Topics in Catalysis

Imidazolium-based ionic liquids as non-conventional media for alcohol dehydrogenase-catalysed reactions

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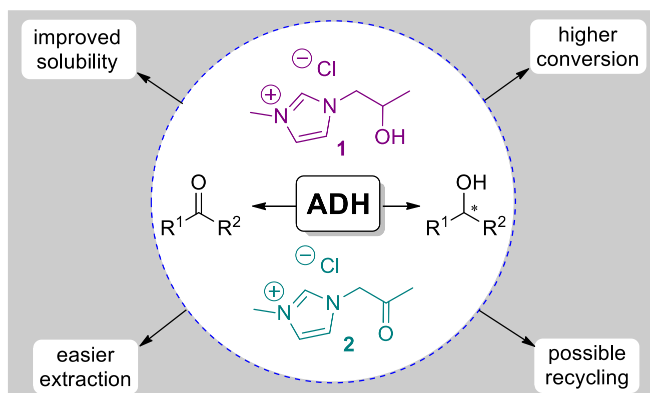


Abstract

Imidazolium-based ionic liquids were designed and synthesised for their use as non-conventional media in alcohol dehydrogenase (ADH)-catalysed reactions. Screenings with several ADHs and various ketone or alcohol substrates for their selective reductions or oxidations, respectively, showed that when containing up to 50% of the ionic liquid the overall conversion of the reactions could be improved in some cases, while the stereoselectivity of the enzyme remained unaltered. Attempts at using these ionic liquids as co-substrates for the recycling of the nicotinamide cofactor led to promising results, opening a new possibility for a substrate-coupled recycling system.

Keywords Alcohol dehydrogenase, Enzyme catalysis, Ionic liquids, Non-conventional media, Secondary alcohols,

1 Introduction

Enzyme-catalysed redox reactions have gained major attention in recent years for the production of enantiopure compounds, whether used for pharmaceutical purposes or in industrial processes, due to the high selectivity, mild reaction conditions and lower environmental impact displayed with oxidoreductases [1]. In particular, alcohol dehydrogenases (ADHs, NAD-dependent EC 1.1.1.1 and NADP-dependent EC 1.1.1.2) perform stereoselective carbonyl reductions and enantioselective alcohol oxidations [2−8]. Enantiopure chiral secondary alcohols are commonly used in the pharmaceutical and food industry as well as for flavour, fragrances and liquid crystals [9-10]. These high added-value compounds can be synthesised by the asymmetric reduction of the corresponding prochiral ketones using several methods, thus affording a 100% theoretical yield of the enantiopure product. Metal-catalysed hydrogenation [11], hydrosilylation [12], and hydrogen-transfer (Meerwein-Ponndorf-Verley reduction) [13−15] reactions are the most commonly used strategies to selectively and catalytically reduce ketones. On the other hand oxidation processes can also provide the enantiopure alcohols, albeit with a maximum yield of 50% through kinetic resolution. In this context, the metal-catalysed Oppenauer oxidation can be seen as a complementary methodology to the ADH protocols. Nevertheless, these

chemical routes still present several drawbacks mainly emerging from an environmental point of view with the presence of toxic reagents and harsh reaction conditions. Alternatively, the use of ADHs to perform redox reactions, which has been extensively studied in past years [1−8], generally appears as a greener synthetic pathway, thus giving rise to valuable industrial applications [16−19].

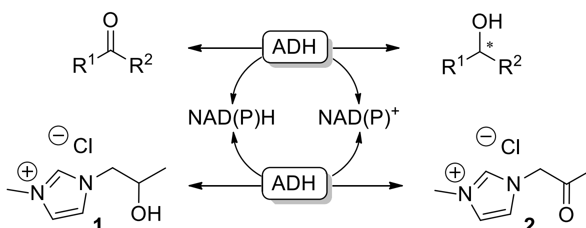
ADHs catalyse redox reactions through a well-known mechanism involving the nicotinamide cofactor NAD(P)H. Common challenges encountered with their use include substrate solubility, product recovery and costly NAD(P)H unless a regeneration system is employed. In principle, an ideal cofactor recycling method should meet the following criteria: (i) stable and inexpensive enzymes, (ii) high specific enzymatic activity, (iii) simple and inexpensive reagents that do not interfere with the isolation of the desired product or with the stability of the enzyme, (iv) high turnover number (TON), and (v) a reachable favourable equilibrium for product formation [20]. The most important strategies that have been developed for the *in situ* regeneration of nicotinamide cofactors with ADHs are: (i) using another enzyme or co-substrate in an "enzyme-coupled" or "substrate-coupled" system, (ii) engineering whole cells to overexpress both the ADH and the cofactor regenerating enzyme, (iii) electrochemical, and (iv) chemical [21−26]. Among all these methodologies, the "substrate-coupled" system is highly convenient for economic reasons. In this case, the same enzyme transforms the substrate of interest and recycles the cofactor at the expense of a cheap co-substrate such as 2-propanol or acetone. Due to the reversibility of ADH-catalysed reactions, a large molar excess of the co-substrate is often added to shift the equilibrium.

2 Ionic liquids in ADH-catalysed reactions

Ionic liquids (ILs) have been increasingly used in homogeneous, heterogeneous and biocatalysis [27]. The use of ILs in biotransformations is still expanding to enzymes such as oxidoreductases [28−34]. In this manner, to increase the solubility of hydrophobic substrates and improve the biocatalytic yield by minimising the substrate or product inhibition, organic solvents and ionic liquids can be used: (i) as co-solvents with the aqueous medium, (ii) as a second phase in a biphasic system or (iii) alone as non-aqueous solvents (e.g. with lipases). However, organic solvents and ionic liquids can inhibit or inactivate the enzyme at high concentrations. Mainly, biphasic systems have been applied with oxidoreductases [35]. Thus, the organic phase contains the hydrophobic substrates, whereas the aqueous phase contains the enzyme(s) and cofactor(s). Notwithstanding, adding organic solvents or ILs (imidazolium or amino-based) can sometimes increase the enzyme activity, stability and stereoselectivity in specific examples with certain enzymes [36−38].

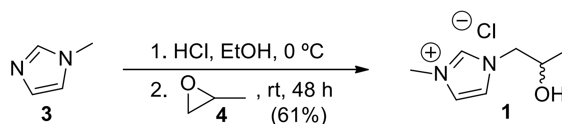
For instance, an extensive study has been performed on the stability of ADH-A (from *Rhodococcus ruber*) with various amounts of IL, demonstrating that ADH-A can in fact resist high concentrations of IL and afford reasonable conversions, especially with those that are hydroxy-functionalised [36]. Additionally, LBADH (from *Lactobacillus brevis*) has been used in the presence of several water-miscible ILs to reduce hardly water-soluble ketones such as 2-nonanone and 2-decanone, and also with an IL in a two-phase system to efficiently achieve the bioreduction of 2-octanone due to favourable partition coefficients of the co-substrate (2-propanol) utilised and the co-product (acetone) obtained [39,40]. More importantly, the use of these co-solvents has led to the development of efficient systems to obtain high added-value compounds in a selective manner [41−43].

In this study we envisioned the regeneration of the nicotinamide cofactors in ADH-catalysed redox reactions using imidazolium-based ILs as non-conventional media and also as co-substrates. As previous results have shown that ADHs can be highly stable with hydroxy-functionalised ILs [36], the synthesis of water-miscible hydroxylated imidazole-based ILs was envisaged to use them as: (i) an appropriate co-solvent for the enzymatic reactions, allowing the use of higher substrate concentrations and a simpler recovery of the final products from the reaction mixture through extraction, and (ii) as a possible co-substrate for the ADH-catalysed transformations, allowing its reuse over several cycles and avoiding the employment or formation of 2-propanol that could interfere in the purification of interesting volatile products (Scheme 1).



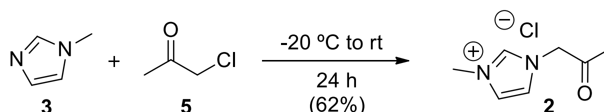
Scheme 1 Proposed ILs for a nicotinamide cofactor recycling system in ADH-catalysed reactions

Therefore, 1-(2-hydroxypropyl)-3-methylimidazolium chloride (IL **1**) and 3-methyl-1-(2-oxopropyl)imidazolium chloride (IL **2**) were chosen to achieve these goals. In a first set of experiments, 2-propanol or acetone were used as co-substrates and the IL as an additive to observe whether the latter affected the enzymatic reaction outcome. These ILs were also chosen based on their straightforward synthesis without purification. Additionally, the chloride anion is (i) smaller than a bromide, (ii) makes the IL more soluble in an aqueous medium such as buffer, contrarily to other anions (e.g. NTf_2, BF_4), and (iii) provides a stable IL, as opposed to other anions (e.g. PF_6). IL **1** was simply obtained from commercially available *N*-methyl imidazole (**3**) with hydrochloric acid in ethanol at 0 ºC and propylene oxide (**4**, Scheme 2) [44]. The product was obtained pure after several washes with diethyl ether to remove the excess of **3**.



Scheme 2 One-pot two-step synthesis of racemic IL **1**

IL **2** was obtained through the nucleophilic substitution of freshly distilled chloroacetone (**5**) with **3** at -20 ºC (Scheme 3) [45]. Again, the only purification required was an extraction with diethyl ether to remove any excess of **3**.

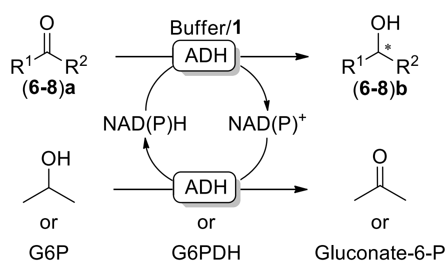


Scheme 3 Synthesis of IL **2**

3 ADH-catalysed bioreductions with IL 1

Once synthesised, the effect of these ILs was studied in several ADH-catalysed bioreduction processes with commercially available and overexpressed enzymes. The following enzymes were used in this study: ADH-A from *Rhodococcus ruber*, LBADH from *Lactobacillus brevis*, LKADH from *Lactobacillus kefir*, RasADH from *Ralstonia* sp., SyADH from *Sphingobium yanoikuyae*, TeSADH from *Thermoanaerobacter ethanolicus*, ADH-T from *Thermoanaerobium* sp. and CPADH from *Candida parapsilosis* (see Supporting Information for more details), and for each of them a model substrate was used in order to understand the influence of the ILs in the enzyme reactivity. Data are collected in Table 1.

Table 1 ADH-catalysed bioreductions of ketones (**6−8**)**a** employing racemic IL **1** as additive[a]



6a R^1 = Ph, R^2 = CH_3 **6b** R^1 = Ph, R^2 = CH_3

7a R^1 = Ph, R^2 = CH_2CH_3 **7b** R^1 = Ph, R^2 = CH_2CH_3

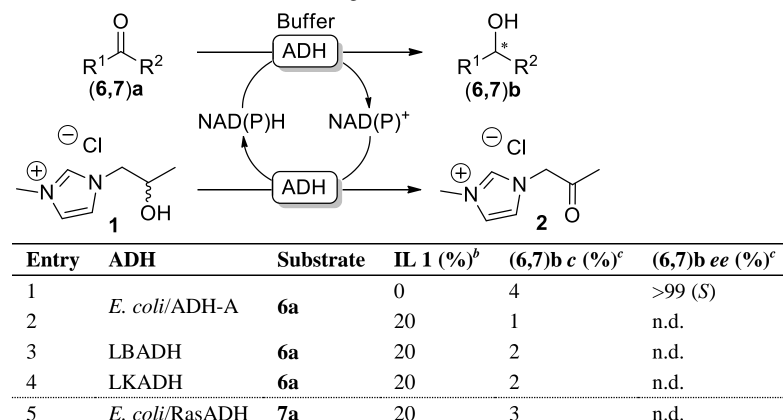
8a R^1 = $(CH_2)_5CH_3$, R^2 = CH_3 **8b** R^1 = $(CH_2)_5CH_3$, R^2 = CH_3

Entry	ADH	Substrate	IL 1 (%)[b]	(6−8)b *c* (%)[c]	(6-8)b *ee* (%)[c]
1			0	76	>99 (*S*)
2			10	80	>99 (*S*)
3	*E. coli*/ADH-A	**6a**	20	83	>99 (*S*)
4			50	87	>99 (*S*)
5			90	1	n.d.
6			0	75	>99 (*S*)
7	ADH-A	**6a**	10	87	>99 (*S*)
8			20	91	>99 (*S*)
9			50	87	>99 (*S*)
10			0	86	>99 (*R*)
11			10	85	>99 (*R*)
12	LBADH	**6a**	20	89	>99 (*R*)
13			50	74	>99 (*R*)
14			90	4	>99 (*R*)
15			0	78	>99 (*R*)
16			10	72	>99 (*R*)
17	LKADH	**6a**	20	>99	>99 (*R*)
18			50	79	>99 (*R*)
19			90	<1	n.d.
20			0	77	>99 (*S*)
21			10	79	>99 (*S*)
22	*E. coli*/RasADH	**7a**	20	99	>99 (*S*)
23			50	86	>99 (*S*)
24			90	<1	n.d.
25			0	80	>99 (*S*)
26	*E. coli*/SyADH	**7a**	10	22	>99 (*S*)
27			20	3	>99 (*S*)
28			50	1	n.d.
29			0	81	>99 (*S*)
30	*E. coli*/TeSADH	**8a**	10	89	>99 (*S*)
31			20	58	>99 (*S*)
32			50	30	>99 (*S*)
33			0	83	>99 (*S*)
34	*E. coli*/ADH-T	**8a**	10	80	>99 (*S*)
35			20	36	>99 (*S*)
36			50	27	>99 (*S*)
37			0	92	>99 (*S*)
38	CPADH	**8a**	10	76	>99 (*S*)
39			20	58	>99 (*S*)
40			50	34	>99 (*S*)

[a] Reaction conditions: [substrate] = 40 mM, lyophilised cells of *E. coli*/ADH (20 mg) or 3 U of commercial ADH, Tris-HCl buffer [50 mM, pH 7.5 (final volume = 0.6 mL), 1 mM NAD(P)H] and IL **1**, 2-propanol (30 μL, 5% v/v) or glucose-6-phosphate (80 mM) and G6PDH (5 U), shaken at 250 rpm at 30 ºC for 24 h. [b] IL was added in % w/v. [c] Measured by GC; absolute configuration in parenthesis; n.d. = not determined

Pleasingly, in several cases, the conversions of the ADH-catalysed bioreduction reactions displayed higher values when increasing the percentage of IL **1** up to 20% w/v (entries 3, 8, 12, 17 and 22) and even at 50% w/v (entries 4, 9, 18 and 23). The best results were obtained with *E. coli*/ADH-A at 50% w/v (87%, entry 4), ADH-A at 50% w/v (87%, entry 9), LBADH at 20% w/v (89%, entry 12), LKADH at 20% w/v (>99%, entry 17) and *E. coli*/RasADH at 20% w/v (99%, entry 22) of IL **1**. The other ADHs, especially *E. coli*/SyADH, fared less well, negatively affected by the presence of this IL. Pleasingly, the excellent stereoselectivities shown by all the biocatalysts in these processes remained unchanged even at high concentrations of IL **1**. Next, the reactions affording the best results were run in the absence of isopropanol or G6P/G6PDH, therefore with IL **1** as the sole potential hydrogen donor for NAD(P)H recycling (Table 2).

Table 2 Bioreductions with ADHs using racemic IL **1** as co-substrate and 1 mM of the corresponding cofactor[a]

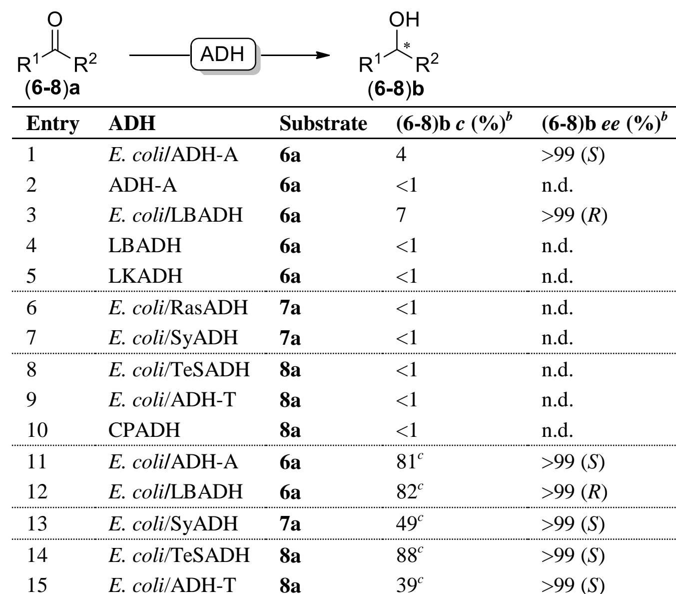


Entry	ADH	Substrate	IL 1 (%)[b]	(6,7)b *c* (%)[c]	(6,7)b *ee* (%)[c]
1	*E. coli*/ADH-A	**6a**	0	4	>99 (*S*)
2			20	1	n.d.
3	LBADH	**6a**	20	2	n.d.
4	LKADH	**6a**	20	2	n.d.
5	*E. coli*/RasADH	**7a**	20	3	n.d.

[a] Reaction conditions: [substrate] = 40 mM, lyophilised cells of *E. coli*/ADH (20 mg) or 3 U of commercial ADH, Tris-HCl buffer [50 mM, pH 7.5 (final volume = 0.6 mL), 1 mM NAD(P)H] and IL **1**, shaken at 250 rpm at 30 °C for 24 h. [b] IL was added in % w/v. [c] Measured by GC; n.d. = not determined.

Unfortunately, IL **1** was not suitable as a co-substrate for these ADHs to recycle NAD(P)H, affording close to no conversion (entries 2 to 5). Nevertheless, in the blank reaction without IL, 4% of the alcohol was obtained (entry 1). This value can be explained by the added cofactor (1 mM) in combination with the known presence of NAD(P)H in the *E. coli* cells, thus allowing the reduction reaction. This observation was confirmed when running the reactions without the addition of any external nicotinamide cofactor (Table 3, entries 1 and 3). Indeed, with the addition of a proper hydrogen donor source such as 2-propanol (entries 11 to 15), high conversions of the enantiopure products could be attained without any additional external nicotinamide cofactor.

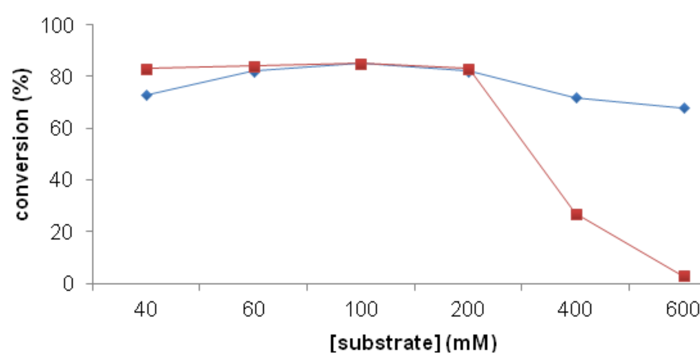
Table 3 Bioreductions without external addition of cofactor[a]



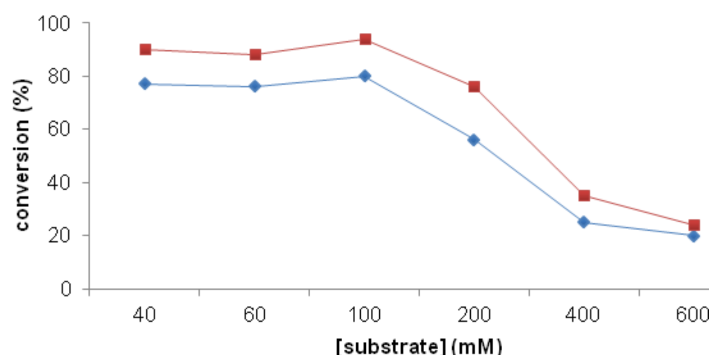
Entry	ADH	Substrate	(6-8)b *c* (%)[b]	(6-8)b *ee* (%)[b]
1	*E. coli*/ADH-A	**6a**	4	>99 (*S*)
2	ADH-A	**6a**	<1	n.d.
3	*E. coli*/LBADH	**6a**	7	>99 (*R*)
4	LBADH	**6a**	<1	n.d.
5	LKADH	**6a**	<1	n.d.
6	*E. coli*/RasADH	**7a**	<1	n.d.
7	*E. coli*/SyADH	**7a**	<1	n.d.
8	*E. coli*/TeSADH	**8a**	<1	n.d.
9	*E. coli*/ADH-T	**8a**	<1	n.d.
10	CPADH	**8a**	<1	n.d.
11	*E. coli*/ADH-A	**6a**	81[c]	>99 (*S*)
12	*E. coli*/LBADH	**6a**	82[c]	>99 (*R*)
13	*E. coli*/SyADH	**7a**	49[c]	>99 (*S*)
14	*E. coli*/TeSADH	**8a**	88[c]	>99 (*S*)
15	*E. coli*/ADH-T	**8a**	39[c]	>99 (*S*)

[a] Reaction conditions: [substrate] = 40 mM, lyophilised cells of *E. coli*/ADH (20 mg) or 3 U of commercial ADH, Tris-HCl buffer (50 mM, pH 7.5, final volume = 0.6 mL), shaken at 250 rpm at 30 ºC for 24 h. [b] Measured by GC; n.d. = not determined. [c] With 2-propanol (30 µL, 5% v/v).

With the promising results seen in Table 1 with IL **1** as co-solvent in the ADH-catalysed bioreductions, attempts to increase the substrate concentration were made, using *E. coli*/ADH-A and *E. coli*/RasADH in the presence or absence of the IL. The IL maintained or increased the conversion up to 200 mM substrate concentration with *E. coli*/ADH-A (Graph 1), whereas with *E. coli*/RasADH the IL was beneficial up to a substrate concentration of 400 mM (Graph 2).



Graph 1 *E. coli*/ADH-A-catalysed bioreduction of acetophenone (**6a**). (◆) 0% w/v IL **1**, (■) 20% w/v IL **1**

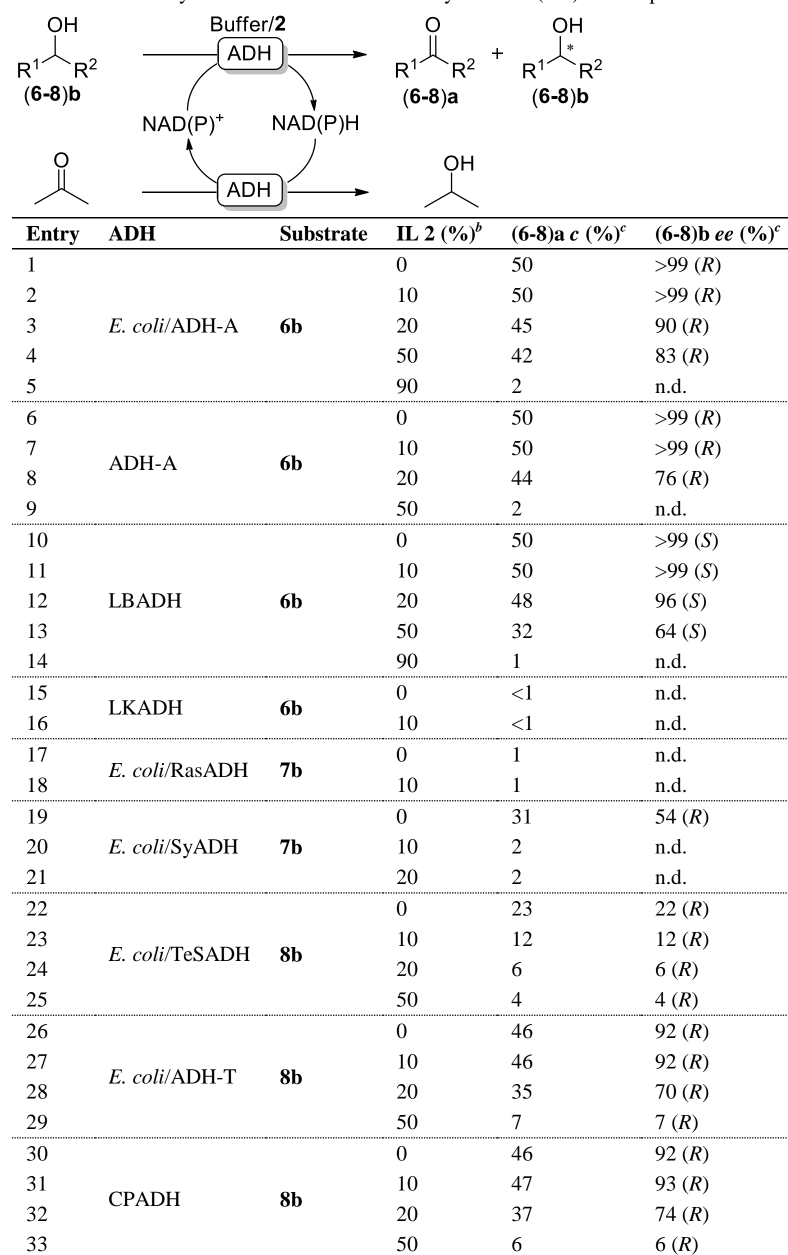


Graph 2 *E. coli*/RasADH-catalysed bioreduction of propiophenone (**7a**). (◆) 0% w/v IL **1**, (■) 20% w/v IL **1**

4 ADH-catalysed biooxidations with IL 2

Subsequently, the opposite biooxidations of various secondary alcohols were studied with several ADHs using acetone as co-substrate at varying amounts of IL **2**, first employed as co-solvent (Table 4). With a maximum conversion of 50% due to the high stereospecificity of the studied enzymes, excellent conversions were again observed with 10 and 20% w/v of IL **2** when combined with ADH-A, LBADH, ADH-T and CPADH, maintaining the excellent selectivity (entries 2, 3, 7, 8, 11, 12, 27 and 31). More importantly, although in some cases 50% w/v of this IL still gave an attractive conversion (entries 4 and 13), **2** appeared to be more toxic for the enzymes since overall lower enzymatic activities were obtained in comparison with IL **1**. LKADH and *E. coli*/RasADH are known to perform poorly with acetone as a co-substrate, and thus as expected did not afford any conversion (entries 15 to 18).

Table 4 ADH-catalysed biooxidations of secondary alcohols (**6-8**)**b** in the presence of IL **2**[a]



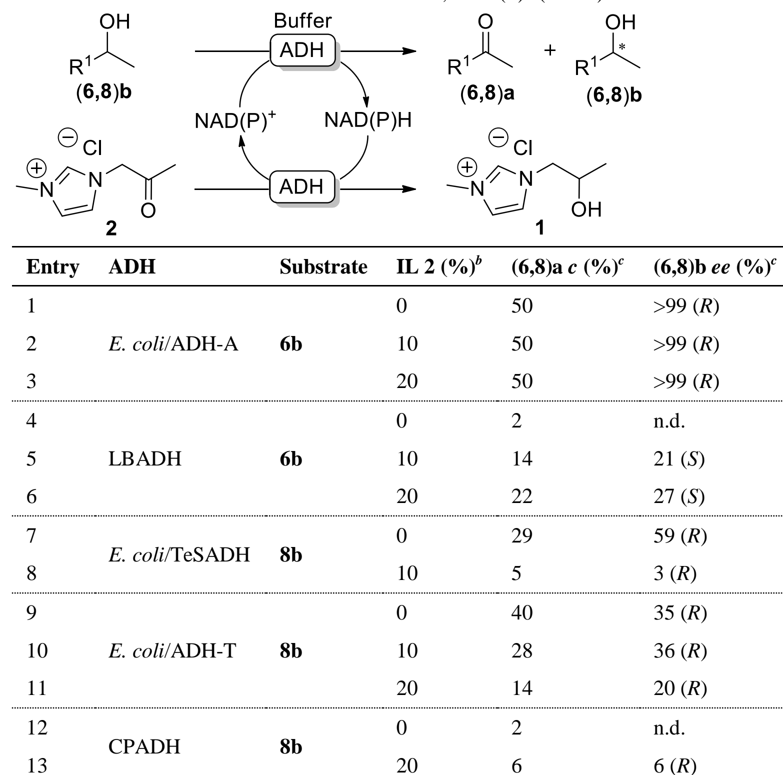
Entry	ADH	Substrate	IL 2 (%)[b]	(6-8)a *c* (%)[c]	(6-8)b *ee* (%)[c]
1	*E. coli*/ADH-A	**6b**	0	50	>99 (*R*)
2			10	50	>99 (*R*)
3			20	45	90 (*R*)
4			50	42	83 (*R*)
5			90	2	n.d.
6	ADH-A	**6b**	0	50	>99 (*R*)
7			10	50	>99 (*R*)
8			20	44	76 (*R*)
9			50	2	n.d.
10	LBADH	**6b**	0	50	>99 (*S*)
11			10	50	>99 (*S*)
12			20	48	96 (*S*)
13			50	32	64 (*S*)
14			90	1	n.d.
15	LKADH	**6b**	0	<1	n.d.
16			10	<1	n.d.
17	*E. coli*/RasADH	**7b**	0	1	n.d.
18			10	1	n.d.
19	*E. coli*/SyADH	**7b**	0	31	54 (*R*)
20			10	2	n.d.
21			20	2	n.d.
22	*E. coli*/TeSADH	**8b**	0	23	22 (*R*)
23			10	12	12 (*R*)
24			20	6	6 (*R*)
25			50	4	4 (*R*)
26	*E. coli*/ADH-T	**8b**	0	46	92 (*R*)
27			10	46	92 (*R*)
28			20	35	70 (*R*)
29			50	7	7 (*R*)
30	CPADH	**8b**	0	46	92 (*R*)
31			10	47	93 (*R*)
32			20	37	74 (*R*)
33			50	6	6 (*R*)

[a] Reaction conditions: [substrate] = 40 mM, lyophilised cells of *E. coli*/ADH (20 mg) or 3 U of commercial ADH, Tris-HCl buffer [50 mM, pH 7.5 (final volume = 0.6 mL), 1 mM NAD(P)$^+$] and IL **2**, acetone (30 μL, 5% v/v), shaken at 250 rpm at 30 ºC for 24 h. [b] IL was added in % w/v. [c] Measured by GC; n.d. = not determined

Similarly as for IL **1**, the oxidation reactions were achieved in the absence of acetone to study the role of IL **2** as co-substrate (Table 5). These results at first appeared promising, since in all cases, and especially with *E. coli*/ADH-A, moderate to excellent conversions and *ee* were obtained even at 20% w/v of IL **2**. Surprisingly, upon closer inspection, the blank biooxidation reactions with *E. coli*/ADH-A lyophilised cells without IL and acetone, also afforded 50% conversion (entry 1), providing the remaining (*R*)-1-phenylethanol (**6b**) with >99% *ee*. Consequently, the conversions obtained with the overexpressed ADHs would be biased due to the ability of the lyophilised *E. coli* cells to internally regenerate the cofactor [46]. For *E. coli*/TeSADH and *E. coli*/ADH-T, adding IL **2** actually decreased the conversion compared to the blank reactions (entries 8 and 10). The only positive result obtained using IL **2** as a hydrogen acceptor was with commercially available LBADH (entries 4 to 6). Thus, in the presence of 20% w/v of IL **2**, the reaction afforded 22% of acetophenone (**6a**) and 27% *ee* for the remaining (*S*)-1-phenylethanol (**6b**). Nonetheless, IL **2** appeared to scarcely regenerate the cofactor with CPADH

(entries 12 and 13), affording only 2% of **8b** without IL **2** in the presence of the cofactor, while obtaining 6% of conversion with 20% w/v of IL **2** present in the reaction.

Table 5 Biooxidations of *sec*-alcohols with ADHs, $NAD(P)^+$ (1 mM) and IL **2** as co-substrate[a]

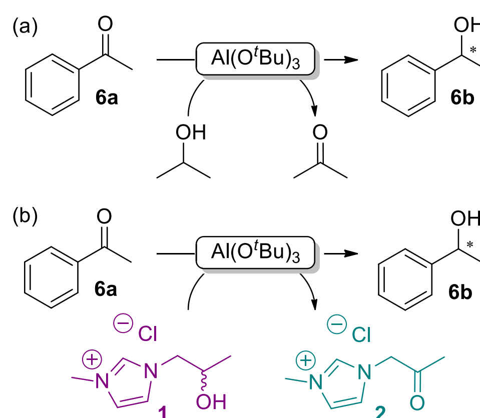


Entry	ADH	Substrate	IL 2 (%)[b]	(6,8)a *c* (%)[c]	(6,8)b *ee* (%)[c]
1			0	50	>99 (*R*)
2	*E. coli*/ADH-A	**6b**	10	50	>99 (*R*)
3			20	50	>99 (*R*)
4			0	2	n.d.
5	LBADH	**6b**	10	14	21 (*S*)
6			20	22	27 (*S*)
7	*E. coli*/TeSADH	**8b**	0	29	59 (*R*)
8			10	5	3 (*R*)
9			0	40	35 (*R*)
10	*E. coli*/ADH-T	**8b**	10	28	36 (*R*)
11			20	14	20 (*R*)
12	CPADH	**8b**	0	2	n.d.
13			20	6	6 (*R*)

[a] Reaction conditions: [substrate] = 40 mM, lyophilised cells of *E. coli*/ADH (20 mg) or 3 U of commercial ADH, Tris-HCl buffer [50 mM, pH 7.5, 1 mM $NAD(P)^+$] and IL **2** (final volume = 0.6 mL), shaken at 250 rpm at 30 ºC for 24 h. [b] IL was added in % w/v. [c] Measured by GC; n.d. = not determined

5 Metal-catalysed reduction of ketones with an ionic liquid

Because these ILs did not act as an appropriate hydrogen donor/acceptor with ADHs due to possible destabilizing interactions with the enzyme, a hydrogen transfer reaction with the aluminium *tert*-butoxide catalyst $Al(O^tBu)_3$ was envisaged (Scheme 4). Previous results with this catalyst have shown that when using 2-propanol for the reduction of ketones (Scheme 4a), or acetone for the oxidation of secondary alcohols, similar conversions to that observed with LBADH and ADH-A as biocatalysts were achieved [47].

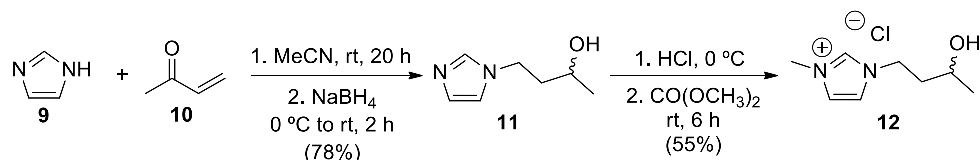


Scheme 4 Proposed metal-catalysed reduction of ketones catalysed by $Al(O^tBu)_3$ with: (a) 2-propanol, and (b) IL **1** as hydrogen donor

Hence, whereas the reduction of **6a** to **6b** using 2-propanol as co-substrate with the aluminium catalyst afforded 86% of conversion, in the same reaction conditions with IL **1** only 7% of conversion was achieved

(Scheme 4b). IL **1** was therefore not efficient to perform hydrogen transfer reactions, which would explain the poor results obtained in the ADH-catalysed reactions as well.

Due to the possible steric hindrance of the imidazole core in **1**, another IL with an additional methylene group to avoid having the imidazole ring at the α-position was envisaged as possible hydrogen donor in this aluminium-catalysed system (IL **12**, Scheme 5). As the ketone obtained from imidazole (**9**) and methyl vinyl ketone (**10**) was unstable (retro-Michael reaction), the hydroxylated derivative **11** was directly produced by *in situ* reduction of the carbonyl group with $NaBH_4$ at 0 °C with an overall yield of 78% for both steps. The imidazole ring was then methylated with hydrochloric acid and dimethylcarbonate, affording IL **12** in 55% yield.



Scheme 5 Three-step synthesis of racemic ionic liquid **12**

6 Conclusions

Imidazolium-based ionic liquids were synthesised and used, first as co-solvents, and then as co-substrates in ADH-catalysed redox reactions employing a "coupled-substrate" approach. Although these ILs did not prove to be suitable hydrogen donors/acceptors, they were successfully used in bioreduction and biooxidation reactions as co-solvents. Best results were attained in the bioreduction of prochiral ketones with a broad range of ADHs and hydroxylated IL **1** leading to comparable or higher conversions with respect to the reactions carried out in aqueous medium, even up to 50% w/v IL concentration, thus enabling the use of higher substrate loadings (up to 200 mM for ADH-A and 400 mM for RasADH) without any detrimental effect on the enzyme selectivity. Interestingly, carbonylic IL **2** could be accepted to some extent by LBADH as hydrogen acceptor for the biooxidation of 1-phenylethanol. Although still in a preliminary phase, these results are a promising starting point to obtain novel derivatives that could be better accepted by ADHs and other redox enzymes.

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