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## 🏤 Bioreductions Catalyzed by an Alcohol Dehydrogenase in **Non-aqueous Media**

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Highly productive biocatalytic reductions were established using an isolated alcohol dehydrogenase (ADH) under waterdeficient conditions. First, a solvent-free system was evaluated for the reduction of 2-butanone catalyzed by ADH evo-1.1.200 promoted by the "smart cosubstrate" 1,4-butanediol. ADH evo-1.1.200 excelled by its activity and stability under high reagent concentrations and hence was the enzyme of choice. However, conversion of 2-butanone was limited to <1% in 10 days under the solvent-free conditions. Therefore, water-immiscible organic solvents were evaluated whereby the highest conversions were achieved in MTBE and toluene. MTBE was chosen as its different boiling point compared to other reaction components (e.g., 2-butanone, 2-butanol, diol cosubstrate, and lactone coproduct) would simplify the downstream processing. Further on, by tuning substrate loading, the productivity of the ADH evo-1.1.200 was successfully increased to a turnover number (TON) of 64000.

Biotransformations in organic/non-aqueous media are enjoying great interest, as most of the synthetically interesting substrates and products are not soluble in water and product recovery from the aqueous medium is often tedious. Indeed, examples of lipase-catalyzed reactions in organic solvents can be often found, whereas the use of alcohol dehydrogenases (ADHs) in non-aqueous media has been limited.<sup>[1]</sup> Hence, ADHcatalyzed biotransformations often run at relatively low substrate loadings and can still be considered economically unattractive. In addition, from an environmental point of view, biotransformations with relatively low substrate loadings suffer from the enormous amount of wastewater generated.

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Scheme 1. ADH-catalyzed reductions running in non-aqueous media through coupling of 1,4-butanediol as cosubstrate.

In the 1980s and 1990s, extensive studies on the use of ADHs in organic solvents were done by Klibanov and co-workers.<sup>[2]</sup> However, since then very few examples of bioreductions in predominantly organic media by using either whole cells<sup>[3]</sup> or isolated enzymes<sup>[4]</sup> have been reported. As a consequence, it would be highly desirable to investigate ADH-catalyzed reductions in organic media under water-deficient conditions, and hence, we drew our attention to neat substrates and to organic solvents (Scheme 1).

Recently, we reported a "smart cosubstrate" approach to overcome the thermodynamic challenge in ADH-catalyzed reductions.<sup>[5]</sup> Therein, by using 1,4-butanediol (1,4-BD) as a sacrificial electron donor the thermodynamic equilibrium could be shifted to the side of the product. This approach offers a significant environmental advantage, as the waste generated for conversions of >95% is reduced 40-fold. Overall, herein we report ADH-catalyzed reduction reactions under water-deficient conditions (in neat substrates or in a water-immiscible solvent) promoted by the "smart cosubstrate" 1,4-BD.

In a first set of experiments, we analyzed the reduction of 2butanone in neat substrates coupled with 1,4-BD as a cosubstrate. We chose 2-butanone as the substrate because in aqueous media the product 2-butanol would form an azeotrope and, thus, downstream processing would be tedious. The enzyme of choice was the commercial ADH evo-1.1.200, as it initially showed no loss in its activity at elevated diol concentrations up to approximately 4м (Figure S1, Supporting Information); the values of  $V_{max}$  (maximum reaction rate) and  $K_{M}$ (Michaelis–Menten constant) were found to be 2400 Ug<sup>-1</sup> and 84 mм for 1,4-BD, respectively.

Reactions running in neat substrates (2-butanone and 1,4-BD) revealed a linear increase in the concentration of 2-butanol over 10 days, however, with a very limited specific activity (15 U  $g^{-1}$ , Figure S2) and < 1% conversion in 10 days (Figure 1). We attributed this significantly diminished enzyme activity in



**Figure 1.** Conversion of 2-butanone by using 1,4-BD as a cosubstrate running in neat substrates catalyzed by evo-1.1.200. Reaction conditions: *c*(2-butanone) = 7.3 m, *c*(1,4-BD) = 3.7 m, *c*(NAD<sup>+</sup>) = 0.5 mm, 2.5 % (*v*/*v*) external water (50 mm Tris-HCl, pH 7.0), *c*(evo-1.1.200) = 0.3 g L<sup>-1</sup>. Reaction mixtures (1.5 mL) were kept at 30 °C and 1000 rpm.



**Figure 2.** Synthesis of 2-butanol under water-deficient conditions running in organic solvents. Reaction conditions: c(2-butanone) = 1 M, c(1,4-BD) = 0.5 M,  $c(NAD^+) = 0.5$  mM, 84% (v/v) organic solvent, 2.5% (v/v) external water (50 mM Tris-HCl, pH 7.0), c(evo-1.1.200) = 0.3 gL<sup>-1</sup>, 30 °C, 1000 rpm. log *P*-(MTBE) = 1.0, log *P*(DIPE) = 1.4, log *P*(toluene) = 2.5, log *P*(DBE) = 2.9, log *P*-(octane) = 4.5, log *P*(decane) = 5.6.

neat substrates to the relatively high hydrophilicity of 1,4-BD ( $\log P = -0.81$ , logarithmic value of octanol-water partition coefficient),<sup>[6]</sup> which may strip the essential "structural water"<sup>[1a]</sup> of the enzyme, and this would result in limited molecular flexibility.

In our further studies, we focused our attention on organic solvents, as we could avoid the aforementioned removal of "structural water" by means of a water-immiscible organic solvent. We chose MTBE (methyl *tert*-butyl ether), DIPE (diisopropyl ether), toluene, DBE (di-*n*-butyl ether), decane, and octane, as these have been successfully applied in ADH-catalyzed reactions.<sup>[7]</sup> In addition, we chose these solvents on the basis of their significantly different polarities (log P = 1.0-5.6). Among



**Figure 3.** Conversion of 2-butanone catalyzed by evo-1.1.200 at different substrate loadings in MTBE (5 mM dodecane) under water-deficient conditions. Reaction conditions: c(2-butanone) = 0.5-2 M, c(1,4-BD) = 0.25-1 M,  $c(\text{NAD}^+) = 0.5 \text{ mM}$ , 71-91% (v/v) MTBE (5 mM dodecane), 2.5% (v/v) external water (50 mM Tris-HCl, pH 7.0),  $c(\text{evo-1.1.200}) = 0.3 \text{ gL}^{-1}$ , 30 °C, 1000 rpm. 2-Butanone and 1,4-BD: 0.5 and 0.25 (**a**), 0.75 and 0.375 (**b**), 1 and 0.5 (**A**), 2 and 1 M (**•**).



**Figure 4.** Specific activity of evo-1.1.200 depending on the concentration of 2-butanone in MTBE. Reaction conditions: c(2-butanone) = 0.5-2 M, c(1,4-BD) = 0.25-1 M,  $c(NAD^+) = 0.5 \text{ mM}$ , 2.5% (v/v) external water (50 mM Tris-HCl, pH 7.0), 71–91% (v/v) MTBE,  $c(evo-1.1.200) = 0.3 \text{ gL}^{-1}$ , 30 °C, 1000 rpm.

the organic solvents screened, MTBE and toluene gave the highest conversions (Figure 2), whereby  $\log P(MTBE) = 1.0$  and  $\log P(toluene) = 2.5$  (Table S2). Thus, in the present context,  $\log P$  cannot be a direct criterion to choose the organic solvent.<sup>[7]</sup> Owing to its lower boiling point, which also differs significantly from the boiling points of the other reaction components (Table S3), we chose MTBE as the solvent for our further investigations.

Next, we evaluated the effect of substrate loading on conversion (Figure 3). The specific activities in MTBE  $(80-150 \text{ Ug}^{-1})$  were determined to be up to 10-fold higher than those in neat substrates  $(15 \text{ Ug}^{-1})$ , Figure S3). Our results showed significantly decreased enzyme activity at a 2-butanone concentration of

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**Table 1.** Conversion and *ee* values of alcohol products 1b-11b after 24 h and 14 days synthesized under water-deficient conditions (2.5% *v*/*v* water) in MTBE (5 mm dodecane) catalyzed by evo-1.1.200.<sup>[a]</sup>

Substrate	Product	24 h Conv. [%]	ee [%]	14 d Conv. [%]	ee [%]
o=	OH	22.6	99.0	40.7	96.5
		19.1	99.7	36.9	98.4
MeO MeO	MeO	9.8	>99.9 <sup>[b]</sup>	18.4	97.8 <sup>[b]</sup>
3a O Cl	3 b OH CI	12.4	> 99.9 <sup>[b]</sup>	42.1	> 99.9 <sup>[b]</sup>
4a 0 —	4b OH	45.8	-	89.3	_
5a 0 0 F F F	5b OH O F F	39.2	> 99.9 <sup>[b]</sup>	99.9	>99.9 <sup>[b]</sup>
6a J	6b OH	14.8	95.0 <sup>[b]</sup>	39.4	93.5 <sup>[b]</sup>
7a 0	7b OH	15.1	21.1	95.6	7.0
8a O	8b OH	12.9	76.5 <sup>[b]</sup>	62.5	49.9 <sup>[b]</sup>
9a 	9b OH	11.2	98.0 <sup>[b]</sup>	34.9	96.7 <sup>[b]</sup>
10a 0 11a	10b OH 11b	9.4	96.8 <sup>[b]</sup>	31.2	96.2 <sup>[b]</sup>

[a] Reaction conditions: c(substrate) = 0.5 M, c(1,4-BD) = 0.25 M,  $c(NAD^+) = 0.5 \text{ mM}$ ,  $c(evo-1.1.200) = 0.3 \text{ gL}^{-1}$ , 30 °C, 1000 rpm. Conversions were determined by GC. Absolute configurations were confirmed by authentic standards. [b] Absolute configurations were assumed because of similar chromatographic behavior; for **4b** and **6b**, the configuration was reversed because of the switch in substituent priorities according to Cahn–Ingold–Prelog rules.

 $2 \times (Figure 4)$ ; however, the initial rates were essentially the same up to  $1 \times 2$ -butanone (Figure 4). The turnover numbers (TONs) for the enzyme and for the cofactor were calculated to be 64000 and 960, respectively. However, the reactions were stopped at a point at which the enzyme was still active; hence, the total turnover numbers (TTNs) would be still higher.

Owing to the imperfect enantioselectivity of evo-1.1.200 towards the reduction of 2-butanone (see Table 1, entry 8), we focused our further studies on screening the substrates. Among the substrates screened (Table 1), the highest conversions were achieved for cyclohexanone (**5** a) and ethyl-4,4,4,-trifluoroacetoacetate (6a). Moderate conversions were obtained for acetophenone (1 a), and notably, the conversions decreased with Cl (see 2a), and MeO (see 3 a) substituents in the para position. Aliphatic ketones (see 8a-11 a), however, were accepted to a lesser extent, and conversions decreased with increasing chain length. The complete conversion of 6a and 2-butanone (8a) was achieved in a maximum of 14 days (Figure S5). The enantiomeric excess (ee) values of evo-1.1.200 for the products 2-chlorophenylethanol (4b) and ethyl-4,4,4-trifluoro-3-hydroxybuta-

noate (6b) were always at a maximum (>99.9%*ee*). The *ee* values of phenylethanol (1 b) and its para-substituted derivatives **2b** and **3b** were  $\geq$  99% *ee*; however, these values slightly decreased over the course of the reaction. The ee values of secondary aliphatic alcohols increased with the chain length. Most interestingly, in all cases we observed a significant decrease in the ee values with increasing conversions. In fact, the ee values decreased steeply if they were low already at the beginning, as shown in the case of 2butanol (8b) and 2-pentanol (9b, Figure S6). We attribute this decrease in the ee values of the alcohol products to enzyme-catalyzed racemization through reoxidation of the alcohol products.<sup>[8]</sup> In the oxidation of 1,4-butanediol, this might be due to the "half-reversibility" of the reaction; hence, the first oxidation step of 1,4-butanediol to the

corresponding hydroxy aldehyde might still be regarded as "quasireversible". In fact, this might be the case if the second oxidation step is rate limiting. At the beginning of the reaction, only 2-butanone and 1,4-butanediol, as substrate and cosubstrate, respectively, are present in the reaction mixture, and hence, the *ee* value of (R)-2-butanol is determined by the enantioselectivity of evo-1.1.200. However, with time a mixture of (R)- and (S)-2-butanol, the hydroxy aldehyde intermediate, and a lactone coproduct is formed, and consequently, there is a competition between the (R)- and (S)-alcohol for their reoxidation, which determines the *ee* of the alcohol product. To

sum up, as long as tiny amounts of "unpreferred" enantiomer are present, the racemization will take place, as it is a thermodynamically downhill process.<sup>[8b]</sup> The essential information for the course of the reactions can be found in the Supporting Information (Figure S6).

In this study, we demonstrated that ADHs applied in substrate-coupled reductions can go far beyond the traditional substrate loadings. For the reduction of 2-butanone coupled with 1,4-butanediol as a cosubstrate in neat substrates, conversion of <1% was detected, still with a significant TON of approximately 7800. Productivity of the enzyme was further optimized by using MTBE as the organic solvent, whereby a TON of 64000 was achieved.

Notably, a suspension of the enzymes in non-aqueous media resembles heterogeneous catalysis (Figure S7); thus, the solid enzyme aggregate can be recycled without immobilization. Ongoing research in our laboratories focuses on recycling the enzyme and application of this approach.

## **Experimental Section**

The chemicals used in this study were commercially obtained in analytical-grade quality and were used as received. The ADH evo-1.1.200, recombinantly expressed in *Escherichia coli*, was commercially available from evocatal GmbH (Monheim am Rhein, Germany). A detailed description of the experimental procedures as well as the analytical protocols is given in the Supporting Information.

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