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Preparative asymmetric reduction of ketones in a biphasic medium with an (*S*)-alcohol dehydrogenase under in situ-cofactor-recycling with a formate dehydrogenase

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Abstract—The substrate range of a novel recombinant (*S*)-alcohol dehydrogenase from *Rhodococcus erythropolis* is described. In addition, an enzyme-compatible biphasic reaction medium for the asymmetric biocatalytic reduction of ketones with in situ-cofactor regeneration has been developed. Thus, reductions of poorly water soluble ketones in the presence of the alcohol dehydrogenase from *R. erythropolis* and a formate dehydrogenase from *Candida boidinii* can be carried out at higher substrate concentrations of 10–200 mM. The resulting (*S*)-alcohols were formed with moderate to good conversion rates, and with up to >99% ee.

1. Introduction

The enzymatic asymmetric reduction with an in situ-NADH-cofactor regeneration using a formate dehydrogenase (FDH) from *Candida boidinii* (Scheme 1, part (a)) is an interesting technology for the production of optically active alcohols which are valuable specialty chemicals.[1] A related process, namely the enzymatic reductive amination by means of an enzyme-coupled in situ-cofactor regeneration according to Scheme 1, part (b) has been shown to be technically feasible, and is a powerful tool for the preparation of enantiomerically pure α-amino acids. This process runs on the ton scale in the industrial synthesis of L-*tert*-leucine (Scheme 1, part (b)).[2] The extension of this attractive enzymatic concept[3,4] towards the large scale synthesis of optically active alcohols[5–7] is highly desirable but still some drawbacks exist.

Among the main limitations are the lacks of isolated NAD-dependent ADHs commercially available on a technical scale, and the lack of suitable reaction media, which guarantee a high ketone solubility. Thus, reactions are usually carried out at a substrate concentration in the range of 5–10 mM or below due to the low solubility of hydrophobic ketones in water. The presence of an organic solvent could improve the solubility of poorly water-soluble ketones, but generally causes significant enzyme deactivation. In particular, this is known for the formate dehydrogenase from *C. boidinii*[8] which is sensitive to organic solvents.[9] The best solution so far is represented by a continuous process with an enzyme-membrane reactor.[10] Albeit good space–time yields in the range of 60–104 g/(L d) can be obtained,[10a,b] the reaction is limited by the solubility of the ketone in water, which is often below 5–10 mM. Thus, the development of alternative reaction concepts for the asymmetric reduction with isolated enzymes still is a challenge, as well as the access to NAD-dependent ADHs, which cover a broad substrate range. They preferably should be cloned and overexpressed in *Escherichia coli* under conditions showing a high production potential.

In this contribution, we report the investigation of the synthetic scope of the recently developed (*S*)-ADH from *Rhodococcus erythropolis* overexpressed in *E. coli*, as well as the first example of an asymmetric enzymatic reduction of poorly water soluble ketones including an in situ-recycling of the cofactor NADH with a FDH, which runs in

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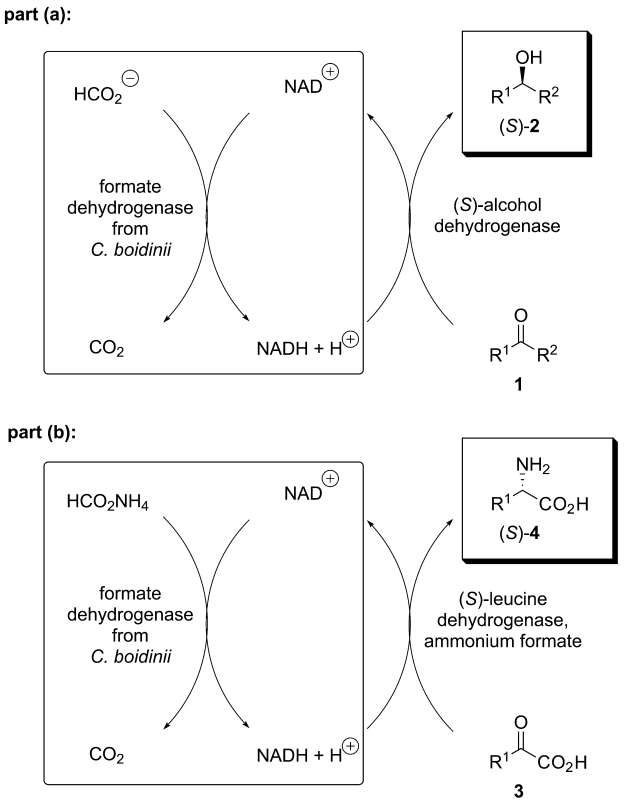


Scheme 1. Production of chiral compounds with NAD-dependent dehydrogenase coupled with formate dehydrogenase for NADH regeneration. (a) Chiral alcohols with an (*S*)-specific ADH; (b) L-amino acids via reductive amination catalyzed by an amino acid dehydrogenase.

the 'direct' presence of an organic solvent at high substrate concentrations.[11]

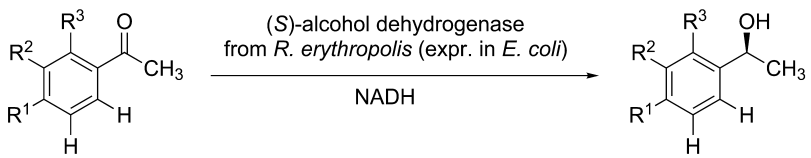
2. Results and discussion

2.1. The scope of a new (*S*)-alcohol dehydrogenase from *R. erythropolis*

We recently developed an *E. coli* expression system for the NAD-dependent (*S*)-alcohol dehydrogenase from *R. erythropolis*,[3k] since previous studies[12] revealed that this enzyme possess a potential for general applicability. This new alcohol dehydrogenase (ADH) isolated from *R. erythropolis* shows a tetrameric structure, it has a molecular weight of 36,206 kDa per subunit, and belongs to the group of zinc-containing medium-chain ADHs.[3k,11a] The developed expression in *E. coli* is efficient and has a high production potential. Thus, this *E. coli* strain should be suited to make the desired enzyme available on a larger scale. With this efficient ADH in hand,[3k,11a] we carried out a detailed substrate screening of various ketones in order to evaluate its general utility.

In general, a broad range of substances represent suitable substrates, and a graphical survey of the photometer-based investigation of the substrate range is presented in Tables 1–3.[13] Acetophenone was used as a reference substrate, and its activity was defined as 100%. With respect to a good preparative applicability, an activity, which is at least in the range of 50–100% is desirable. To start with the aromatic ketone substrates bearing one substituent (Table 1), acetophenones with an interesting variety of substitution pattern are well accepted. Although the acceptance of any type of substituent in the *para*-position is evident (entries 2–9), the type of substituent significantly determine the degree of activity. Any *p*-halogenated acetophenone derivatives (entries 2–4), as well as *p*-methyl- and *p*-methoxysubstituted analogues (entries 5, 8), gave satisfactory to excellent activities in the range of 194–1333% compared to acetophenone as a reference substrate. Notably, the *p*-bromo- and *p*-chloro-derivatives led to the highest activities whereas, the corresponding fluoro-derivative showed a lower activity. In contrast, very low activities of only 19 and 17% were found for *p*-hydroxyacetophenone and *p*-aminoacetophenone (entries 7, 9). An activity of 469% was observed for *p*-(*n*-butyl)acetophenone (entry 6). These latter examples might indicate that electronic properties of the substituent rather than its steric effects are determining the suitability of a substrate. The +I- or −I effect does not appear to have a significant impact. However, the non-satisfactory results for the *p*-hydroxy and *p*-amino-substituted acetophenone indicate that nucleophilicity of the substituent and the ability to form hydrogen bond interactions might play a significant role.

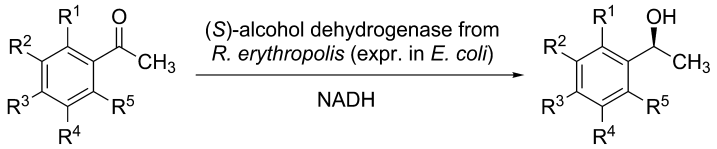
Table 1. Enzymatic activities for monosubstituted acetophenones (determined by photometrical assay)



Entry[a]	R^1	R^2	R^3	Activity (%)	Entry[a]	R^1	R^2	R^3	Activity (%)
1	H	H	H	100	10	H	Cl	H	2384
2	F	H	H	194	11	H	OH	H	47
3	Cl	H	H	1198	12	H	OCH_3	H	394
4	Br	H	H	1333	13	H	NH_2	H	44
5	CH_3	H	H	640	14	H	H	Cl	85
6	n-C_4H_9	H	H	469	15	H	H	CH_3	16
7	OH	H	H	19	16	H	H	OH	9
8	OCH_3	H	H	231	17	H	H	OCH_3	9
9	NH_2	H	H	17					

[a] The photometrical assay is described in Section 3.

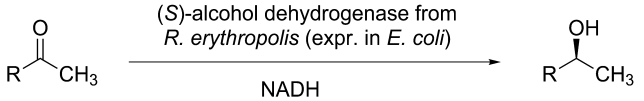
Table 2. Enzymatic activities for di- and trisubstituted acetophenones (determined by photometrical assay)



Entry[a]	R^1	R^2	R^3	R^4	R^5	Activity (%)
1	H	OCH_3	OCH_3	H	H	48
2	H	OCH_3	OCH_3	OCH_3	H	21
3	OH	H	OH	H	H	6
4	OCH_3	H	OCH_3	H	H	7
5	F	H	F	H	H	305

[a] The photometrical assay is described in Section 3.

Table 3. Enzymatic activities for aliphatic ketones (determined by photometrical assay)



Entry[a]	R	Activity (%)	Entry[a]	R	Activity (%)
1	n-C_4H_9	1096	5	CH_2OPh	4180
2	n-C_5H_{11}	3328	6	$CH_2C(O)OCH_3$	134
3	n-C_8H_{17}	2521	7	$CH_2C(O)OC_2H_5$	1020
4	CH_2Cl	119			

[a] The photometrical assay is described in Section 3.

In addition, the influence of the *o*-, *m*-, and *p*-position on the activity was studied. In general, acetophenone derivatives with substituents in *o*- or *m*-position represent suitable substrates, too. However, it appears to be a general tendency that *o*-substituted acetophenones gave less satisfactory results compared with their *m*- or *p*-substituted analogues. For example, the *p*-chloroacetophenone and the *m*-analogue gave high activities of 1198 and 2384%, respectively, whereas an activity of only 85% was detected for *o*-chloroacetophenone (entries 3, 10, 14). A comparable tendency can be found for other types of substituents, for example, methyl- and methoxy-substituents (see entries 5, 8, 15, 17).

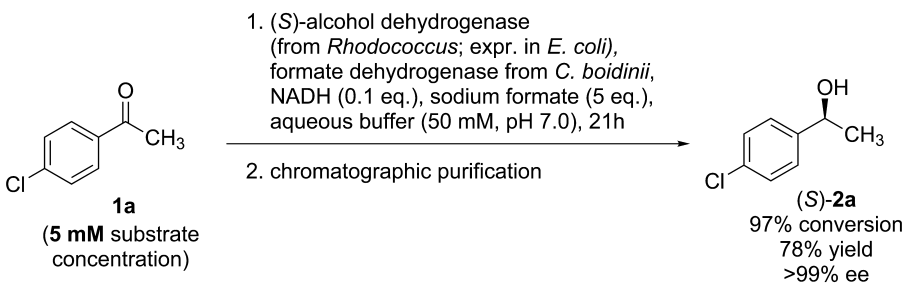
Acetophenones bearing two substituents can also represent suitable substrates in dependence on the substitution pattern (Table 2). For example, 2,4-difluoroacetopheonone gave an activity of 305% whereas a remarkably decreased activity of 7% only was determined when using a 2,4-dimethoxy-acetophenone (entries 4, 5). A significant decrease of the activity was achieved when using acetophenones bearing three substituents. Thus, trimethoxyacetophenone gave a low activity of 21% (entry 2).

In addition, several types of 2-alkanones also serve as good substrates (Table 3). High activities were observed in particular when using longer-chain alkanones, for example, 2-hexanone, 2-heptanone, and 2-decanone (entries 1–3). Furthermore, β-keto esters are good substrates, in particular when bearing an ethoxy group (entry 7). The presence of such an ethyl ester group gave noticeably higher results compared to the methyl ester group (entries 6, 7). The study of ester substrates is ongoing. Further good substrates are substituted acetone derivatives, in particular phenoxy-acetone with an activity of 4180% (entry 5). It should be added at this stage, that in general substrates bearing an aceto functionality are good substrates.

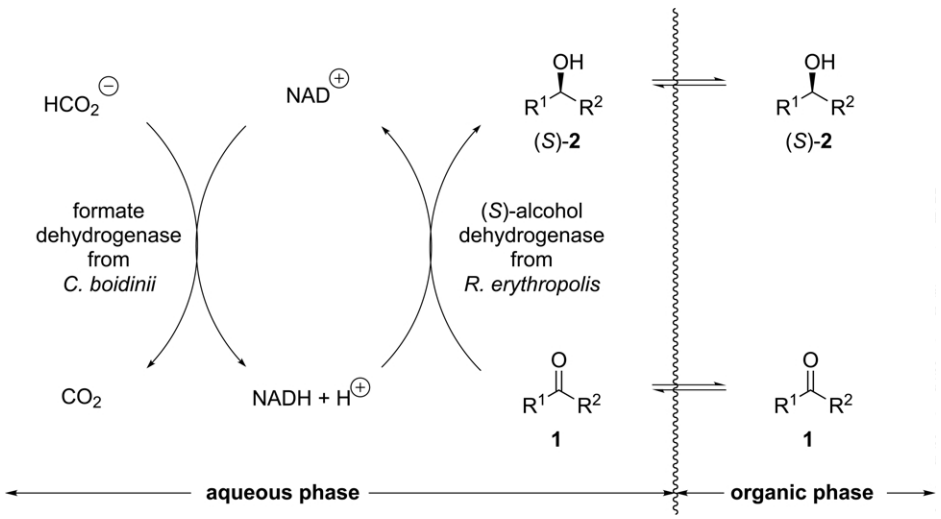
Thus, this new recombinant (*S*)-alcohol dehydrogenase from *R. erythropolis* shows both a broad substrate acceptance and high specific activities.[14] Subsequently, the preparative potential of this (*S*)-alcohol dehydrogenase was studied. An initial reduction on a preparative scale with formate and a formate dehydrogenase for cofactor-regeneration was carried out using *p*-chloroacetophenone, **1a**, as a substrate in homogeneous aqueous solution at low substrate concentration (5 mM). According to these standard reaction conditions the product (*S*)-**2a** was obtained with a conversion rate of 97% after a reaction time of 21 h. The subsequent chromatographic purification gave the product, (*S*)-**2a**, in 78% yield, and with an enantioselectivity of >99% ee (Scheme 2).

2.2. Discovery of a new reaction medium

Albeit this reduction works well in principle, the main problem of these standard conditions was the low substrate



Scheme 2. Enzymatic reduction in aqueous medium at low substrate concentration (10 mM).

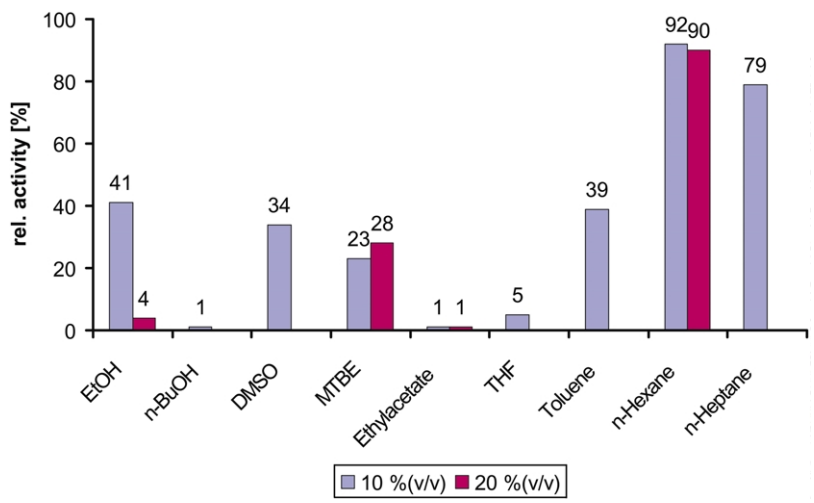


Scheme 3. Concept of an enzymatic reduction in biphasic media.

concentration of the hydrophobic ketones leading to non-satisfactory volumetric productivities (Scheme 2). Thus, the next key step was the development of a suitable aqueous-organic solvent reaction medium according to Scheme 3 which fulfils the following criteria: (i) both enzymes, namely the recombinant (*S*)-ADH and FDH from *C. boidinii* and mutants thereof, remain stable in the presence of the organic solvent; (ii) good solubility of poorly water-soluble ketones which led to high substrate concentrations of up to 200 mM; (iii) simple reaction protocol for lab scale applications, which allows a flexible and fast preparation of chiral alcohols; (iv) robust process with potential for technical scale applications in the future.

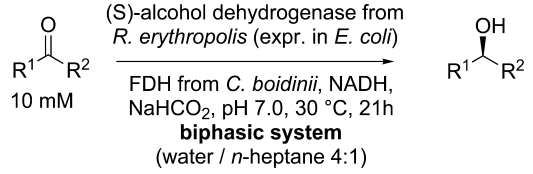
As mentioned above, the stability of the formate dehydrogenase (FDH) from *C. boidinii* as the most sensitive enzyme in this system turned out to be in particular a critical issue. We focused on this enzyme (or mutants thereof) for cofactor-regeneration due to several advantages such as favorable economic data, the large scale availability, and a proven technical applicability.[8] A screening among polar solvents showed a strong deactivation of the FDH (mutant C23S, C262A)[8] even in the presence of only 10% (v/v) of the organic solvent component (Scheme 4).[9] In addition, typical solvents for a biphasic medium failed. For example, MTBE led to a remarkable decrease of the activity after 2 days. A rapid loss of activity was found when ethylacetate was used. In this case a (nearly) complete deactivation was observed within 6 h only. Non-polar aromatic hydrocarbons, for example, toluene, deactivate FDH remarkably, too. However, in the presence of 10% (v/v) of *n*-hexane a long-term stabilization was achieved (Scheme 4). Even after 66–69 h, an activity of 92 and 90% remained for aqueous solutions containing 10% (v/v) and 20% (v/v), respectively, of *n*-hexane. Notably, other aliphatic hydrocarbons, for example, *n*-heptane also showed a positive effect (with 79% activity after ca. 3 days when using 10% (v/v)), and the stability was maintained at increased amount of the organic solvent of up to 60% (v/v). For this study, we have chosen *n*-hexane and in particular *n*-heptane as an organic solvent component. The high degree of stability independent of the amount of organic solvent guarantees sufficient flexibility with respect to the fine-tuning of the reaction later in the process development step.

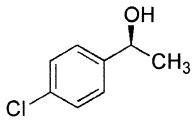
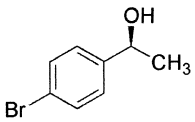
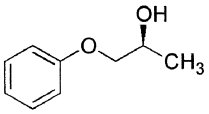
In addition, the (*S*)-selective alcohol dehydrogenase from *R.*



Scheme 4. Stability of the FDH in aqueous-organic media (the activities were measured after ca. 3 days for *n*-hexane, 2 days for *n*-heptane, and 2 days or less for the other solvents).

Table 4. Reduction in a biphasic medium with different substrates

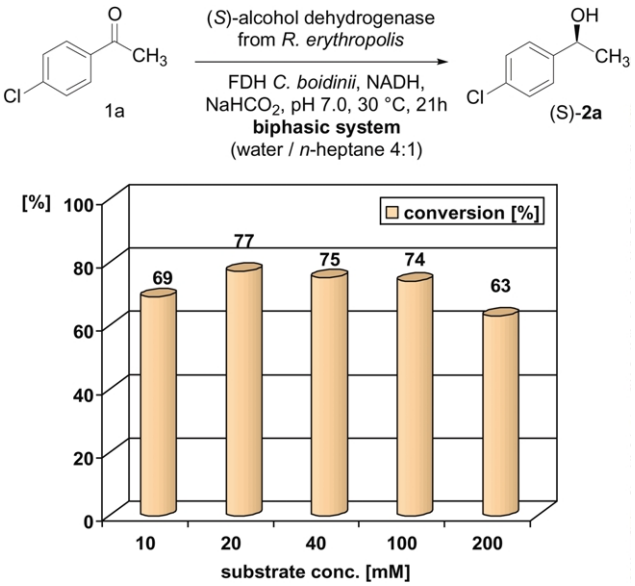


Entry	Product	Conversion (%)	ee (%)[a]
1		69	>99
2		65	97
3		>95	>99

[a] The enantiomeric excess (ee) was determined by GC chiral chromatography.

erythropolis turned out to be stable under the same conditions. In particular, the stability in heptane and hexane—which turned out to be very compatible with the FDH—is high. The activity of the (*S*)-ADH within 48 h remains nearly unchanged in the presence of 20% (v/v) as well as 60% (v/v) of *n*-heptane indicating a good stability in the biphasic medium consisting of an aqueous phase and heptane. Thus, a suitable solvent system was found in which both enzymes remain stable.

With this enzyme-compatible reaction medium in hand, preparative conversions were carried out as a next step. Good conversion rates accompanied by excellent enantioselectivities were obtained with a variety of aromatic ketone substrates. Selected examples are shown in Table 4. In the presence of the (*S*)-ADH from *R. erythropolis*, *p*-chloroacetophenone was converted into the optically active (*S*)-enantiomer, (*S*)-**2a**, with >99% ee with a conversion of 69% (entry 1). A conversion of 65%, and a slightly lower

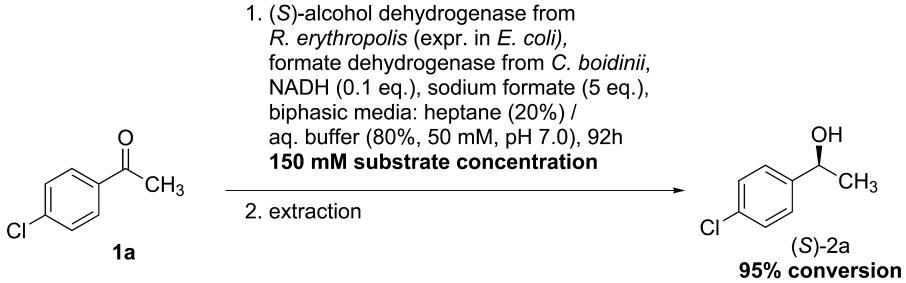


Scheme 5. Conversions of *p*-chloroacetophenone at increased substrate concentrations.

enantioselectivity of 97% ee was observed when using *p*-bromoacetophenone (entry 2). The asymmetric reduction of phenoxyacetone proceeded quantitatively under formation of intermediate (*S*)-**2c** with an enantioselectivity of >99% ee (entry 3).

These studies, which proved the applicability of the new reaction system for preparative conversions were followed by further investigation of the new reaction media at higher substrate concentrations in order to reach high volumetric productivities. As a model reaction, the reduction of *p*-chloroacetophenone, **1a**, in the presence of the (*S*)-ADH from *R. erythropolis*[3k] was investigated. Increased substrate concentrations from 10 mM (69% conversion) up to 100 mM led to comparable conversion rates (Scheme 5). At 20 and 40 mM, slightly improved conversion rates of 77 and 75%, respectively, were obtained. Even at 100 mM, a good conversion of 74% was still achieved. An analogous reaction with a subsequent chromatographical purification of the crude product gave the desired product (*S*)-**2a** with a yield of 67%. A further increase of the substrate concentration up to 200 mM gave a still satisfactory conversion rate of 63%. Thus, higher volumetric productivities can be obtained with this new reaction medium compared to the previous ADH/FDH-coupled reductions in pure aqueous medium.

The reaction conditions were further optimized, for



Scheme 6. Quantitative conversion in enzymatic reduction of *p*-chloroacetophenone in a biphasic medium.

example, with respect to the preferred enzyme/substrate ratio for a high conversion of >90%. Using improved reaction conditions (pH 7.0, 30 °C, (*S*)-ADH and FDH amount of 60 U/mmol of substrate), a conversion of 95% was observed at a substrate concentration of 150 mM (Scheme 6).

In conclusion, the substrate range of a novel recombinant (*S*)-alcohol dehydrogenase from *R. erythropolis* was studied, and a practical and efficient reaction medium for the reduction of ketones via enzymatic in situ-cofactor-regeneration was found. The conversions proceed at high substrate concentrations in the 'direct' presence of an organic solvent. The general applicability of this reaction system as well as the simple access to optically active alcohols on a lab scale are further advantages. Currently, further process development is in progress.

3. Experimental

The measurements of the enzymatic activity were carried out using the spectrophotometer JASCO V-530 UV/VIS. The solution of the (*S*)-alcohol dehydrogenase from *R. erythropolis* (overexpressed in *E. coli*) was prepared according to Refs. 3k and 11a. The solution of the formate dehydrogenase from *C. boidinii* (mutant: C23S, C262A; overexpressed in *E. coli*) was prepared according to Ref. 8. The ketones, cofactors, sodium formate and the solvents are commercially available and were used in the activity tests and preparative conversions without further purification.

3.1. Measurement of enzyme activity (typical protocol; according to Tables 1–3)

The NAD-dependent ADH activity was determined spectrophotometrically measuring the oxidation of NADH at 340 nm (ε_{340}=6.22 mM^{-1} cm^{-1}) in the presence of the ketone. The activity was measured at 30 °C in a cuvette (1 mL) containing 1.45 mM of the ketone in 100 mM sodium phosphate buffer (pH 6.0), and 0.25 mM of NADH. The reaction was started by the addition of the recombinant (*S*)-alcohol dehydrogenase from *R. erythropolis* (10 μL of the (*S*)-ADH solution described below as a crude extract or partially purified by ion exchange chromatography, which was diluted by a factor of 100). One unit of ADH activity was defined as the amount of enzyme that converted 1 μmol of NADH per minute. The activity tests described in entries, 1–5, 8, 10, 14 in Table 1, and in all entries in Table 3, are based on the use of an (*S*)-ADH solution with an activity of 13.4 U/mL (for acetophenone); the activity tests described in entries, 6, 7, 9, 11–13, 15–17 in Table 1, and in all entries in Table 2, are based on the use of an (*S*)-ADH solution with an activity of 43.3 U/mL (for acetophenone).

3.2. Enzymatic reduction in aqueous media (according to Scheme 2)

At a reaction temperature of 30 °C, 10 U of the (*S*)-alcohol dehydrogenase, and 10 U of the formate dehydrogenase was 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 analyzed with respect to the conversion rate (97% according to HPLC), dried over magnesium sulfate, and evaporated in vacuo. After chromatographical purification (eluent: ethyl acetate/*n*-hexane 25:75) of the resulting crude product, the desired isolated product (*S*)-**2a** was obtained in 78% yield (64.0 mg), and with an enantioselectivity of >99% ee (determined by HPLC).

3.3. Measurement of the enzymatic activity of the formate dehydrogenase (FDH) from *C. boidinii* (mutant: C23S, C262A; overexpressed in *E. coli*; see also Ref. 8) after storage in aqueous organic solvent mixtures

At first, 100 μL of a formate dehydrogenase solution (formate dehydrogenase (FDH) from *C. boidinii* (mutant: C23S, C262A; overexpressed in *E. coli*; see also Ref. 8) were dissolved in 10 mL of a solvent mixture containing 10–20% (v/v) of an organic solvent (an overview about the investigated solvents is given in Scheme 4). The samples were stored for a duration of up to 3 days, and regularly samples of 100 μL are taken from the solution. These samples were analyzed with respect to the enzymatic activity by spectrophotometrically measuring the reduction of NAD^+ to NADH at 340 nm (ε_{340}=6.22 mM^{-1} cm^{-1}) with sodium formate (spectrophotometer: JASCO V-530 UV/VIS). The activity was measured at 30 °C in a cuvette (1 mL) containing 500 μL of a sodium formate solution (0.8 M; pH 8.2; 0.1 M phosphate buffer), and 500 μL of a NAD^+-solution (0.4 mM). The reaction was started by the addition of the enzyme solution (10 μL of the 100 μL solution). One unit of FDH activity was defined as the amount of enzyme that converted 1 μmol of NAD^+ per minute. The resulting enzymatic activities of the FDH in dependency of the storage time in the presence of organic solvents are described in Scheme 4.

3.4. Enzymatic reduction with different types of substrates (according to Table 4)

A general procedure is as follows: At a reaction temperature of 30 °C, 10 U of the (*S*)-alcohol dehydrogenase from recombinant *R. erythropolis* (see also Ref. 3k), and 10 U of the formate dehydrogenase from *C. boidinii* (mutant: C23S, C262A; expressed in *E. coli*; see also Ref. 8) are added to a solution of 0.5 mmol of the ketone component (83.9 mg), 2.5 mmol sodium formate (171.6 mg), and 0.1 mmol of NADH (70.2 mg) in a solvent mixture, consisting of 10 mL of *n*-heptane, and 40 mL of a phosphate buffer (50 mM; pH 7.0). After stirring the reaction mixture for 21 h, the organic phase was separated and the aqueous phase is extracted with 3×50 mL of methyl *tert*-butyl ether. The collected organic phases are dried over magnesium sulfate, and after filtration and evaporation of the volatile components in vacuo the resulting oily crude product was analyzed with respect to the conversion rate (via NMR, HPLC). The ee was determined by chiral GC chromatography using a chiral column 'CP-Chirasil-DEX CB' (length: 25 cm; diameter: 25 μm; 1.3 mL/min; gas: He) from the company Chrompack.

3.5. Enzymatic reduction at higher substrate concentrations (according to Scheme 5) exemplified for the reaction with a substrate concentration of 100 mM

At a reaction temperature of 30 °C, 10 U of the recombinant (*S*)-alcohol dehydrogenase from *R. erythropolis* (see also Ref. 3k), and 10 U of the formate dehydrogenase from *C. boidinii* (mutant: C23S, C262A; expression in *E. coli*; see also Ref. 8) were added to a solution of 0.5 mmol of *p*-chloroacetophenone (83.9 mg), 2.5 mmol sodium formate (171.6 mg), and 0.1 mmol of NADH (70.2 mg) in a solvent mixture, consisting of 1 mL of *n*-heptane, and 4 mL of a phosphate buffer (50 mM; pH 7.0). After stirring the reaction mixture for 21 h, the organic phase was separated and the aqueous phase was extracted with 3×5 mL of methyl *tert*-butyl ether. The collected organic phases were dried over magnesium sulfate, and after filtration and evaporation of the volatile components in vacuo the resulting oily crude product was analyzed with respect to the conversion rate (via NMR, HPLC).

In addition, for the asymmetric reduction at a substrate concentration of 100 mM the product (*S*)-**2a** has been purified from the crude product and isolated via TLC-chromatography (eluent: ethylacetate/*n*-hexane (25:75); thin-layer-chromatography plate: Merck TLC plate 20×20 cm, silica gel 60 F254; R_f-value: 0.21; yield: 67%).

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13. The determination of enzyme activities were done using a cell-free crude extract of *E. coli* with the recombinant (*S*)-ADH enzyme from *R. erythropolis*. In addition, activity studies were also done with a (partially) purified form of the enzyme. The presence of other dehydrogenases causing a background activity was not observed in the experimental investigation. The specific activities of this (*S*)-ADH from *R. erythropolis* expressed in *E. coli* is in the range of 100–1000 U/mg of purified protein depending of the structure to be converted, see also Ref. 3k. For comparison, horse liver alcohol dehydrogenase as one of the most widely applied (*S*)-alcohol dehydrogenase so far shows an activity in the range of 1–3 U/mg only, see Ref. 1a.
14. 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), see also Ref. 11a.