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An Exceptionally DMSO-Tolerant Alcohol Dehydrogenase for the Stereoselective Reduction of Ketones

Iván Lavandera,[b] Alexander Kern,[c] Martina Schaffenberger,[a] Johannes Gross,[b] Anton Glieder,[c] Stefaan de Wildeman,[d] and Wolfgang Kroutil*[a]

A novel short-chain alcohol dehydrogenase from Paracoccus pantotrophus DSM 11072, which is applicable for hydrogen transfer, has been identified, cloned, and overexpressed in E. coli. The enzyme stereoselectively reduces several ketones in a sustainable substrate-coupled approach using 2-propanol (5% v/v) as hydrogen donor. The enzyme maintained its activity in organic co-solvents in biphasic as well as monophasic systems and was even active in micro-aqueous media (1% v/v aqueous buffer). In general, a higher conversion was observed at higher log P values of the solvent, however, DMSO, which exhibits the lowest log P value of all solvents investigated, was not only tolerated but led to a higher conversion and relative activity (110–210%). For example, the conversion after 24 h in 15% v/v DMSO was double that for the reaction performed in buffer. This tolerance to DMSO may be attributed to the ability of the wild-type strain to adapt and grow in media with high sulfur content.

Introduction

The asymmetric reduction of ketones by alcohol dehydrogenases (ADHs) requires a nicotinamide cofactor (NADH or NADPH) as hydride source. Recycling of the cofactor can most efficiently be achieved in a sustainable substrate-coupled approach,[1] where the ADH performing the desired reduction also recycles the cofactor, sacrificing small alcohol molecules such as 2-propanol or ethanol in a hydrogen-transfer-like fashion. This bioorganic protocol resembles a very simple and elegant method in contrast to the enzyme-coupled approach, whereby a second enzyme is needed to recycle the cofactor.[2]

In this context, it would be expected that most of the ADHs should be able to convert the small molecule 2-propanol and work according to the substrate-coupled approach. However, only few ADHs are able to do so; one reason is probably that most enzymes of this type show very low stability in the presence of 2-propanol, acetone, ethanol or acetaldehyde, as stability at elevated concentrations of these compounds are needed (at least 2–3% v/v) for the reaction. Thus, a high concentration of the corresponding alcohols is used to drive the reaction equilibrium to the product side. The first example of a substrate-coupled system was shown by Klibanov and coworkers using ethanol and horse liver ADH.[3] Subsequently, other ADHs stable to 2-propanol obtained from *Lactobacillus kefir*,[4] *Lactobacillus brevis*,[5] *Candida parapsilosis*,[6] *Thermoanaerobacter brockii*,[5c] *Pseudomonas fluorescens*,[7] *Thermoanaerobacter ethanolicus*,[8] *Leifsonia* sp.,[9] and *Rhodococcus ruber*[10] were described. In particular, the latter tolerates very high concentrations of 2-propanol (up to 80% v/v).[11] This enzyme has been overexpressed successfully in *E. coli*[10,12] and reduces a broad spectrum of ketones usually with perfect stereoselectivity.[13]

All these alcohol-stable enzymes could in principle be good candidates to perform reductions in the presence of other organic co-solvents. The use of non-aqueous solvents for the stereoselective reduction of ketones provides the advantage of solubilizing the substrates, as most of them are highly hydrophobic and thus display low solubility in aqueous media. However, owing to the instability of most of these ADHs in non-conventional environments this technique has been scarcely applied for biocatalytic redox processes.[14]

Here, we present a novel short-chain dehydrogenase obtained from *Paracoccus pantotrophus* which can reduce a broad set of ketones with very high stereoselectivity when used in combination with 2-propanol (5% v/v) as hydrogen donor in a biocatalytic hydrogen transfer reaction. In addition, its stability at elevated concentrations of organic solvents is reported, with special emphasis on dimethyl sulfoxide (DMSO).

Results and Discussion

To identify novel ADHs working in a substrate-coupled approach, we tested more than 100 lyophilized bacterial and yeast strains for the bioreduction of acetophenone at elevated

[a] *M. Schaffenberger, Prof. W. Kroutil*
Department of Chemistry, Organic and Bioorganic Chemistry
University of Graz, Heinrichstrasse 28, 8010 Graz (Austria)
Fax: (+43) 316-380-9840
E-mail: wolfgang.kroutil@uni-graz.at

[b] *Dr. I. Lavandera, J. Gross*
Research Centre Applied Biocatalysis
c/o Department of Chemistry, Organic and Bioorganic Chemistry
University of Graz, Heinrichstrasse 28, 8010 Graz (Austria)

[c] *Dr. A. Kern, Prof. A. Glieder*
Research Centre Applied Biocatalysis
c/o Institute for Molecular Biotechnology, Graz University of Technology
Petersgasse 14/2, 8010 Graz (Austria)

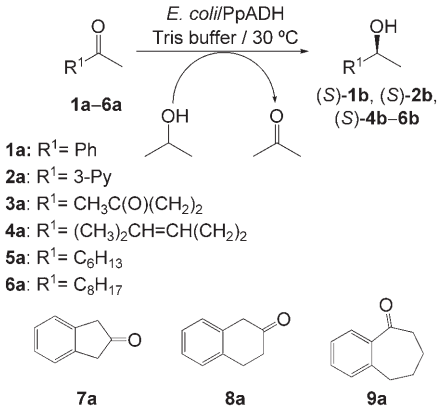
[d] *Dr. S. de Wildeman*
DSM Pharmaceutical Products
P.O. Box 18, 6160, MD Geleen (The Netherlands)

substrate concentration (5–10 g L^{-1}) employing 2-propanol (10% v/v) as hydrogen source. Special emphasis was laid on microorganisms capable of growing in polluted or chemically stressed environments. *Paracoccus pantotrophus* DSM[15] 11072, which is reported to be able to grow on carbon disulfide and is involved in the degradation of sulfur compounds,[16] was identified as a hit in the screening.

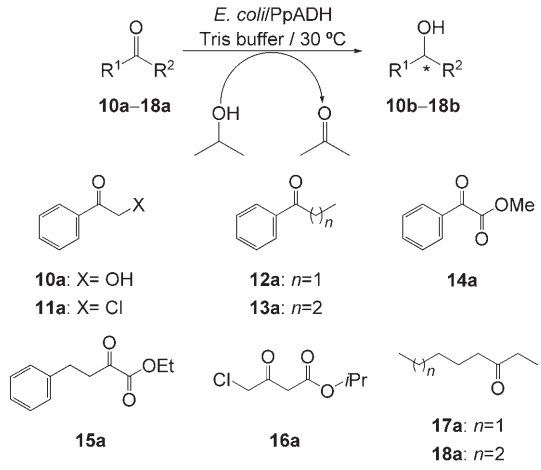
Subsequently, a gene library from *Paracoccus pantotrophus* was constructed[17] and screened on filter paper for increased NADH fluorescence[18] as a result of the oxidation of 2-propanol. Positive clones were then sequenced and analyzed by comparison with databases using the blastx algorithm for putative alcohol dehydrogenases or related genes. A short-chain alcohol dehydrogenase gene was identified, amplified by PCR and cloned into the plasmid pEamTA.[19] The enzyme was subsequently overexpressed in *E. coli* Top 10F′.

In a typical reduction experiment, lyophilized cells of *E. coli* containing the recombinant *Paracoccus pantotrophus* ADH (denoted as *E. coli*/PpADH) were employed for the reduction of the ketones (5 g L^{-1}) with 2-propanol (5% v/v) in Tris-HCl buffer (50 mM, pH 7.5, 1 mM NADH; Scheme 1, Table 1). Several methyl and cyclic ketones such as aromatic (**1a**, **2a**), aliphatic (**4a**–**6a**) and cyclic ketones (**7a**–**9a**) as well as a diketone (**3a**) were investigated to study the substrate spectrum of this enzyme. We observed good activity and excellent stereoselectivity, with the enantiopure *S*-configured alcohols obtained in the case of aromatic and aliphatic methyl ketones. Unfortunately, in the case of the diketone 2,5-hexanedione (**3a**), PpADH did not show any activity. In the case of cyclic substrates, the five-membered substrate **7a** was not converted while the six-membered homologue substrate β-tetralone (**8a**) was transformed with good activity and selectivity. The seven-membered homologue benzosuberone (**9a**) was reduced with excellent stereoselectivity although at a moderate rate.

Most chiral alcohols, which are building blocks for bioactive alcohols, are derived from more sterically demanding ketones (Scheme 2, Table 2). For example, derivatives of chloro alcohol **11b** are employed as intermediates in the synthesis of pharmaceuticals for the treatment of obesity and depression,[20] while alcohol **15b** is a valuable intermediate to obtain ACE antagonists.[21] When a hydroxy group was present in the α-position (**10a**), no activity was detected, however, to our delight ω-chloroacetophenone (**11a**) with a chlorine at the α-position was reduced to the corresponding enantiopure alcohol (*R*)-**11b**. Ethyl (**12a**) or propyl (**13a**) ketones were also reduced with good activity and selectivity. The presence of longer alkyl



Scheme 1. Biocatalytic asymmetric hydrogen transfer (Py = pyridyl).



Scheme 2. Biocatalytic reduction of bulky ketones.

Table 1. Reduction of methyl ketones and cyclic ketones employing lyophilized cells of *E. coli*/PpADH and 2-propanol (5% v/v).[a]

Substrate	Relative activity[b]	*ee* [%][c]
1a	24	>99 (*S*)
2a	51	>99 (*S*)
3a	<1	n.a.
4a	5	>99 (*S*)
5a	4	>99 (*S*)
6a	2	>99 (*S*)
7a	<1	n.a.
8a	44	90 (*S*)
9a	13	>95 (*S*)

[a] Substrate concentration: 5 g L^{-1}. [b] 100% activity corresponds to 7.8 nmol substrate converted per mg per minute. [c] Calculated from gas chromatogram (n.a. = not applicable).

Table 2. Reduction of sterically demanding ketones employing lyophilized cells of *E. coli*/PpADH and 2-propanol (5% v/v).[a]

Substrate	Relative activity[b]	*ee* [%][c]
10a	<1	n.a.
11a	93	>99 (*R*)[d]
12a	44	>99 (*S*)
13a	12	78 (*S*)
14a	100	94 (*S*)[d]
15a	55	>99 (*R*)[d]
16a	12	67 (*R*)[d]
17a	1.5	n.d.
18a	1.2	n.d.

[a] Substrate concentration: 5 g L^{-1}. [b] 100% activity corresponds to 7.8 nmol substrate converted mg per minute. [c] Calculated from gas chromatogram. [d] Switch in Cahn–Ingold–Prelog priority (n.a. = not applicable, n.d. = not determined).

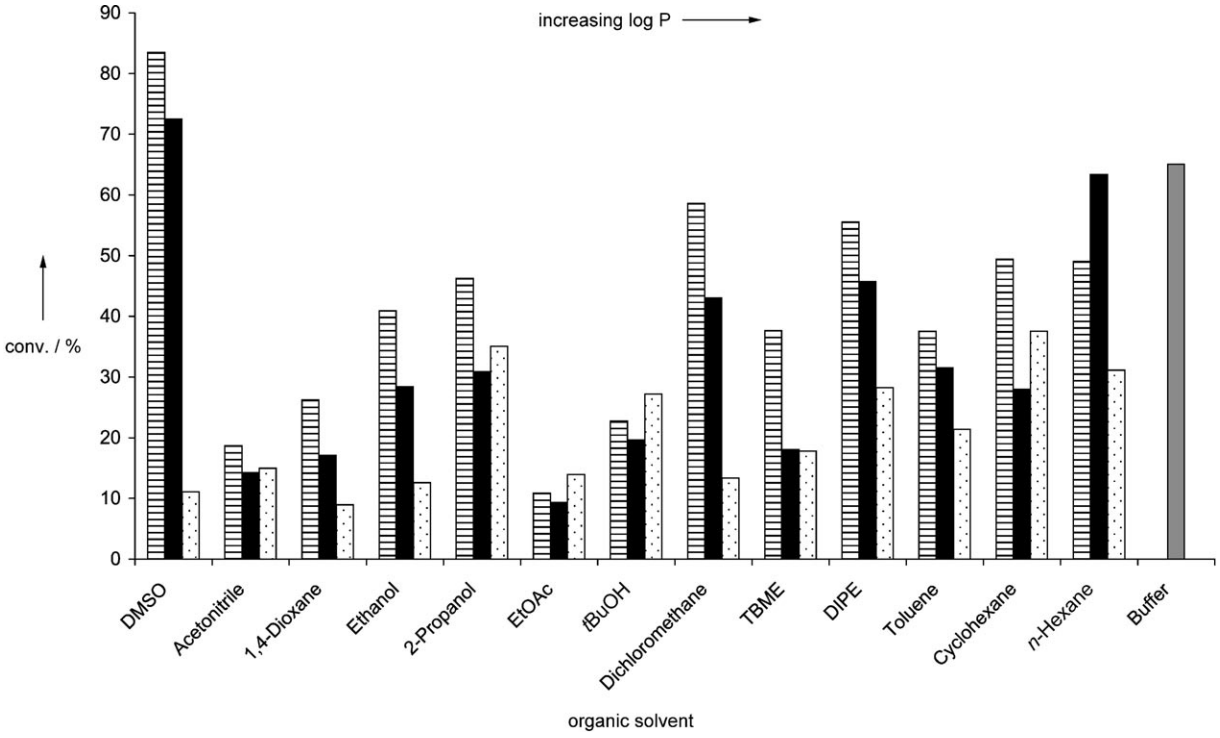


Figure 1. Effect of the concentration of organic solvent on the conversion of the *E. coli*/PpADH-catalyzed reduction of **14a** (10 g L^{-1}) in the presence of 2-propanol (5% v/v) after a reaction time of 24 h. The striped bars correspond to 20% v/v organic solvent; the black bars correspond to 50% v/v organic solvent; and the spotted bars correspond to 99% v/v organic solvent. The organic solvents are listed according to increasing log P (P = partition coefficient) value from left to right. TBME: *tert*-butyl methyl ether; DIPE: diisopropyl ether.

chains (butyl or pentyl) led to a clear decrease in activity. For the two aromatic α-ketoesters (**14a**, **15a**), we experienced a surprise: the two related substrates were reduced with opposite stereopreference. Thus, ketone **14a** gave the *S* enantiomer with 94% *ee* whereas substrate **15a** was transformed to enantiopure (*R*)-**15b**. Therefore, the two ketoesters may be attached in opposite binding mode in the active site of the enzyme. Finally, two 3-alkanones (**17a**, **18a**) were reduced to the corresponding alcohols but with low activity, emphasizing that this ADH prefers aromatic substrates to aliphatic ones.

As *E. coli*/PpADH proved to be stable in the presence of 2-propanol which is required in the substrate-coupled approach, the conversion in the presence of different organic co-solvents was studied. Ketone **14a** was chosen as a model substrate for this study (Figure 1). The conversion was investigated at 20 and 50% content of organic solvent as well as in a micro-aqueous environment.[22]

In mono- or biphasic aqueous–organic solvent systems using 20% or 50% v/v of organic co-solvent, lyophilized cells of *E. coli*/PpADH were partially or fully active in all systems under these conditions. In most cases, a higher conversion was achieved with 20% v/v organic solvent than with 50% v/v of organic solvent except for *n*-hexane. In contrast to all expectations, DMSO which exhibits the lowest log P value of all solvents investigated caused an apparent activation or stabilization of the catalyst. Even at 50% v/v DMSO content, the conversion in the reduction of **14a** was comparable to that achieved in buffer alone. Although DMSO might act as a solubilizer for the substrate in the aqueous solution leading to higher availability of the substrate to the enzyme, the enzyme clearly shows an outstanding tolerance to this solvent, as other water-miscible solvents such as 1,4-dioxane did not have a similar impact on the conversion. Similar alcohol dehydrogenase preparations, for example, *E. coli*/ADH-"A" showed a clear diminished activity in the presence of DMSO.[14a] For all organic solvents investigated, the stereoselectivity of the enzyme remained completely intact leading to (*S*)-**14b** with similar stereoselectivity, emphasizing the robustness of this catalyst.

In micro-aqueous organic solvent systems (99% v/v organic solvent), all water-miscible solvents such as DMSO, acetonitrile, 1,4-dioxane or EtOH led to significant deactivation of the enzyme (10–20% of the remaining activity). The only exception was 2-propanol, which also acted as a co-substrate. Although hydrophilic (water-miscible) organic solvents ($\log P < 0$) led to a decrease in the conversion, better biocompatibility was found for hydrophobic solvents ($\log P > 2$) such as toluene, cyclohexane and hexane. These results are in line with our previous observations obtained with an alcohol dehydrogenase from *Rhodococcus ruber* (ADH-"A").[14a]

In general, DMSO is not considered as an appropriate co-solvent for alcohol dehydrogenases.[14c,23] To study the effect of DMSO in more detail, conversions were measured at varied DMSO concentrations for the reduction of substrate **14a** (10 g L^{-1}) after 1 h (Figure 2). The conversions of **14a** were comparable at DMSO contents between 5 and 15% v/v. On the other hand, a notable decrease in conversion was detected

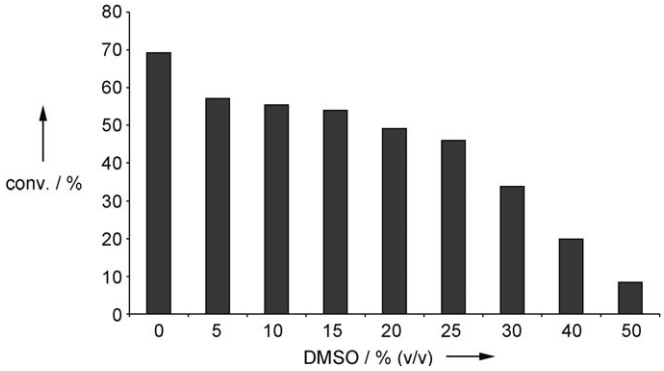


Figure 2. Influence of the amount of DMSO on the reduction ($t = 1$ h) of **14a** (10 g L^{-1}) catalyzed by *E. coli*/PpADH in the presence of 2-propanol (5% v/v) as hydrogen donor.

when the concentrations of DMSO exceeded 30% v/v. After a reaction time of 1 h the highest conversion was obtained in buffer solution alone, however, after 24 h this was surpassed by the conversions obtained in the presence of DMSO (Figure 1). This could be an indication that the positive effect of DMSO may be attributed to stabilization of the enzyme than to activation.

Finally, by employing 15% v/v DMSO, the conversions for the reduction of selected substrates were determined and compared with those in buffer solution alone (Figure 3). As expected from the results above, a positive effect of DMSO on

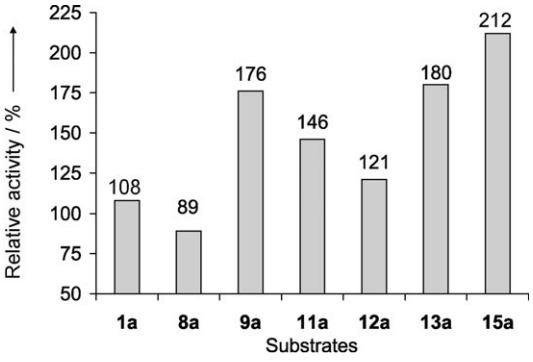


Figure 3. Comparison of activity in buffer solution alone and a mixture of buffer/DMSO (85/15 v/v) of the *E. coli*/PpADH-catalyzed reduction of different substrates in the presence of 2-propanol (5% v/v). The activity in buffer solution alone for each substrate corresponds to 100%.

the PpADH-catalyzed reactions was observed in most cases. In the case of ketone **15a**, the conversion even doubled as compared to that in buffer alone.

In all the cases studied, the stereoselectivity of the enzyme was not affected and the corresponding alcohols were obtained with comparable enantiomeric excesses.

Conclusions

A new short-chain dehydrogenase from *Paracoccus pantotrophus* DSM 11072 (PpADH) was identified and overexpressed. The enzyme reduces ketones to the corresponding enantiopure secondary alcohols through sustainable biocatalytic hydrogen transfer employing 2-propanol as hydrogen donor. Lyophilized *E. coli* cells containing the overexpressed PpADH were shown to be an excellent catalyst for biocatalytic hydrogen transfer, allowing the synthesis of versatile building blocks. *E. coli*/PpADH was successfully employed in non-conventional aqueous–organic solvent systems and tolerated even microaqueous media composed of a hydrophobic organic solvent (99% v/v) and buffer.

Of all the organic solvents investigated, DMSO proved to be special. For most substrates, the identified PpADH led to higher conversions in 50% v/v DMSO than in buffer alone after 24 h. Furthermore, with 15% v/v DMSO higher conversions were obtained compared to the corresponding reactions without organic solvent. An explanation for this unexpected observation might be that *Paracoccus pantotrophus* DSM 11072 can grow on carbon disulfide and is involved in the degradation of sulfur compounds.[16] Thus, it can be expected that if this microorganism can survive in such nasty environments, it can tolerate a certain extent of sulfur derivatives, for example, DMSO. Therefore, enzymes coming from this species should be especially well adapted to tolerate them. This study shows that it is advantageous to make a preselection of organisms for screening reactions. For instance, testing microorganisms capable of growing in an organic-contaminated environment increases the chance to find enzymes that are also stable in the presence of organic compounds.

Experimental Section

General: Ketones and racemic alcohols were commercially available either from Sigma–Aldrich–Fluka (Vienna, Austria) or Lancaster (Frankfurt am Main, Germany). Racemic compounds **2b**, **9b**, **11b** and **16b** were synthesised by conventional reduction from the corresponding ketones ($NaBH_4$, MeOH, room temperature).[13b] All other reagents and solvents were of the highest quality available. Conversions, enantioselectivities and absolute configurations were determined using GC analysis as previously described.[10,13,14a] Conversions measured by GC-FID were corrected using calibration curves. The data shown are the means of double measurements.

The construction of the gene library from *Paracoccus pantotrophus* was performed according to reported methods.[17] The transformation of *E. coli* Top 10F′ with the resulting library was carried out by electroporation. The colonies were then transferred to a filter paper and screened for increased NADH fluorescence[18] owing to the oxidation of 2-propanol. Positive clones were sequenced and analyzed by blastx for putative alcohol dehydrogenases or related genes. A putative short-chain dehydrogenase gene was identified, amplified by PCR and cloned into the plasmid pEamTA.[19]

Sequence of the identified PpADH (Accession number: EU427522):
atgagcaattccgtcgaaggccgcgtggtcatcgtcaccggcgccggccgcggcatcgg-
ccgctccatcgccgaggggctggcgcaggccggcgcccgggtggtgatcgccgacatcg-
ccgccgataccgccgaaaccaccgccgccgagatccgcgaggcgggcgggcaggccat-

cggccttgccgtcgatgtgaccgaccgcgccagcacgcgcgcgctgatcgcccgcaccgtcgccgaacatggccggctggatgccatgttcaacaatgccggcatcgcccaggtcaagccgttcaacgacatcaccgaggacgactggcaccgcgtcatggacgtgaacgccatgggcgtgctgatcggcatccaggaggcggcacggcagttcatcgcccagggcggcggcggcaagatcgtcaacaccgcctcgatcgccggcaagcagggctacgagcccttggcgcattactcggccagcaagttcgccgtggtggcgctgacccaggccgcggcccgcgccttcggcaagcacggcatctgcgtcaacgccatctgccccggcgtggtcgccaccgacatgtggaagctgatcgacaagggcttcaaggacgaggggctgaccagccgcgacaacgaggctttcgaggcttttccgccgacatcctgctgggccgcccctcgcgccccgaggatctggccggcgtctcgatcttcctcgcctcggccgggtcggactacatgaccgggcaaagcctggtcgtcgacggcggcatggtgctgctatga.

Optimized batch procedure for preparation of lyophilized cells of *E. coli*/PpADH: *E. coli* Top 10F′+PpADH was stored at −86 °C in a solution of glycerol/luria broth ampicillin (LB-amp; 15:85). Prior to use, it was plated on LB-amp (100 mg L^{-1}) and incubated for 48 h at room temperature. A single colony was plated again on LB-amp (48 h, cultivation at room temperature), and a loop of cells was used to inoculate LB-amp medium (250 mL) in 1-L baffled shake flasks. After incubation for 24 h at 30 °C at 130 rpm, the expression of PpADH was induced by the addition of isopropyl thiogalactoside (IPTG; 450 mg L^{-1}, 2 mM final concentration). Again, additional ampicillin sodium salt (100 mg L^{-1}) was added. The incubation was performed at 20 °C to avoid the formation of inclusion bodies for 24 h at 130 rpm. The cells were harvested by centrifugation (8000 rpm, 20 min, 4 °C), the medium was decanted, and the cells were resuspended in water, shock-frozen (liquid nitrogen) and lyophilized.

General protocol for biocatalytic hydrogen transfer: Lyophilized cells of *E. coli* Top F′+PpADH (15 mg) were rehydrated in buffer (600 μL, 50 mM Tris-HCl, 1 mM NADH, pH 7.5) for 30 min at 30 °C at 130 rpm in Eppendorf tubes (1.5 mL). The substrate (5 g L^{-1}) and 2-propanol (32 μL, 5% v/v) were added, and the mixture was agitated at 30 °C and 130 rpm for 24 h. Afterwards, the reaction was stopped by addition of ethyl acetate (2×0.5 mL). The organic layer was separated from the aqueous phase by centrifugation (2 min, 13000 rpm) and dried over Na_2SO_4. Conversions and enantioselectivities were determined by using GC analysis.

General procedure for biocatalytic reduction employing *E. coli*/PpADH in buffer/organic solvent systems. Lyophilized cells of *E. coli* Top F′+PpADH (15 mg), stored at 4 °C, were rehydrated in the corresponding volume of buffer (50 mM, 1 mM NADH, pH 7.5) for 30 min at 30 °C and 130 rpm on a rotary shaker in an Eppendorf vial (1.5 mL). Organic solvent (final volume of 0.6 mL), 2-propanol (32 μL, 5% v/v) and ketone (10 g L^{-1}) were added. The mixtures were shaken at 30 °C and 130 rpm for 24 h, and the reaction was stopped by extraction with ethyl acetate or diethyl ether (2× 0.5 mL). The organic layer was separated from the cells by centrifugation (2 min, 13000 rpm) and dried over Na_2SO_4. Conversions and enantiomeric excesses of the alcohol were determined by GC analysis.

General procedure for the biocatalytic reduction of **14a** employing *E. coli*/PpADH in micro-aqueous media. Lyophilized cells of *E. coli*/Top F′+PpADH (15.0 mg), stored at 4 °C, were rehydrated in buffer (6 μL, 50 mM Tris-HCl, 100 mM NADH, pH 7.5) for 30 min at 30 °C and 130 rpm on a rotary shaker in an Eppendorf vial (1.5 mL). The corresponding anhydrous organic solvent (0.6 mL), 2-propanol (32 μL, 5% v/v) and substrate **14a** (10 g L^{-1}) were added, and the reaction mixtures were shaken at 30 °C and 130 rpm for 24 h and stopped. Afterwards, water (300 μL) was added and the mixture was extracted with ethyl acetate or diethyl ether (2×0.5 mL). The organic layer was separated from the cells by centrifugation (2 min, 13000 rpm) and dried over Na_2SO_4. Conversions and enantiomeric excesses of **14b** were determined by GC analysis.

Influence of DMSO concentration on the *E. coli*/PpADH-catalyzed reduction of **14a**. Lyophilized cells of *E. coli* Top F′+PpADH (15 mg), stored at 4 °C, were rehydrated in the corresponding volume of buffer (50 mM Tris-HCl, 1 mM NADH, pH 7.5) for 30 min at 30 °C and 130 rpm on a rotary shaker in an Eppendorf vial (1.5 mL). Then, DMSO (final volume of 0.6 mL), 2-propanol (32 μL, 5% v/v) and **14a** (10 g L^{-1}) were added. The mixtures were shaken at 30 °C and 130 rpm for 1 h, and the reactions were stopped by extraction with ethyl acetate (2×0.5 mL). The organic layer was separated from the cells by centrifugation (2 min, 13000 rpm) and dried over Na_2SO_4. Conversions and enantiomeric excesses of **14b** were determined by GC analysis.

Acknowledgements

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Keywords: biocatalysis · enzymes · ketones · reduction · sulfur

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