Towards a Large-Scale Asymmetric Reduction Process with Isolated Enzymes: Expression of an (*S*)-Alcohol Dehydrogenase in *E. coli* and Studies on the Synthetic Potential of this Biocatalyst

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Received: June 26, 2002; Accepted: October 18, 2002

Dedicated to Prof. Dr. Dieter Seebach on the occasion of his 65th birthday, and Dr. Stefan Weiss on the occasion of his retirement.

Abstract: An NAD-dependent and widely applicable (*S*)-alcohol dehydrogenase is isolated and described as a novel enzyme. It is expressed in an *E. coli* strain with a high production potential. The application of this enzyme in asymmetric biocatalytic reduction is presented as well. This enzyme shows a broad substrate range comprising aliphatic and aromatic ketones as well as β-keto esters. The synthetic application of this new (*S*)-alcohol dehydrogenase led to optically active alcohols in high conversion rates accompanied by enantioselectivities of up to >99% ee. Compared to the wild-type enzyme significant advantages are observed besides the improved availability, such as a high enantioselectivity independent of the reaction time. Furthermore, the alcohol dehydrogenase was cloned and successfully overexpressed in *E. coli* resulting in a biocatalyst with a potential to be available on technical scale in the future. The development of a large-scale available and widely applicable (*S*)-alcohol dehydrogenase is one prerequisite to use isolated enzymes for asymmetric reduction processes as a valuable tool.

Keywords: alcohols asymmetric catalysis; biotransformations; enzymes; enzyme catalysis; reduction

The asymmetric reduction of ketones in the presence of isolated alcohol dehydrogenases based on the concept of an enzymatic *in situ*-cofactor recycling according to Scheme 1, equation (a), represents a favoured approach to optically active (*S*)-alcohols. These compounds are key building blocks for the fine chemicals industry.[1] In contrast to the well-established asymmetric hydrogenation of ketones in the presence of metal-catalysts,[2] and the biocatalytic reduction using cells,[3] respectively, such an approach using isolated enzymes has not been reported yet on an industrial scale although a multitude of biocatalysts were discovered for this reaction.[4,5] The use of isolated enzymes, however, would be desirable because there are significant advantages to be anticipated.[6] Furthermore, the basic concept of this type of reaction, namely an enzymatic synthesis *via* cofactor regeneration *in situ*, has already proven to be technically feasible. At Degussa AG recently the first successful implementation of a biocatalytic process under cofactor-regeneration has been realised on a tons scale and applied in the production of L-amino acids, e.g., L-*tert*-leucine [see Scheme 1, equation (b)].[7] Our studies are directed to extend this concept towards an efficient and technically feasible production of optically active alcohols according to Scheme 1, equation (a). One prerequisite, however, remains the development of suitable isolated alcohol dehydrogenases which have to be used instead of the α-amino acid dehydrogenases.[8]

Unfortunately, there are two main limitations of this enzymatic approach for optically active alcohols so far with respect to the type of alcohol dehydrogenase. First of all, numerous alcohol dehydrogenases such as those isolated from *Thermoanaerobium brockii*,[9] *T. ethanolicus*[10] or *Lactobacillus brevis*[4a] require the use of NADPH instead of NADH as a co-factor. These enzymes cannot be taken in account for technical

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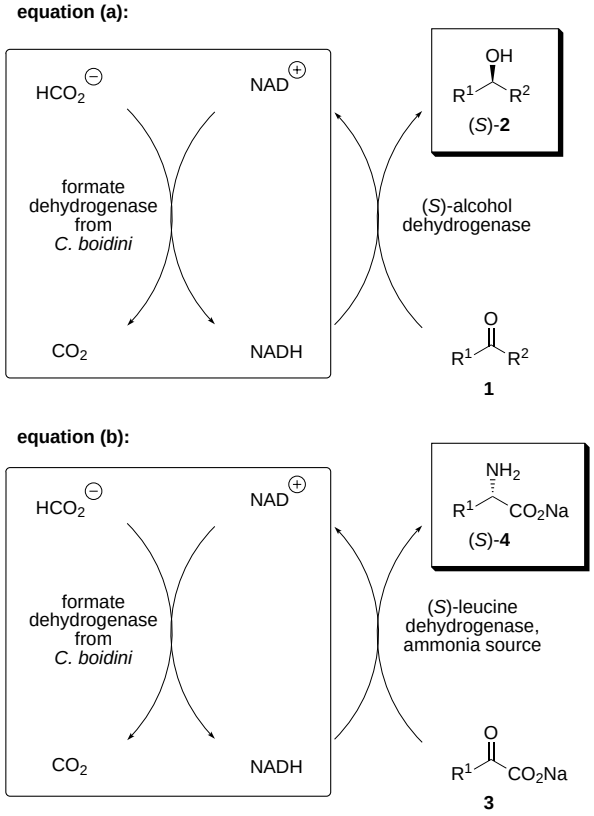
reactions due to the very high price of NADPH. Thus, for large-scale enzymatic processes under *in situ* cofactor regeneration, the development and application of alcohol dehydrogenases which are based on the remarkably cheaper co-factor NADH is necessary in order to achieve economically favourable data.[11] A further limitation is the availability of isolated NAD-dependent alcohol dehydrogenases on a technical scale. As far as we know, there are currently no commercially available isolated NAD-dependent alcohol dehydrogenases which are produced economically on a large scale, e.g., *via* an expression in *E. coli*. Thus, the expression of alcohol dehydrogenases in *E. coli* under conditions showing a high production potential which would guarantee a large-scale availability and cheap access is still a challenge as well as its application in the enzymatic production of optically active alcohols according to Scheme 1, equation (a).

In this communication we report the preparation of an isolated NAD-dependent and widely applicable (*S*)-alcohol dehydrogenase, which is available by expression in an *E. coli* strain showing a high production potential. The latter criterion is necessary to achieve a large-scale availability in the future. In addition, we present our study concerning the synthetic potential of this enzyme, e.g., the substrate range and its suitability as an efficient (bio-)catalyst for the asymmetric reduction of ketones. In particular, we have developed this new (*S*)-alcohol dehydrogenase biocatalyst with respect to the following criteria: (i) production *via* an efficient expression system; (ii) NADH-dependency, (iii) broad substrate range; (iv) high specific activity, hence demanding a low amount of protein for biocatalytic reductions; (v) synthesis of optically active alcohols in good yields, and with high enantioselectivities in the asymmetric reduction reactions.

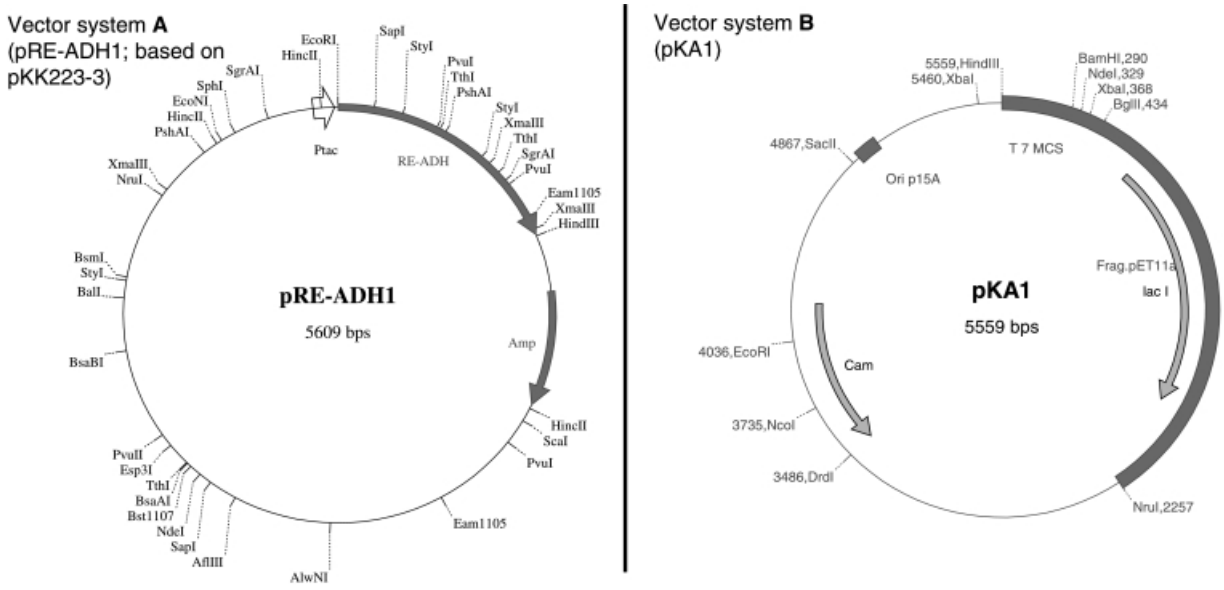
As the target enzyme for our expression experiments, we chose the (*S*)-alcohol dehydrogenase from *Rhodococcus erythropolis* (READH) which was identified in previous studies[12] as an interesting enzyme with potential for general applicability. The first step consists of the isolation of the protein and its related DNA. Starting from the crude extract of *R. erythropolis*, a highly purified alcohol dehydrogenase was obtained in high yield in three steps. The new alcohol dehydrogenase (ADH) isolated from *R. erythropolis* shows a tetrameric structure and has a molecular weight of 36,206 kDa per subunit as calculated from the gene or amino acid sequence, respectively. According to the determined amino acid sequence this alcohol dehydrogenase belongs to the group of medium-chain alcohol dehydrogenases.

Subsequently, an efficient expression system was developed. Amino acid sequencing followed by isolation and subsequent cloning of the genes and overexpression in *E. coli* represented the first steps. Transformation and expression of the nucleic acid sequence in



Scheme 1.

E. coli was carried out *via* cloning of the alcohol dehydrogenase gene into the standard vector pKK223-3 (Amersham Pharmacia Biotech). In the cell-free crude extract of *E. coli*, the recombinant enzyme was found with an activity of 6 U/mg with respect to *p*-chloroacetophenone, whereas the crude extract of *R. erythropolis* shows an activity of 2.5 U/mg only (with respect to the same substrate). This expression system based on vector **A** (Scheme 2) was stable, and the resulting recombinant enzyme was used for the subsequent studies on the synthetic potential. Additionally, in order to improve the expression rate a new vector pKA1 has been developed. The concept of this new vector **B** is also given in Scheme 2. In the presence of a plasmid containing this vector **B**, the expression rate has been increased up to 70 U/mg (Scheme 2) but the storage stability of this strain is limited so far, and needs further optimisation. Notably, a high expression rate of 1600 U per g harvested cells can be achieved with the recombinant strain **A**. The amount of cells per L of fermentation broth, however, is still in the range of 5 g only (due to the use of typical laboratory standard conditions using LB complex media), and is currently under optimisation by applying fermentation conditions better accommodated to technical processes. Due to its high production potential, this *E. coli* strain should be suited to make the desired enzyme available on a large scale.[13] An overview about the vectors is given in Scheme 2.



Scheme 2. Overview about the vectors pRE-ADH1 and pKA1.

As a next step, we carried out a detailed study of the scope and limitations of the new recombinant (*S*)-alcohol dehydrogenase with respect to the substrate range in order to evaluate its general utility. A broad range of substrates is accepted in combination with a specific activity of > 100 U/mg of purified ADM protein for many of these substrates [for comparison, the horse liver alcohol dehydrogenase as the (probably) most widely applied (*S*)-alcohol dehydrogenase so far shows an activity in the range of 1 – 3 U/mg[6b] only]. A graphical survey of the photometer-based investigation of the substrate range is presented in Scheme 3. As can be seen, aromatic ketones with an interesting variety of substitution patterns are well accepted. In particular, the acceptance of any type of substituents in the *p*-position is evident. Thus, any halogenated acetophenone derivatives, **1d** – **f**, as well as *p*-methyl- and *p*-methoxy-substituted analogues, **1g** and **1h**, gave good to excellent activities in the range of 194 to 1333% (compared to acetophenone as a reference substrate). Notably, the bromo- and chloro-derivatives, **1d** and **e**, led to the highest activities whereas the corresponding fluoro-derivative **1f** showed a somewhat lower activity of 194%. Acetophenone derivatives with substituents in the *o*- or *m*-positions represent suitable substrates, too. A remarkably high activity of 2384% was found with *m*-chloroacetophenone, **1c**. In addition, 2-alkanones also serve as good substrates. High activities were observed in particular when using 2-decanone, **1m**, and 2-heptanone, **1l**, with activities of 2521%, and 3328%, respectively. Furthermore, β-keto esters are good substrates with high specific activities for the compounds **1i** – **j** of up to 1020%. Obviously, the activity strongly depends on the alkyl ester group as can be seen in Scheme 3. The presence of an ethyl ester group gave noticeably higher

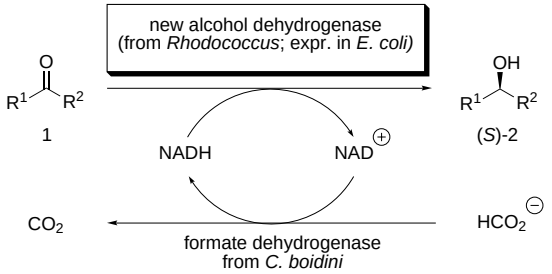
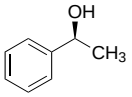
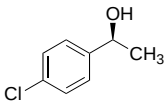
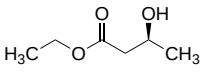
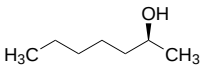
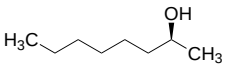
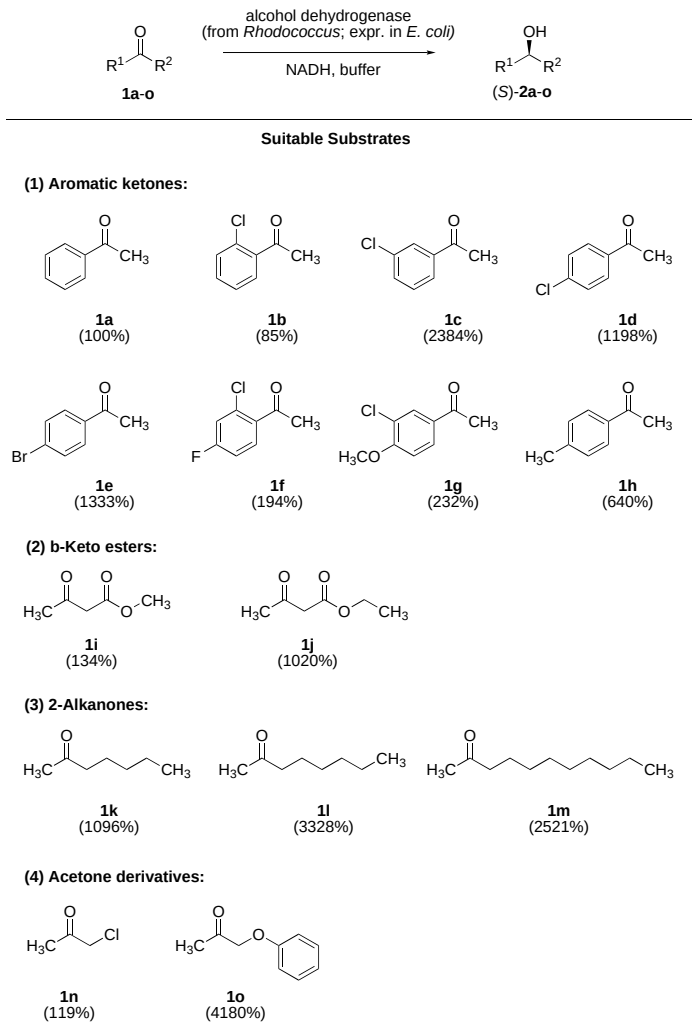


Table 1. Synthesis of optically active alcohols (*S*)-**2** using the new ADH[a]

Entry	Product		Conversion [%]	ee [%]
1		**2a**	>95	>99
2		**2d**	>95	>99
3		**2j**	>95	>99
4		**2k**	>95	>99
5		**2l**	>95	>99

[a] For synthetic details, see experimental section.



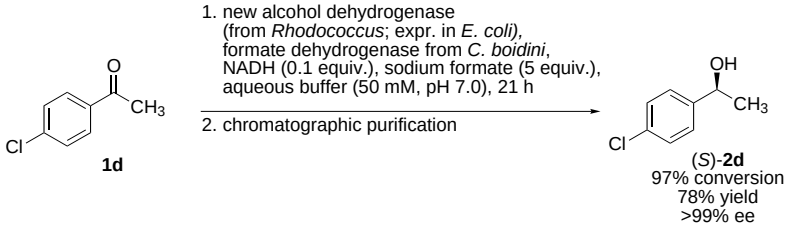
Scheme 3. Investigation of the activity towards different type of substrates **1**.

results (1020%) as compared to the methyl ester group (134%). Further very good substrates are substituted acetone derivatives such as chloroacetone, **1n** (119%), and phenoxyacetone, **1o** (4180%). These high activities are remarkable since acetone itself does not represent a suitable substrate. Thus, this new (*S*)-alcohol dehydrogenase expressed in *E. coli* shows both a broad generality and high specific activity. It is to be noticed that several differences in substrate range were observed in comparison with the corresponding wild-type alcohol dehydrogenases (from crude extracts).[14]

In the course of our further studies, we started to carry out preliminary synthetic conversions (Table 1). As the cofactor regenerating system we focused on the formate dehydrogenase-based route according to Scheme 1, equation (a). The experiments were carried out with aromatic ketones **1a**,**d**, aliphatic ketones **1k**,**l**, and a β-keto ester, **1j**, as a substrate, respectively. The reactions proceeded well for all used substrates independent of the type of ketone. The conversion rates were in the range of $>95\%$. As can be further seen from Table 1, in all cases enantioselectivities of $>99\%$ ee were obtained for the products **2a**,**d**,**k**,**l**, and **2j**. Thus, applying the new (*S*)-alcohol dehydrogenase in enzymatic syntheses based on the *in situ* cofactor regeneration concept led to the desired optically active alcohols of type (*S*)-**2** in high conversion rates and with high enantioselectivities.

A reduction on a preparative scale with subsequent isolation of the product was carried out using *p*-chloroacetophenone, **1d**, as a substrate in pure aqueous solution at pH 7.0 according to standard reaction conditions (for details, see Experimental Section). The (*S*)-alcohol dehydrogenase, and formate dehydrogenase for NAD-regeneration were used in a catalytic amount of 20 U per mmol of substrate. After a reaction time of 21 h, the desired product, (*S*)-**2d**, was obtained with a conversion rate of 97%, and after subsequent chromatographic purification, the product, (*S*)-**2d**, has been isolated in 78% yield, and with an enantioselectivity of $>99\%$ ee. The reaction is graphically summarised in Scheme 4.

A further advantage of the alcohol dehydrogenase expressed in *E. coli* compared to the corresponding wild-type whole-cell biocatalyst was found when we studied the course of the enantioselectivity during the reaction. The reduction of *p*-chloroacetophenone, **1d** with the wild-type whole-cell biocatalyst (immobilised with alginate) resulted in a quantitative conversion to (*S*)-*p*-chloro-2-phenylethanol, **2d**, but showed a high ee value in the first stage of the reaction only. However, after 10 h a remarkable decrease of enantioselectivity was observed at an unchanged high conversion rate (e.g., 70% ee after 25h, and 5% ee after 50 h).[15] This is probably due to the presence of a second alcohol



Scheme 4. Asymmetric enzymatic reduction of **1d** on a preparative scale.

dehydrogenase in the wild-type whole cell biocatalyst. In contrast, the reduction with the new alcohol dehydrogenase from *E. coli* led to the desired product (S)-*p*-chloro-2-phenylethanol, **2d**, in high conversion, and with strict enantioselectivity independent of the reaction time (since no other competing alcohol dehydrogenases are present). Furthermore, a formation of the opposite enantiomer was not observed, even at higher substrate concentrations. These results again affirm the usefulness of the recombinant enzyme. In the meantime, we have also achieved an immobilisation of the enzyme to allow a multi-use, and efficient recycling.[16]

In conclusion, we report the development of an isolated NAD-dependent and widely applicable, new (*S*)-alcohol dehydrogenase, which is available by expression in an *E. coli* strain showing a high production potential. The enzyme shows a broad substrate range comprising aliphatic and aromatic ketones as well as β-keto esters. Preliminary synthetic applications led to optically active alcohols in high conversion rates accompanied by enantioselectivities of $>99\%$ ee. Compared to the wild-type enzyme, significant advantages are observed, such as high enantioselectivity independent of the reaction time or high volume and specific activities, respectively. The alcohol dehydrogenase was cloned and successfully overexpressed, both steps being important prerequisites to achieve an availability on a technical scale in the future. Currently, the optimisation and up-scaling of the fermentation protocol for the production of this new isolated (*S*)-alcohol dehydrogenase from *Rhodococcus erythropolis* are in progress. In addition to the availability of alcohol dehydrogenase enzymes, the development of suitable reaction conditions for enzymatic reductions with high space-time yields are an important criteria and still a challenge in order to realise a valuable and large-scale feasible alternative to the existing commercially applied technologies, namely metal-catalysed asymmetric hydrogenation and biocatalytic whole-cell-based reduction. Experimental studies addressing these issues are also currently in progress.

Experimental Section

Bacterial Strains, Vectors, and Culture Conditions

Rhodococcus erythropolis strain DSM 43 297 was grown on medium DSM 65 containing 0.4% (w/v) glucose, 0.4% (w/v) yeast extract and 1% (w/v) malt extract, pH 7.2 in a rotary shaker incubator (120 rpm) for 72 h at 30 °C. Cells were collected by centrifugation (4 °C, 15 min at 10,000 rpm) and used as the source of crude cell extract. *E. coli* cells JM 105 (harbouring recombinant pKK223-3) and BL21(DE3) (containing the recombinant pKA1) were cultivated at 30 °C in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract and 1% NaCl, pH 7.5) to which 100 μg/mL ampicillin was added. The induction of the genes under the control of the *tac* and T7 promotors was achieved using 1 mM and 25 μM, respectively, of isopropyl β-D-thiogalactoside (IPTG) at an OD_{600} of 0.3. Cells were exposed to the inducer for 18 h and then collected by centrifugation (4 °C, 15 min at 10,000 rpm).

Preparation of cell extract: aliquots of the frozen cells were thawed and resuspended in a 100 mM potassium phosphate buffer, pH 6.0 (1 g cell per 2.5 mL of buffer). The *Rhodococcus erythropolis* cell suspension was mixed with glass beads (diameter 0.3 mm) in a volume ratio of 1:2. Cell lysis was achieved in a glass bead mill (SCP-Disintegrator, Innomed-Konsult AB, Sweden) for 15 min. Cell debris was removed by centrifugation at 13,000 rpm for 15 min at 4 °C. *E. coli* cells JM 105 and BL21(DE3) were disrupted with an ultrasonic oscillator (Sonopuls HD 60; Bandelin) at 4 °C for 4 min. The cell debris was removed by centrifugation (13,000 pm, 15 min). Partial purification of the enzyme was performed for the wild-type and recombinant enzymes by applying a heat-treatment step incubating the crude extract for 15 min at 65 °C. The supernatant was used for enzyme assays and transformation reactions.

Measurement of Enzyme Activity (Typical Protocol; According to Scheme 3)

NAD-dependent ADH activity was determined spectrophotometrically measuring the oxidation of NADH at 340 nm ($\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$) using the standard substrate *p*-chloroacetophenone. Activity was measured at 30 °C in a cuvette (1 mL) containing 1.45 mM of *p*-chloroacetophenone in 100 mM sodium phosphate buffer, pH 6.0, and 0.25 mM of NADH. The reaction was started by the addition of the enzyme (10 μL of partially purified enzyme). One unit of ADH activity was defined as the amount of enzyme that converted 1 μmol of NADH per minute.

General Procedure for the Enzymatic Reduction (According to Table 1)

The (*S*)-alcohol dehydrogenase from *Rhodococcus erythropolis* expressed in *E. coli* was partially purified by ion exchange chromatography, and the activities (U/mL) were determined photometrically with substrates **1a – o** (for results, see Scheme 3; for the protocol of the assay in detail, see protocol "measurement of enzyme activity"). The substrates **1a – o** were purchased from commercial sources, and used without further purification. The reduction reactions (according to Table 1) were carried out by preparing a mixture of a ketone (in general in the range of 1 to 10 mM, dependent on the solubility), NAD (0.5 mM), sodium formate (100 mM), and formate dehydrogenase (1 U/mL), followed by the subsequent addition of the (*S*)-alcohol dehydrogenase (0.5 U/mL; units are generally referring to the activity determined with *p*-chloroacetophenone as a substrate). After a reaction time of 0 min, 5 min, 10 min, and 30 min, a sample of 100 μL was taken from the reaction mixture, and extracted with 100 μL of chloroform. The organic phase was directly analysed by gas chromatography with respect to conversion rate and enantioselectivity. The chiral column CP-Chirasil-DEX CB (length: 25 cm; diameter: 25 μm; 1.3 mL/min; gas: He) purchased from the

Chrompack company was applied for this GC analysis. The temperature program included: 5 min at 60 °C, followed by an increase of 5 °C/min until a temperature of 190 °C was reached (for hexanone/hexanol: 30 min at 60 °C, followed by an increase of 10 °C/min until a temperature of 195 °C was reached).

Enzymatic Reduction on a Preparative Scale (According to Scheme 4)

At a reaction temperature of 30 °C, 10 U of the (*S*)-alcohol dehydrogenase (expressed in *E. coli* cells JH105/pRE-ADH1), and 10 U of the formate dehydrogenase were added to a solution of 0.5 mmol *p*-chloroacetophenones (83.9 mg), 2.5 mmol sodium formate (171.6 mg), and 0.1 mmol of NADH (70.2 mg) in 100 mL of a phosphate buffer (50 mM; pH 7.0). After stirring the reaction mixture for 21 h, the aqueous phase was extracted with 3 × 100 mL of methyl *tert*-butyl ether. The collected organic phases were analysed with respect to the conversion rate (97% according to HPLC), dried over magnesium sulfate, and evaporated under vacuum. After chromatographic purification (eluent: ethyl acetate/*n*-hexane, 25:75) of the resulting crude product, the desired isolated product (*S*)-**2d** was obtained; yield: 64 mg (78%) with an enantioselectivity of > 99% ee (determined by HPLC).

Acknowledgements

Many thanks are due to Mr. Hendrik Hüsken for carrying out the preparative conversion, and technical assistance. In addition, we thank Dr. Oliver May, Dr. Stefan Verseck, Dr. Andreas Karan, Dr. Stefan Buchholz, and Dr. Michael Schwarm for interesting discussions, their support, and many helpful comments. This work was supported by the Bundesministerium für Bildung und Forschung (Biotechnologie 2000 – Nachhaltige BioProduktion; Project: "Entwicklung eines biokatalytischen und nachhaltigen Verfahrens zur industriellen Herstellung enantiomerenreiner Amine und Alkohole unter besonderer Berücksichtigung der Atomökonomie"). Part of this work (W.H.) was financially supported by the Deutsche Forschungsgemeinschaft (SFB 380), which is gratefully acknowledged.

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[8] Other prerequisites which have to be fulfilled in order to realise a valuable and large-scale feasible reduction with isolated enzymes are, e. g., high yields, high enantioselectivities, and in particular good space-time yield (so far often enzymatic reductions are typically carried out at low substrate concentrations of *ca.* 10 mM).
[9] a) Y. Korkhin, A. J. Kalb, M. Peretz, O. Bogin, Y. Burstein, F. Frolow, *J. Mol. Biol.* **1998**, *278*, 967 – 981; b) O. Bogin, M. Peretz, Y. Burstein, *Protein Sci.* **1997**, *6*, 450 – 458.
[10] P. J. Holt, R. E. Williams, K. N. Jordan, C. R. Lowe, N. C. Bruce, *FEMS Microbiol. Lett.* **2000**, *190*, 57 – 62.
[11] According to ref.[6b] NAD is about seven times cheaper compared with NADP.
[12] a) W. Hummel, C. Gottwald, *DOS* 4,209,022, **1993**; *Chem. Abstr.* **1994**, *120*, 29495; b) T. Zelinski, J. Peters, M.-R. Kula, *J. Biotechnol.* **1994**, *33*, 283 – 292.
[13] A detailed description of these molecular biological studies covering the plasmid concept, and a new vector system in detail will be published independently from this contribution in due course; a brief experimental summary is already included in the experimental section.
[14] For example, compared with the wild-type ADH, the new alcohol dehydrogenase expressed in *E. coli* shows a preference for the substituted acetophenones **1d** towards the acetoacid esters **1j**. The new alcohol dehydrogenase expressed in *E. coli* also led to a higher activity for *p*-chloroacetophenone compared with *p*-methylacetophenone. In case of the wild-type ADH the opposite effect was observed with higher activities for the latter substrate.
[15] W. Hummel, K. Abokitse, unpublished results.
[16] These results will be published in detail in due course elsewhere.

CORRIGENDUM

In the communication by W. Hummel, K. Abokitse, K. Drauz, C. Rollmann, and H. Gröger in Issue 1 + 2, 2003, pp. 153 – 159, the structures of compounds **1f, 1g, 1k, 1l,** and **1m** were drawn incorrectly in **Scheme 3**; likewise, structures **2k** and **2l** in **Table 1** are incorrect. The correct compounds are as follows:

p-fluoroacetophenone (**1f**), *p*-methoxyacetophenone (**1g**) 2-hexanone (**1k**), 2-heptanone (**1l**) 2-decanone (**1m**), (*S*)-2-hexanol (**2k**), and (*S*)-2-heptanol (**2l**).

The authors apologize for these errors.