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# Practical Asymmetric Enzymatic Reduction through Discovery of a Dehydrogenase-Compatible Biphasic Reaction Media†

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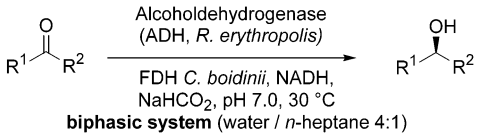
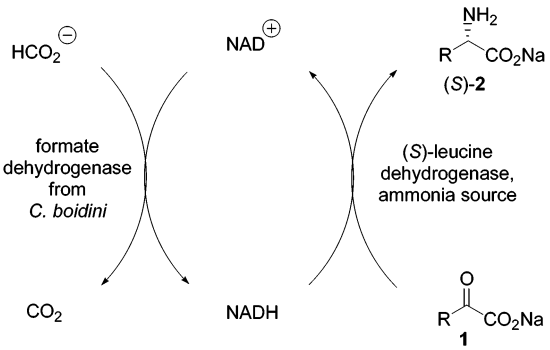
## ABSTRACT




**An enzyme-compatible biphasic reaction media for the asymmetric biocatalytic reduction of ketones with in situ cofactor regeneration has been developed. In this biphasic reaction media, which is advantageous for reactions at higher substrate concentrations, both enzymes (alcohol dehydrogenase and FDH from *Candida boidinii*) remain stable. The reductions with poorly water-soluble ketones were carried out at substrate concentrations of 10–200 mM, and the optically active (*S*)-alcohols were formed with moderate to good conversions and with up to >99% ee.**

The enzymatic reductive amination by means of an enzyme-coupled in situ cofactor regeneration according to Scheme 1 is a powerful tool for the preparation of enantiomerically pure α-amino acids.[1] This has been demonstrated by Bommarius et al. in the industrial synthesis of L-*tert*-leucine and related α-amino acids, which runs on a ton scale.[2] A key feature of this process is the use of the formate dehydrogenase (FDH) from *Candida boidinii* for the in situ regeneration of the cofactor NADH. The extension of this attractive

**Scheme 1.** Production of L-*tert*-Leucine via Reductive Amination

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(1) (a) Hummel, W.; Schütte, H.; Schmidt, E.; Wandrey, C.; Kula, M.-R. *Appl. Microbiol. Biotechnol.* **1987**, *26*, 409−416. (b) Krix, G.; Bommarius, A. S.; Drauz, K.; Kottenhahn, M.; Schwarm, M.; Kula, M.-R. *J. Biotechnol.* **1997**, *53*, 29−39.

(2) (a) Bommarius, A. S.; Drauz, K.; Hummel, W.; Kula, M.-R.; Wandrey, C. *Biocatalysis* **1994**, *10*, 37−47. (b) Schultz, C.; Gröger, H.; Dinkel, C.; Drauz, K.; Waldmann, H. In *Applied Homogeneous Catalysis with Organometallic Compounds*, 2nd ed.; Cornils, B., Herrmann, W. A., Eds.; VCH−Wiley: Weinheim, Germany, 2002; Vol. 2, Chapter 3.2.1.

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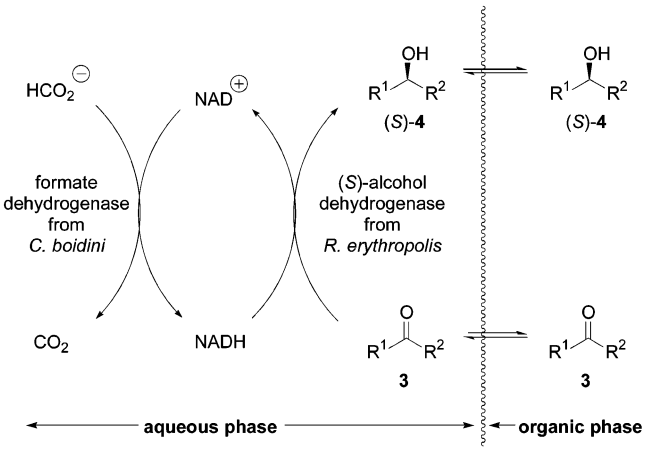
enzymatic concept[3,4] toward the synthesis of optically active alcohols[5−7] is highly desirable, but still some drawbacks exist that limit practicability and large-scale applicability. This method falls short of others although numerous efficient alcohol dehydrogenases with excellent (*R*)- and (*S*)-enantiospecificities have been developed[3,4] and are, at least in part, already commercially available.

A main limitation is the low solubility of hydrophobic ketone substrates in aqueous media. Therefore, reactions are usually carried out at a very low substrate concentration of ≤5−10 mM, which leads to nonsatisfactory volumetric productivities. Consequently, even on a lab scale, the practicability of the reduction of hydrophobic ketones via enzymatic in situ cofactor regeneration is obviously limited despite its high potential. The presence of an organic solvent could improve the solubility of poorly water-soluble ketones but generally causes severe enzyme deactivation. In particular, this is known for the formate dehydrogenase from *C. boidinii*[8] which is sensitive to organic solvents.[9] Several kinds of alcohol dehydrogenases were found to be unstable in the presence of organic solvents, too. The best solution so far is represented by a continuous process with an enzyme−membrane reactor.[10] This efficient "three-loop" concept is based on an enzymatic reaction in pure aqueous media, a separation of the aqueous phase from the enzyme via ultrafiltration, and subsequent continuous extraction of the aqueous phase with an organic solvent. Organic and aqueous phases are separated by a hydrophobic membrane. However, although good space−time yields in the range of 60−104 g $L^{-1}$ $day^{-1}$ are 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 represents a challenge.

In this communication, we report the first example of an asymmetric enzymatic reduction of poorly water-soluble ketones, including an in situ recycling of the cofactor NADH with FDH, which runs in the "direct" presence of an organic solvent at higher substrate concentrations. Such suitable biphasic reaction media allowing reductions at higher substrate concentrations represent a significant process improvement. The principles of this process are summarized in Scheme 2. This novel type of a biocatalytic synthesis of optically active (*S*)-alcohols possesses the following criteria: (i) an aqueous−organic solvent system in which both enzymes (carbonyl reductase and FDH from *C. boidinii* for the cofactor regeneration) remain stable; (ii) good solubility of poorly water-soluble ketones, which led to high substrate concentrations of up to 200 mM; (iii) a simple reaction protocol for lab-scale applications, comprising an economically attractive processing procedure, which allows a simple, flexible, and fast preparation of optically active alcohols; and (iv) a robust process with potential for technical-scale applications in the future.

**Scheme 2.** Concept of Enzymatic Reduction in Biphasic Media




The first key step was the development of a suitable reaction medium that guarantees a high solubility of ketones, as well as a high stability of the enzymes. As mentioned above, the stability of the FDH from *C. boidinii* as the most sensitive enzyme component turned out to be a critical issue.

(3) It should be mentioned at this stage that numerous efficient (*S*)- and (*R*)-specific alcohol dehydrogenases have already been found. For selected contributions, see: (a) Bradshaw, C. W.; Hummel, W.; Wong, C.-H. *J. Org. Chem.* **1992**, *57*, 1532. (b) Bogin, O.; Peretz, M.; Burstein, Y. *Protein Sci.* **1997**, *6*, 450−458. (c) Korkhin, Y.; Kalb, A. J.; Peretz, M.; Bogin, O.; Burstein, Y.; Frolow, F. *J. Mol. Biol.* **1998**, *278*, 967−981. (d) Holt, P. J.; Williams, R. E.; Jordan, K. N.; Lowe, C. R.; Bruce, N. C. *FEMS Microbiol. Lett.* **2000**, *190*, 57−62. (e) Matsuda, T.; Harada, T.; Nakamura, K. *Chem. Commun.* **2000**, 1367−1368. (f) Riebel, B.; Hummel, W. *Biotechnol. Lett.* **2001**, *23*, 231−234 (g) Schubert, T.; Hummel, W.; Kula, M.-R.; Müller, M.; *Eur. J. Org. Chem.* **2001**, 4181−4187. (h) Stampfer, W.; Kosjek, B.; Moitzi, C.; Kroutil, W.; Faber, K. *Angew. Chem.* **2002**, *114*, 1056−1059. (i) Hummel, W.; Abokitse, K.; Drauz, K.; Rollmann, C.; Gröger, H. *Adv. Synth. Catal.* **2003**, in press.

(4) For reviews about biocatalytic reduction in general, see: (a) Hummel, W. *Adv. Biochem. Eng./Biotechnol.* **1997**, *58*, 146−184. (b) Faber, K. *Biotransformations in Organic Chemistry*, 4th ed.; Springer-Verlag: Berlin, 2000; Chapter 2.2.3, pp 192−194.

(5) For selected contributions of an alternative approach via asymmetric metal-catalyzed hydrogenation of ketones, see: (a) Burk, M. J.; Hems, W.; Herzberg, D.; Malan, C.; Zanotti-Gerosa, A. *Org. Lett.* **2000**, *2*, 4173−4176. (b) Ohkuma, T.; Koizumi, M.; Yoshida, M.; Noyori, R. *Org. Lett.* **2000**, *2*, 1749−1751. (c) Ohkuma, T.; Takeno, H.; Honda, Y.; Noyori, R. *Adv. Synth. Catal.* **2001**, *343*, 369−375. (d) Review: Noyori, R.; Okhuma, T. *Angew. Chem., Int. Ed.* **2001**, *40*, 40−73.

(6) For selected contributions of an alternative approach via asymmetric whole-cell biocatalytic reduction of ketones, see: (a) Yasohara, Y.; Kizaki, N.; Hasegawa, J.; Wada, M.; Kataoka, M.; Shimizu, S. *Tetrahedron: Asymmetry* **2001**, *12*, 1713−1718. (b) See ref 3e. (c) See ref 3h.

(7) For selected contributions of an alternative approach via asymmetric reduction of ketones based on substrate-coupled cofactor regeneration, see: (a) Wolberg, M.; Ji, A. G.; Hummel, W.; Müller, M. *Synthesis* **2001**, 937−942. (b) Schubert, T.; Hummel, W.; Müller, M. *Angew. Chem.* **2002**, *114*, 656−659.

(8) Properties of the cofactor-regenerating enzyme FDH from *C. boidinii* have been improved remarkably by the Kula group using protein engineering. Thus, a stable and efficient formate dehydrogenase is now available on a large scale for NADH regeneration; see: Slusarczyk, H.; Felber, S.; Kula, M.-R.; Pohl, M. *Eur. J. Biochem.* **2000**, *267*, 1280−1289.

(9) Despite its great technical potential and applicability, the FDH from *C. boidinii* was found to be sensitive to the presence of organic solvents. The general problem of rapid deactivation of enzymes other than hydrolases in the presence of organic solvents has been previously described in several contributions, e.g.: In Kruse, W.; Kragl, U.; Wandrey, C. DE 4436149, 1996.

(10) (a) Liese, A.; Seelbach, K.; Wandrey, C. *Industrial Biotransformations*; Wiley-VCH: Weinheim, Germany, 2000; pp 103−106. (b) Kruse, W.; Hummel, W.; Kragl, U. *Recl. Trav. Chim. Pays-Bas* **1996**, *115*, 239−243. (c) For a review of this method and other large-scale applications of oxidoreductases, see: Hummel, W. *TIBTECH* **1999**, *17*, 487−492.

We focused on this specific enzyme as the preferred FDH for cofactor regeneration due to its low price, large-scale availability, and proven technical applicability.[8] A screening of 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 (Figure 1). In addition, typical solvents for a biphasic media failed, e.g, MTBE, which led to a remarkable decrease of the activity after 2 days. A very rapid loss of activity was found when ethyl acetate was used. In this case, a (nearly) complete deactivation was observed within only 6 h. Nonpolar aromatic hydrocarbons, e.g., toluene, deactivate FDH remarkably, too.

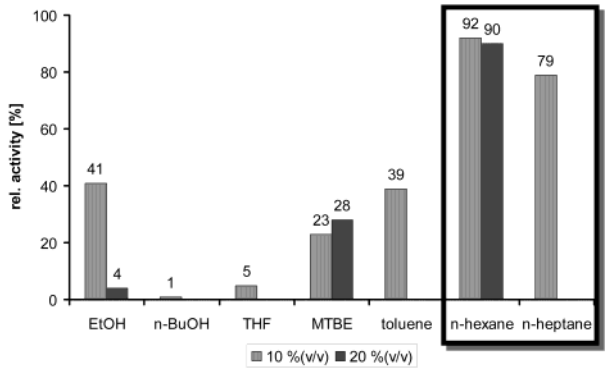


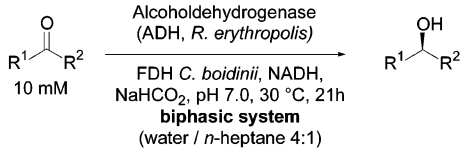

**Figure 1.** Stability of the FDH in aqueous−organic media (activities were measured after ca. 3 days for *n*-hexane, 2 days for *n*-heptane, and 2 days or less for the other solvents; for details, see Supporting Information).

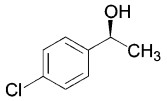
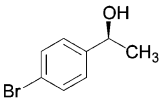
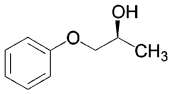
However, we were pleased to find that in the presence of 10% (v/v) *n*-hexane, a long-term nondestabilization was achieved (Figure 1). Even after 66−69 h, activities of 92 and 90% remained for aqueous solutions containing 10 and 20% (v/v) *n*-hexane, respectively. Notably, other aliphatic hydrocarbons, e.g., *n*-heptane, also were found to be suitable organic solvents, and the stability was maintained at an increased amount of the organic solvent, e.g., *n*-heptane, of up to 60% (v/v).

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 large-scale available (*S*)-selective alcohol dehydrogenase (ADH) from *Rhodococcus erythropolis*, which we have developed very recently,[3i] turned out to be stable for long periods under the same conditions. Thus, a suitable solvent system was found in which both enzymes remain stable.

With this enzyme-compatible reaction media in hand, preparative conversions were carried out as a next step. We were pleased to find that good conversions accompanied by excellent enantioselectivities were obtained with a variety of aromatic ketone substrates. Examples are shown in Table 1. In the presence of an (*S*)-ADH from *R. erythropolis*, *p*-chloroacetophenone was converted into the optically active (*S*)-enantiomer, (*S*)-**4a**, with >99% ee (69% conversion; entry 1).

**Table 1.** Reduction in Biphasic Media with Different Substrates




| entry[a] | product | | conversion [%] | ee [%][b] |
|---|---|---|---|---|
| 1 |  | (*S*)-**4a** | 69 | >99 |
| 2 |  | (*S*)-**4b** | 65 | 97 |
| 3 |  | (*S*)-**4c** | >95 | >99 |

[a] General procedure: at a reaction temperature of 30 °C, 10 U of (*S*)-alcohol dehydrogenase from *R. erythropolis* (expression in *E. coli*; see also ref 3i) and 10 U of formate dehydrogenase from *C. biodinii* (mutant: C23S, C262A; expression in *E. coli*; see also ref 8) were added to a solution of 0.5 mmol of the ketone component, 2.5 mmol of 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 the reaction mixture was stirred 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 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 (via NMR and HPLC). [b] Enantiomeric excess (ee) was determined by chiral GC chromatography.

A conversion of 65% and a slightly lower enantioselectivity of 97% ee were observed when using *p*-bromoacetophenone as a starting material (entry 2). The asymmetric reduction of phenoxyacetone proceeded quantitatively under formation of (*S*)-**4c** with an enantioselectivity of >99% ee (entry 3). Compounds of type **4c** or derivatives thereof are known to be biologically active, thus representing pharmaceutically interesting molecules.

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 in the presence of the (*S*)-ADH from *R. erythropolis*[3i] was investigated. Increased substrate concentrations from 10 mM (69% conversion) up to 100 mM led to comparable conversions (Figure 2). At 20 and 40 mM concentrations, slightly improved conversions of 77 and 75%, respectively, were obtained. Even at a 100 mM concentration, a good conversion of 74% was still achieved. An analogous reaction with subsequent chromatographic purification of the crude product gave the desired product (*S*)-**4a** with a yield

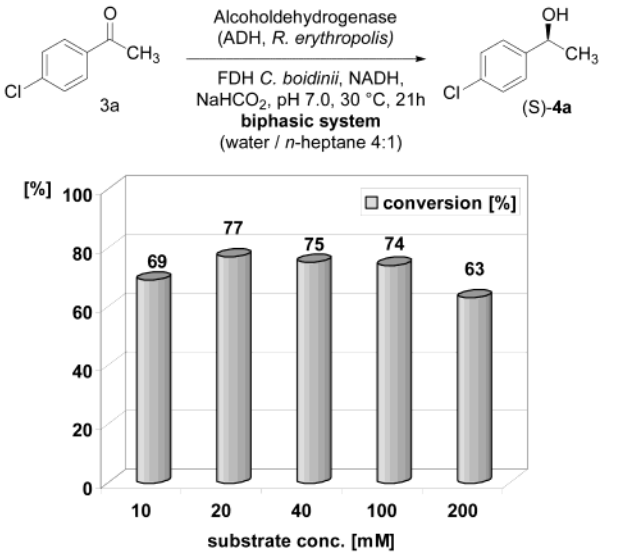



**Figure 2.** Conversions at increased substrate concentrations.

of 67% (for experimental details, see Supporting Information). A further increase of the substrate concentration up to 200 mM gave a still satisfactory conversion of 63%. Thus, higher volumetric productivities can be obtained with this new reaction medium.

In conclusion, a practical and efficient asymmetric reduction of ketones via enzymatic in situ cofactor regeneration was found that runs 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 (and possibly on a technical scale in the future) are further advantages. Currently, several downstream process improvements comprising recycling issues are under investigation.

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**Supporting Information Available:** Protocol for the investigation of the enzymatic stabilities in aqueous−organic solutions (according to Figure 1) as well as experimental protocols and data referring to Table 1 and Figure 2, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

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