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# Asymmetric biocatalytic reduction of ketones using hydroxy-functionalised water-miscible ionic liquids as solvents

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**Abstract**—Bi- and monophasic ionic liquid (IL)/buffer systems were successfully employed for the biocatalytic reduction of ketones catalysed by the alcohol dehydrogenase ADH-'A' from *Rhodococcus ruber* via hydrogen transfer. Two different catalyst preparations were employed, namely recombinant ADH-'A' 'immobilised' in *Escherichia coli* and partially purified ADH-'A'. For biphasic systems conversions were acceptable until 20% v $v^{-1}$ of IL. In contrast, hydroxy-functionalised 'Tris-like'-ILs were successfully employed in monophasic systems up to 90% v $v^{-1}$ IL. The use of these solvents allowed highly stereoselective enzymatic carbonyl reductions at substrate concentrations from 1.2 to 1.5 M.


## 1. Introduction

Biocatalysis applied to industrial processes has been shown to be a very advantageous alternative to conventional chemical methods, and is nowadays widely used for the preparation of enantiomerically pure pharmaceuticals and other compounds of interest.[1] Its applicability increased when it was noticed that enzymes could also work in non-aqueous media such as organic solvents, which allows us to solubilise the mostly highly hydrophobic substrates not soluble in water.[2] Although organic solvents are extensively employed, they suffer from severe drawbacks such as their toxicity to the environment.

In this sense, ionic liquids have recently emerged as a promising new class of biocompatible solvents,[3] although it is still a matter of discussion whether all can be considered as 'green', due to their non-volatility and non-flammable character. Nevertheless, they have shown both high thermal and chemical stability. Furthermore, their application in biocatalytic reactions led to transformations displaying higher regio-[4] or enantioselectivity.[5]

There are only a few examples concerning the use of alcohol dehydrogenases (ADHs)[6] with ionic liquids. The first report is from Howarth et al.[7] describing immobilised Baker's yeast in a mixture at [BMIM]$PF_6$/water. Later, Kragl et al. showed that by employing an ionic liquid [BMIM][$(CF_3SO_2)_2N$] immiscible with water, bioreductions catalysed by the ADH from *Lactobacillus brevis* were shifted to the product-side due to favourable partition coefficients.[8] In the same year, two ADHs—one for recycling of the cofactor and one for the desired oxidation—were combined in ionic liquids.[9] Furthermore, it has been demonstrated that ionic liquids are suitable solvents for performing bioreductions using whole cell systems, like with *Lactobacillus kefir* or *Saccharomyces cerevisiae*, although IL concentrations have never been higher than 20% v $v^{-1}$.[10] Recently, Matsuda et al. have used immobilised *Geotrichum candidum* on a water-absorbing polymer to reduce several ketones employing different ionic liquids and 2-propanol for cofactor recycling.[11] Surprisingly, only a very few isolated ADHs are able to work according to the 'coupled-substrate' approach (Scheme 1), thus employing 2-propanol for cofactor recycling using a single enzyme.[12]

Recently, we identified an alcohol dehydrogenase from *Rhodococcus ruber* DSM 44541 named ADH-'A', which catalyses the reduction of the desired ketone as well as the recycling of the cofactor, thus working according to

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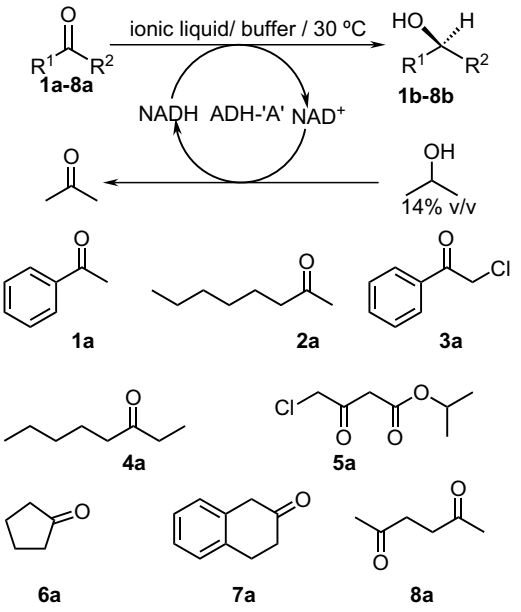
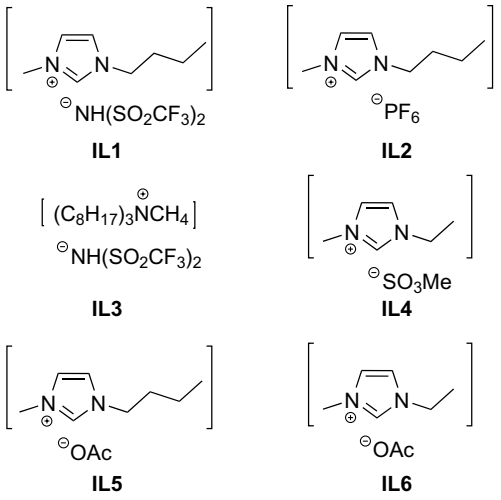



**Scheme 1.** Ionic liquid/buffer as reaction medium for the 'coupled-substrate' approach in the ADH-'A' catalysed reduction of ketones **1a**–**8a**.




**Scheme 2.** Ionic liquids employed in the biocatalytic reduction of ketones catalysed by ADH-'A'.

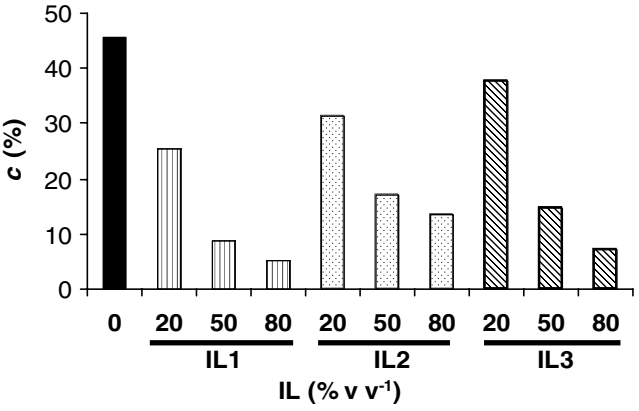



**Figure 1.** Reduction of **1a** ($t = 2$ h) using *E. coli*/ADH-'A' and different proportions of the water-immiscible ionic liquids (**IL1**–**IL3**).

the 'coupled-substrate' approach.[13] This enzyme, highly stable in organic solvents, that is, up to 50% v $v^{-1}$ of acetone or 80% v $v^{-1}$ of 2-propanol, can efficiently be over-expressed in *Escherichia coli* cells,[14] and is now commercially available from Biocatalytics Inc./Codexis.

Herein we report the application of commercial second-generation functionalised ionic liquids, completely miscible with water, for the biocatalytic hydrogen transfer employing ADH-'A' from *R. ruber*.

## 2. Results and discussion

### 2.1. Use of conventional ionic liquids

Three first-generation water-immiscible ionic liquids **IL1**–**IL3** (Scheme 2), which have previously been described to be tolerated by ADHs,[8] were employed in a two phase system with a Tris-buffer. In a typical experiment *E. coli* cells with over-expressed ADH-'A' (*E. coli*/ADH-'A') were employed for the reduction of acetophenone **1a** in the presence of 2-propanol for the recycling of the nicotinamide cofactor (NADH).

*E. coli*/ADH-'A' catalysed the reduction in biphasic systems even at high concentration of IL (80% v $v^{-1}$) leading in all cases to enantiopure (*S*)-1-phenylethanol [(*S*)-**1b**]. The reaction slows down with an increasing amount of IL (Fig. 1) probably due to the deactivation of the enzyme, as well as the lower availability of the substrate in the aqueous phase. Furthermore, the water-immiscible ionic liquids caused severe problems during work-up, since the solvent used for extraction (ethyl acetate) was (at least partially) miscible with the IL leading to difficulties in the subsequent GC analysis. Employing other organic solvents for extraction from the water/IL mixture such as diethyl ether or *n*-hexane did not lead to any improvements.

Trying to avoid these problems, water-miscible and organic solvent-immiscible ionic liquids were investigated. For this purpose, **IL4**–**IL6** (Scheme 1) were tested at different concentrations in a one-phase reaction system for the reduction of acetophenone **1a** catalysed by *E. coli*/ADH-'A' (Fig. 2).

Good conversions were only achieved with **IL5** and **IL6** at low IL concentration (20% v $v^{-1}$). Comparing the results obtained for **IL4** and **IL6**, which differ only in the counter-anion, it can be concluded that the anion acetate **IL6** shows higher compatibility for the enzyme than the anion methanesulfonate **IL4**, since significantly higher conversion was achieved with **IL6** than with **IL4** at 20% v $v^{-1}$.

### 2.2. Hydroxy-functionalised ionic liquids

Since water and, therefore, a protic environment capable of forming OH-bridges is preferred by enzymes, we searched

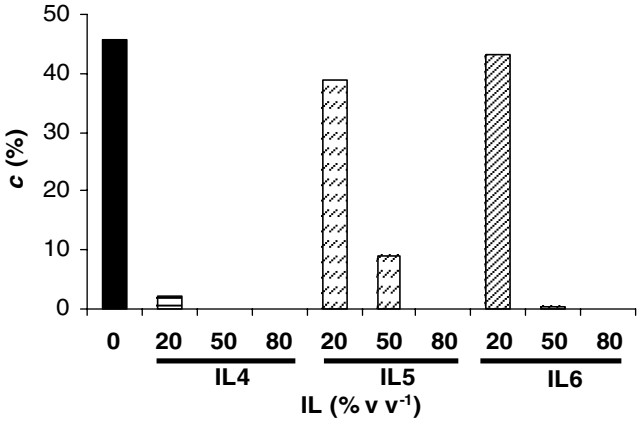
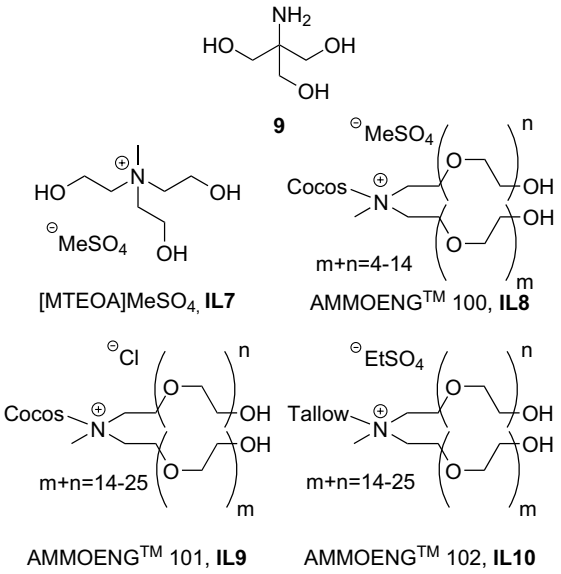



**Figure 2.** Reduction of **1a** ($t = 2$ h) using *E. coli*/ADH-'A' and different proportions of the water-miscible ionic liquids (**IL4**–**IL6**).

for ionic liquids with hydroxy and ether functionalities. A buffer-salt which is commonly employed in enzymatic reactions is TRIS [tris(hydromethyl) aminomethane **9**, Scheme 3] possessing three hydroxy moieties. We speculated that ionic liquids mimicking this salt might also be accepted. For this purpose, tris-(2-hydroxyethyl)-methylammonium methylsulfate [MTEOA]$MeSO_4$ **IL7** and AMMOENG™ 100, 101 and 102 **IL8**–**IL10**[15] were selected as possible solvents. These ionic liquids were found to be perfectly miscible with water and immiscible with EtOAc, thus simplifying the work-up of the enzymatic reactions by extraction with EtOAc.




**Scheme 3.** Hydroxy-functionalised ionic liquids employed in the biocatalytic reduction of ketones catalysed by ADH-'A'.

Our first efforts were dedicated to finding the highest concentration for each ionic liquid at which the alcohol dehydrogenase still showed activity. For a first attempt, *E. coli*/ADH-'A' cells were employed. To our delight, the catalyst accepted rather high concentrations of the ionic liquids up to 90% v $v^{-1}$ (Table 1). Comparing the results achieved with **IL7**–**IL10** at 90% v $v^{-1}$ (Table 1, entry 6), **IL10** caused the most significant loss of activity, while by employing **IL7**, the best results were achieved. Higher concentration of each ionic liquid (99% v $v^{-1}$) led to deactivation of the biocatalyst (entry 7). Furthermore, under all conditions tested, the enzyme gave exclusively the corresponding Prelog alcohol (*S*)-**2b** in enantiopure form (>99% ee), indicating that the stereoselectivity of ADH-'A' was not altered by the ionic liquids to any extent.

**Table 1.** Conversions[a] (%) for the enzymatic reduction of **2a**[b] by *E. coli*/ADH-'A' at various concentration of different ionic liquids

| Entry | IL (% v $v^{-1}$) | IL7 | IL8 | IL9 | IL10 |
|---|---|---|---|---|---|
| 1 | 0 | 84.3 | 84.3 | 84.3 | 84.3 |
| 2 | 50 | 81.3 | 77.3 | 79.2 | 80.1 |
| 3 | 60 | 79.5 | 77.9 | 78.2 | 76.7 |
| 4 | 70 | 79.0 | 77.5 | 78.2 | 69.7 |
| 5 | 80 | 76.4 | 73.1 | 77.4 | 67.1 |
| 6 | 90 | 65.3 | 27.0 | 28.3 | 7.5 |
| 7 | 99 | ⩽1.0 | ⩽1.0 | ⩽1.0 | ⩽1.0 |

[a] Measured by GC.
[b] [**2a**] = 22.8 g $L^{-1}$.

Since the mixtures of IL/buffer at 90% v $v^{-1}$ IL were rather viscous, possible limitations due to inefficient mixing were suspected. In order to overcome this limitation, enzymatic reductions were performed at 37 °C instead of 30 °C with 90% v $v^{-1}$ IL. Indeed, significantly higher conversions were achieved after 24 h: 67.1% for **IL8** (149% increase); 41.6% for **IL9** (47% increase); and 27.4% for **IL10** (265% increase). Since we have previously observed that in a buffer this enzyme does not display higher activity at 37 °C compared to 30 °C,[16] this effect must be attributed to the change of viscosity of the mixtures. Anyway, performing the experiments with the 'micro-aqueous' systems (99% v $v^{-1}$ **IL7**–**IL10**) at 37 °C did not lead to an increase in activity, indicating that more than 1% v $v^{-1}$ aqueous buffer solution was necessary. We have measured the viscosity at 30 °C and 37 °C of all of these different mixtures, but no clear correlation with the conversion pattern was observed. Although viscosity is probably one of the parameters that influences the activity of the process, it is not the main one at 1% v $v^{-1}$ water concentration.

Since the best results were obtained with **IL7**, the time course of the bioreduction of **2a** catalysed by *E. coli*/ADH-'A' at two different concentrations was studied in more detail. Employing 40% v $v^{-1}$ of **IL7,** the reduction was almost equally fast as in a buffer leading to conversions close to 80% in less than 1 h (Fig. 3). At 80% v $v^{-1}$ IL, the apparent initial rate was still approximately 60% of the activity at 40% v $v^{-1}$ IL.

Keeping the concentration of the ionic liquid constant at 90% v $v^{-1}$, the reduction at varied concentrations of 2-octanone **2a** revealed (Fig. 4), that although the conversions decreased with increasing substrate concentration, the actual space time yield (expressed as g of **2b** formed per L of solution per h) increased as a function of ketone concentration, reaching a maximum at 1.2–1.5

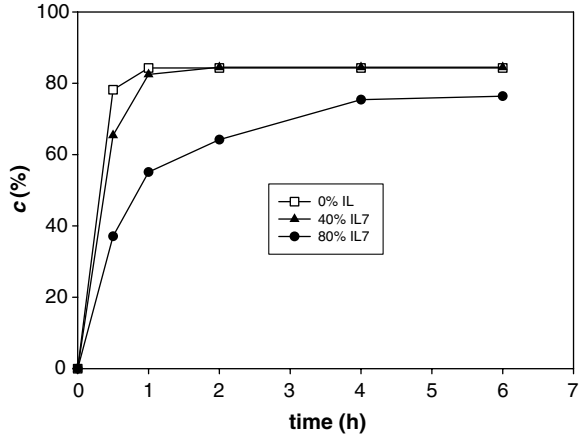



**Figure 3.** Time course of the enzymatic reduction of **2a** catalysed by *E. coli*/ADH-'A' in: (i) buffer (□); (ii) 40% v $v^{-1}$ **IL7** (▲); and (iii) 80% v $v^{-1}$ **IL7** (●), 30 °C.

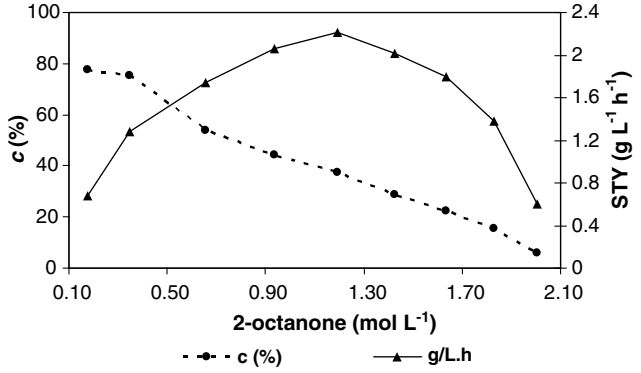



**Figure 4.** Conversion (●) and space time yield (▲, STY) of the reduction at varied **2a** concentration catalysed by *E. coli*/ADH-'A' in 90% v $v^{-1}$ **IL7**, 26 h, 30 °C.

M (154–193 g $L^{-1}$). Even at ketone concentrations close to 2.0 M, a certain amount of enzyme activity was still observed. Again, at all substrate concentrations investigated, the reduction of 2-octanone led to enantiopure (*S*)-**2b** (ee >99%).

To obtain a clearer profile of the activity of the enzyme at various concentrations of **IL7**–**IL10**, the conversion of the reduction was measured after a shorter period of time (4 h) employing a crude preparation of ADH-'A' (1.1 units). As shown in Figure 5, all four ionic liquids can be used up to 50% v $v^{-1}$ IL showing only a negligible (**IL7**, **IL9**) or just a moderately diminished conversion (**IL8**, **IL10**) under the reaction conditions employed. At concentrations above 50%, clear differences were observed. **IL10** led to a faster decrease of activity with an increasing amount of IL compared to **IL7**–**IL9**. The best suitable ionic liquid again proved to be **IL7**, in which the catalyst retained its best activity until 70% v $v^{-1}$. Nevertheless, at 80% v $v^{-1}$ of **IL7** still 80% of apparent residual activity was measured. Up to concentrations close to 90% v $v^{-1}$, **IL7**, was superior to **IL9**, which was better than **IL8**. At 90% v $v^{-1}$, the three ILs **IL7**–**IL9** reached comparable conversions (40% within

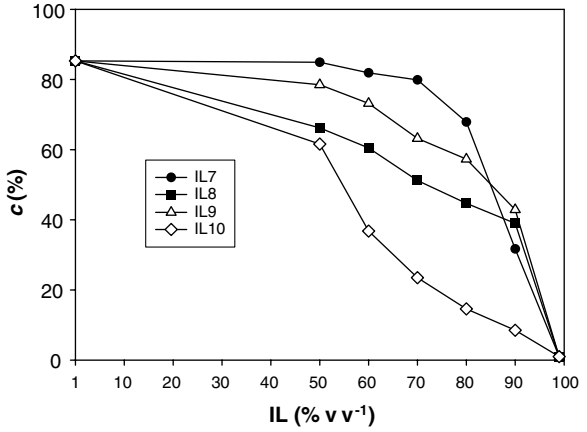



**Figure 5.** Conversion at varied concentrations of different ionic liquids for the reduction of **2a** catalysed by crude ADH-'A' ($t = 4$ h).

4 h). Summarising, the ionic liquids **IL7**–**IL9** can be employed until a concentration of 90% v $v^{-1}$.

Finally, the application of these novel reaction media for the biocatalytic reduction was extended to further ketones, as shown in Table 2. Performing the reductions in 90% v $v^{-1}$ **IL7**, ketones **1a**, **3a**–**5a** and **7a** were reduced to the corresponding enantiopure Prelog *sec*-alcohols **1b**, **3b**–**5b** and **7b**.

**Table 2.** ADH-'A' (crude preparation) catalysed reduction in 90% v $v^{-1}$ **IL7**/ 10% v $v^{-1}$ aqueous buffer

| Ketone[a] | Units | Time (h) | $c^{c}$ (%) | ee (%) |
|---|---|---|---|---|
| **1a** | 1.1 | 24 | 21.6 | >99 (*S*) |
| **3a** | 1.1 | 42 | 13.8 | >99 (*R*)[d] |
| **4a** | 3.3 | 24 | 10.4 | >99 (*S*) |
| **5a** | 1.1 | 30 | 19.7 | >99 (*R*)[d] |
| **6a** | 1.1 | 24 | 45.2 | n.a. |
| **7a** | 3.3 | 30 | 31.7 | >99 (*S*) |
| **8a**[b] | 2.2 | 30 | 26.1 | >99 (*S*,*S*) |

n.a. not applicable.
[a] [Ketone] = 11.4 g $L^{-1}$ except for **4a**, **7a** and **8a** for which the substrate concentration is 22.8 g $L^{-1}$.
[b] No reduction intermediate (hydroxy ketone **8c**) was observed.
[c] Measured by GC.
[d] Change in CIP-priority.

When diketone **8a** was employed as a substrate, the diol was found without traces of the hydroxy ketone; thus, enantiopure (2*S*,5*S*)-hexane diol **8b** was exclusively obtained.

## 3. Conclusions

We have shown that the recently overexpressed[14] organic solvent-tolerant and now commercially available ADH-'A' can successfully be employed in non-conventional one-phase aqueous-ionic liquid solvent systems for the highly enantioselective reduction of a broad set of ketones. Most of the literature examples previously reported employed water-immiscible ionic liquids. ADH-'A' from *R.*

*ruber* proved to be ideal to work in water miscible monophasic media composed of 80–90% v $v^{-1}$ of IL. Furthermore, these systems allow us to employ high substrate concentrations of 1.2–1.5 M. The hydroxy functionalised 'Tris-like' ionic liquid [MTEOA]$MeSO_4$ **IL7** was shown to be highly suited as a co-solvent even at 90% v $v^{-1}$. The applicability of redox-enzymes in such ionic solvents will enable the transformation of water-immiscible substrates, which could otherwise not be accepted.

## 4. Experimental

### 4.1. General

Ketones **1a**–**8a**, alcohol **6b**, racemic alcohols **1b**–**4b** and **7b**, diol **8b** and hydroxyketone **8c** were commercially available either from Sigma–Aldrich–Fluka (Vienna, Austria) or Lancaster (Frankfurt am Main, Germany). Compound **5b** was synthesised by reduction from the corresponding ketone **5a** ($NaBH_4$, MeOH, 5 °C).[17] **IL1**, **IL2**, **IL3**, AMMOENG™ 100, 101 and 102 (**IL8**–**IL10**) were available from Solvent Innovation (Köln, Germany). **IL4**, **IL5**, **IL6** and tris-(2-hydroxyethyl)-methylammonium methylsulfate **IL7** were products from Fluka. All other reagents and solvents were of the highest quality available. TLC plates were run on silica gel Merck 60 $F_{254}$ and visualised by UV or by spraying with a $KMnO_4$ solution.

The preparation of the lyophilised cells[14] and partially purified ADH-'A'[18] has previously been described. ADH-'A' is commercially available from BioCatalytics Inc.

The absolute configurations of the *sec*-alcohols **1b**–**5b**, **7b** and diol **8b** were determined by either (i) comparison of elution order on GC with published data,[18–20] or by (ii) co-injection with commercially available material or independently synthesised chiral reference material.

### 4.2. Experimental procedures

**4.2.1. General method for the biocatalytic reduction of ketones employing *E. coli*/ADH-'A' in buffer/ionic liquid media.** Lyophilised cells of *E. coli* Tuner™ (DE3)/pET22b + -ADH-'A' (5–15 mg), stored at 4 °C, were rehydrated in the corresponding volume of Tris/HCl buffer (50 mM, pH 7.5, 1.0 mM NADH) for 30 min at 30 °C and 120 rpm on a rotatory shaker in an Eppendorf vial (1.5 mL). The ionic liquid (final volume: 0.6 mL), 2-propanol (97 μL, 14% v $v^{-1}$) and the ketone (10–20 μL) were then added. The mixtures were shaken at 30 °C and 120 rpm for the time given and stopped by extraction with diethyl ether or ethyl acetate (2 × 0.5 mL). The organic layer was separated from the cells by centrifugation (2 min, 13,000 rpm) and dried over $Na_2SO_4$. Conversions and enantiomeric excesses of the corresponding alcohols were determined by GC analysis.

**4.2.2. Typical procedure for the enzymatic reduction of 2a in 99% IL7 catalysed by *E. coli*/ADH-'A'.** Lyophilised cells of *E. coli*/ADH-'A' (5 mg), stored at 4 °C, were rehydrated in 6 μL of a 100 mM NADH solution (pH 7.5) for 30 min at 30 °C or 37 °C and 120 rpm on a rotatory shaker in an Eppendorf vial (1.5 mL). **IL7** (0.6 mL), 2-propanol (97 μL, 14% v $v^{-1}$) and ketone **2a** (20 μL) were added. The reaction was shaken at 30 °C and 120 rpm for 24 h and stopped by extraction with ethyl acetate (2 × 0.5 mL). The organic layer was separated by centrifugation (2 min, 13,000 rpm) and dried over $Na_2SO_4$. Conversion and enantiomeric excess of **2b** were determined by GC analysis.

**4.2.3. Temperature effect on the reduction of 2-octanone 2a in IL7-IL10 catalysed by *E. coli*/ADH-'A'.** Lyophilised cells of *E. coli* Tuner™ (DE3)/pET22b + -ADH-'A' (5–15 mg), stored at 4 °C, were rehydrated in 60 μL Tris/HCl buffer (50 mM, pH 7.5, 10 mM NADH) for 30 min at 30 °C or 37 °C and 120 rpm on a rotatory shaker in an Eppendorf vial (1.5 mL). The ionic liquid (540 μL, 90% v $v^{-1}$), 2-propanol (97 μL, 14% v $v^{-1}$) and **2a** (20 μL) were added. The mixtures were shaken at 30 °C or 37 °C and 120 rpm for 24 h and stopped by extraction with ethyl acetate (2 × 0.5 mL). The organic layer was separated from the cells by centrifugation (2 min, 13,000 rpm) and dried over $Na_2SO_4$. Conversion and enantiomeric excess of **2b** were determined by GC analysis.

**4.2.4. Typical method for the biocatalysed reduction of ketones employing partially purified ADH-'A' in mixtures of buffer/ionic liquid IL7–IL10.** Unless otherwise stated, the corresponding ionic liquid (final reaction volume of 0.6 mL), 2-propanol (97 μL, 14% v $v^{-1}$) and substrates **1a**–**8a** (10–20 μL) were added to a solution of partially purified ADH-'A' (50–150 μL, 1.1–3.3 units) in Tris/HCl buffer (50 mM, pH 7.5, 1 mM NADH). The reactions were shaken at 30 °C and 120 rpm for the appropriate time. The mixture was then extracted with ethyl acetate (2 × 0.5 mL). The organic layer was dried over $Na_2SO_4$ and analysed by GC in order to determine the conversions and enantiomeric excesses of the corresponding alcohols **1b**–**8b**.

**4.2.5. Procedure for the reduction of 2-octanone 2a catalysed by partially purified ADH-'A' in 99% IL7.** Partial purified ADH-'A' preparation in Tris/HCl buffer (1.1 units, 50 μL) was lyophilised. The enzyme was rehydrated in 6 μL of a 100 mM NADH solution (pH 7.5) for 30 min at 30 °C and 120 rpm on a rotary shaker in an Eppendorf vial (1.5 mL). **IL7** (0.6 mL), 2-propanol (97 μL, 14% v $v^{-1}$) and **2a** (10 μL) were added to the cells and the reaction was shaken at 30 °C and 120 rpm for 24 h. The mixture was extracted with ethyl acetate (2 × 0.5 mL) and the organic layer was dried over $Na_2SO_4$. Conversion and enantiomeric excess of **2b** were determined by GC analysis.

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