterization of a membrane-bound hydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J. Bacteriol.* **2000**, *182* (12), 3423–3428.

(60) Madani, N. D.; Malloy, P. J.; Rodriguez-Pombo, P.; Krishnan, A. V.; Feldman, D. *Candida albicans* estrogen-binding protein gene encodes an oxidoreductase that is inhibited by estradiol. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 922–926.

(61) Miranda, M.; Ramirez, J.; Guevara, S.; Ongay-Larios, L.; Pena, A.; Coria, R. Nucleotide sequence and chromosomal localization of the gene encoding the old yellow enzyme from *Kluyveromyces lactis*. *Yeast* **1995**, *11*, 459–465.

(62) Khan, H.; Harris, R. J.; Barna, T.; Craig, D. H.; Bruce, N. C.; Munro, A. W.; Moody, P. C. E.; Scrutton, N. S. Kinetic and structural basis of reactivity of pentaerythritol tetranitrate reductase with NADPH, 2-cyclohexenone, nitroesters and nitroaromatic explosives. *J. Biol. Chem.* **2002**, *277*, 21906–21912.

(63) Williams, R. E.; Rathbone, D. A.; Scrutton, N. S.; Bruce, N. C. Biotransformation of explosives by the old yellow enzyme family of flavoproteins. *Appl. Environ. Microbiol.* **2004**, *70*, 3566–3574.

(64) Strassner, J.; Furholz, A.; Macheroux, P.; Amrhein, N.; Schaller, A. A homolog of old yellow enzyme in tomato - spectral properties and substrate specificity of the recombinant protein. *J. Biol. Chem.* **1999**, *274*, 35067–35073.

(65) Schaller, F.; Weiler, E. W. Molecular cloning and characterization of 12-oxophytodienoate reductase, an enzyme of the octadecanoid signaling pathway from *Arabidopsis thaliana*. *J. Biol. Chem.* **1997**, *272*, 28066–28072.

(66) Shimoda, K.; Hirata, T. Biotransformation of enones with biocatalysts - two enone reductases from *Astasia longa*. *J. Mol. Catal. B: Enzym.* **2000**, *8*, 255–264.

(67) Shimoda, K.; Ito, D. I.; Izumi, S.; Hirata, T. Novel reductase participation in the syn-addition of hydrogen to the C=C Bond of enones in the cultured cells of *Nicotiana tabacum*. *J. Chem. Soc., Perkin Trans. 1* **1996**, 355–358.

(68) Hall, M.; Stueckler, C.; Kroutil, W.; Macheroux, P.; Faber, K. Asymmetric bioreduction of activated alkenes using cloned 12-oxophytodienoate reductase isoenzymes OPR-1 and OPR-3 from *Lycopersicon esculentum* (tomato): A striking change of stereoselectivity. *Angew. Chem., Int. Ed.* **2007**, *46*, 3934–3937.

(69) Itoh, N.; Yachi, C.; Kudome, T. Determining a novel NAD$^+$-dependent amine dehydrogenase with a broad substrate range from *Streptomyces virginiae* IFO 12827: Purification and characterization. *J. Mol. Catal. B: Enzym.* **2000**, *10* (1–3), 281–290.