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Enzymatic ketone reductions with co-factor recycling: Improved reactions with ionic liquid co-solvents

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Abstract

Ionic liquids have been applied to two pharmaceutically relevant ketone reductions mediated by isolated enzymes. Alcohol dehydrogenase isolated from *Rhodococcus erythropolis* (ADH RE) was found to mediate the asymmetric reductions of 4′-Br-2,2,2-trifluoroacetophenone to (*R*)-4′-Br-2,2,2-trifluoroacetophenyl alcohol and 6-Br-β-tetralone to its corresponding alcohol (*S*)-6-Br-β-tetralol. Both of these reactions employed a second enzyme, glucose dehydrogenase 103 (GDH 103) to recycle the co-factor NAD through the oxidation of glucose to gluconic acid. In the case of 4′-Br-2,2,2-trifluoroacetophenone the traditional organic co-solvent approaches were limited to a maximum product concentration of 10 g L^{-1} due to substrate deactivation of the biocatalyst. Employing 10% (v/v) [BMP][NTf2], a water immiscible ionic liquid, facilitated conversion of 50 g L^{-1} ketone to the chiral alcohol in less than 24 h. The initial rate of reaction was improved more than four times in the presence of 10% (v/v) ionic liquid compared to no co-solvent and the product could be readily isolated in 85% (w/w) overall yield with an ee of 99%. In the case of 6-Br-β-tetralone the reaction was found to proceed more favorably with both miscible and immiscible ionic liquid co-solvents compared to a number of organic solvents. Again, the product could be readily isolated in 88% (w/w) overall yield with an ee of >99%. For both bioconversions the stability of both the ADH RE and the GDH 103 co-enzyme was found to be enhanced by the presence of certain ionic liquids compared to both organic solvents and aqueous buffer. In the case of the immiscible ionic liquid [BMP][NTf2] present at a volume fraction of 10%, the measured enzyme half lives were 266 and >300 h, respectively. These promising results were obtained after screening only a limited range (11) of representative, commercially available ionic liquids. Critical factors in the screening of ionic liquids for bioconversion applications appear to be the solubility of the substrate, biocatalyst stability in the presence of the ionic liquid co-solvent and mass transfer rates from the ionic liquid into the aqueous phase.

Keywords: Ionic liquid; Ketone reduction; Co-factor recycling; Alcohol dehydrogenase; Glucose dehydrogenase

1. Introduction

The application of biocatalysis is increasingly important for finding efficient routes to enantiomerically pure compounds required for the synthesis of complex pharmaceuticals containing one or more chiral centers [1–4]. An established and widely used reaction is the asymmetric reduction of prochiral ketones to chiral alcohols [5,6]. These can be easily transformed into a variety of functional groups, providing useful chiral building blocks for the synthesis of complex molecules [7]. The asymmetric bioreduction of prochiral carbonyl compounds by whole cells [8] or isolated enzymes provide an established alternative to chemical synthesis methods [1]. Whole cells provide ease of cofactor regeneration but usually operate under conditions providing low substrate concentrations (<2 g L^{-1}) [7]. Isolated enzymes can provide processes capable of operating at industrially relevant substrate concentrations of 50–100 g L^{-1} [9]. The cofactor regeneration required for isolated enzyme bioconversions has been effectively demonstrated at scale using a second enzyme such as formate dehydrogenase or glucose dehydrogenase [9,10]. Processes with isolated enzymes are amenable to rapid process development where speed is a priority for applying biocatalytic processes to early stage pharmaceutical development [11].

Issues faced in the development of enzymatic bioreductions include the poor solubility of hydrophobic non-natural ketone

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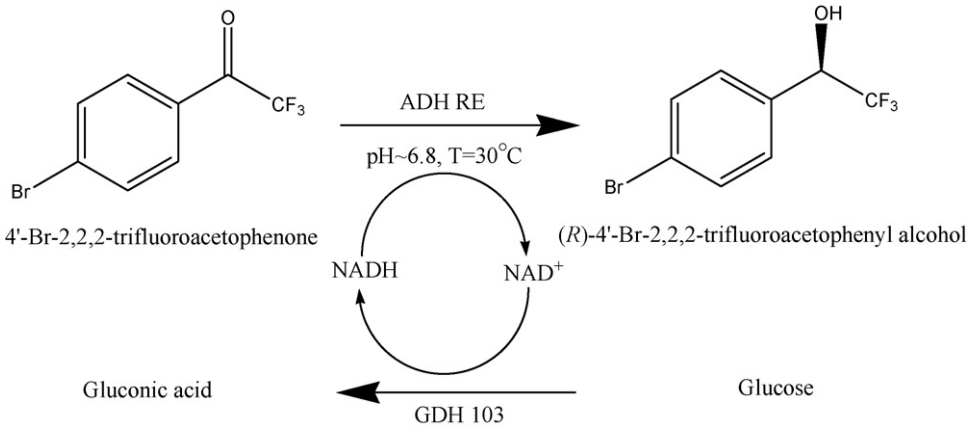


Fig. 1. The asymmetric reduction of 4′-Br-2,2,2-trifluoroacetophenone to (*R*)-4′-Br-2,2,2-trifluoroacetophenyl alcohol by alcohol dehydrogenase isolated from *Rhodococcus erythropolis* (ADH RE) and co-factor rec2ycling by the glucose dehydrogenase 103 (GDH 103) mediated oxidation of glucose.

substrates in aqueous media and possible toxicity of the substrate to the catalyst. Co-solvent can be added to increase aqueous substrate solubility [12] but this must be balanced by any negative impact of the co-solvent itself on the catalyst. Similarly, the toxicity of a substrate in the aqueous phase may be reduced by using a biphasic system in which the organic phase acts as a reservoir for the substrate minimizing exposure of the biocatalyst to the substrate [13]. Again, there is a trade off to be made between effective substrate provision and the stability of the catalyst in the biphasic reaction medium.

A possible alternative to organic co-solvents are ionic liquids, that have the property of being composed entirely of ions but are liquid at, or close to, room temperature. They have practically zero volatility, low flammability, generally reduced toxicity to operators, remain liquid over a broad temperature range (−80 °C to 200 °C), exhibit Newtonian rheology, and are said to have 'tunable' physicochemical properties [14]. Their polarity, hydrophobicity, and solvent miscibility behaviours can be tuned through the appropriate modification of the cation and anion [15]. Typically their polarity is in the range of 0.6–0.7 relative to water [16,17].

Ionic liquids as media for biocatalysis have attracted considerable interest [18]. Much of the work has focused upon isolated enzymes, particularly lipases [19–21], with relatively few involving oxidations [22,23] or reductions [24-26]. The work of Eckstein et al. [24] involved the enantioselective reduction of 2-octanone by alcohol dehydrogenase (ADH) isolated from *Lactobacillus brevis* in the presence of [Bmim][$(CF_3SO_2)_2N$]. However the initial ketone concentration in this study was low at $<1\ g\ L^{-1}$. Where co-factor recycling has previously been employed in the case of redox bioconversions, it was by the addition of co-substrate. The enantioselective reduction of prochiral ketones to alcohols is an important reaction in the synthesis of pharmaceuticals [27], but no other examples of ketone reductions by isolated enzymes in the presence of ionic liquids exist in the literature at the present time.

For biocatalytic applications ionic liquids have been shown to promote improved enzyme stability. The lipase Novozym 435 was shown to retain almost 300% of its initial activity after 24 h incubation in the ionic liquid [MMEP][CH_3CO_2] and 200% in [Bmim][CH_3CO_2] when compared to hexane [19]. The activity of thermolysin was retained after incubation in [Bmim][PF_6] for 144 h whereas almost half of the original activity was lost after similar incubation in ethyl acetate [28]. An esterase isolated from *Bacillus stearothermophilus* was found to be greatly stabilised in the ionic liquid [Bmim][PF_6] with a half-life of >240 h which was a 30-fold increase over hexane and 3 times that in methyl *tert*-butyl ether (MTBE).

This paper shows improvements to bioconversions using ionic liquids as co-solvents for two pharmaceutically important ketone reductions using isolated enzymes and a glucose dehydrogenase for cofactor recycling:

(1) The reduction of 4′-Br-2,2,2-trifluoroacetophenone to (*R*)-4′-Br-2,2,2-trifluoroacetophenyl alcohol (Fig. 1). Reduction of multi-substituted prochiral acetophenones for pharmaceutical synthesis has been previously reported with whole cell biocatalysis [1] and by isolated enzymes [9]. In these cases the acetophenones were either highly soluble in aqueous media [1] or the reaction conditions could be manipulated so that the substrate was readily converted by the biocatalyst of choice. In the particular case of 4′-Br-2,2,2-trifluoroacetophenone the substrate is soluble in aqueous media to concentrations sufficient to drive the reaction but was found to have a deactivating effect on the biocatalyst. The application of room temperature ionic liquids seeks to address this bottleneck in chiral alcohol synthesis.

(2) The reduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol (Fig. 2). 6-Br-β-tetralol is a key chiral intermediate in the synthesis of the antiarrhythmia drug candidate MK-0499 [29]. Chemical methods for this reduction are limited to an ee of 20% for the (*R*) enantiomer [29]. Whole cell routes led to high selectivity for the enantiomer of interest (>99% ee) using either *Trichosporon capitatum* [30] or *Rhodococcus erythropolis* [31]. We have previously found that reactions with both of these whole cell systems proceeded favorably in ionic liquids, but are limited to low substrate concentration of $<2\ g\ L^{-1}$ [32].

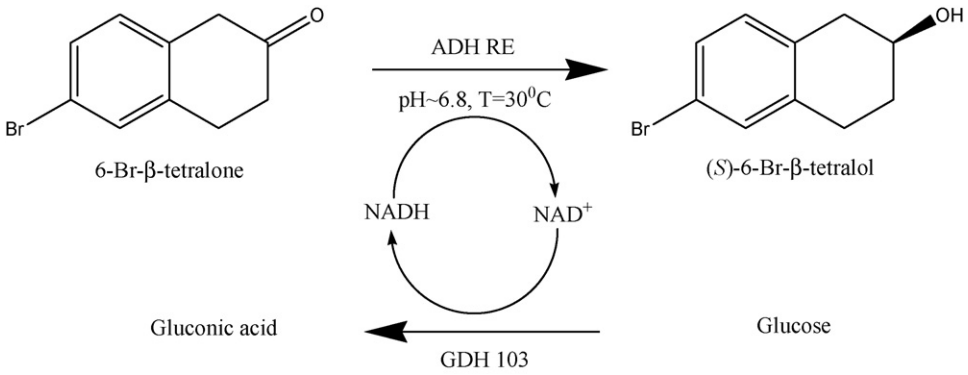


Fig. 2. The asymmetric reduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol by alcohol dehydrogenase isolated from *Rhodococcus erythropolis* (ADH RE) and co-factor recycling by the glucose dehydrogenase 103 (GDH 103) mediated oxidation of glucose.

For both these cases an isolated enzyme approach is pursued in this work in order to improve the substrate concentrations the reactions can operate at to industrially desirable levels of 50 g L^{-1} or above.

2. Materials and methods

2.1. Chemicals and enzymes

All organic solvents were procured from Sigma–Aldrich (USA) and were of >99% purity. Ionic liquids were obtained from Solvent Innovations (Cologne, Germany) and were of >98% purity. Substrates were obtained from Sigma–Aldrich and were of >97% purity. All enzyme preparations and biocatalyst libraries were procured from Biocatalytics (California, USA) and Julich Chiral Solutions (Julich, Germany).

2.2. Solubility studies

500 mg of substrate was weighed into a 1.5 mL HPLC vial and 1 mL of the appropriate solvent added. These were then shaken at 1400 rpm at 30 °C on a thermomixer (Eppendorf) for 24 h. After 24 h the samples were removed and allowed to settle before 50 μL was taken and diluted into 950 μL acetonitrile and assayed for substrate content by reverse phase HPLC. Aqueous solubility curves were generated by taking the appropriate amount of these samples and adding to 100 mM potassium phosphate dibasic (KH_2PO_4) buffer (pH ~7.0) to a total volume of 1 mL. The vials were returned to identical conditions as above and allowed to equilibrate for 24 h. The samples were then allowed to settle before 50 μL was taken and diluted into 450 μL acetonitrile in a dead end filter vial to ensure no solids proceeded to solute analysis by reverse phase HPLC.

2.3. Substrate partitioning study

500 μL of ionic liquid was saturated with substrate as described in Section 2.2 and undissolved solid removed using a dead end filtration vial. 500 μL of various organic solvents were then added and the mixture returned to the thermomixer and allowed to mix for 3 h at 1400 rpm. For immiscible solvents, a sample of both phases was taken, dissolved in acetonitrile and the substrate concentration determined by reverse phase HPLC analysis. The extent of substrate extraction into the aqueous phase was subsequently determined by mass balance.

2.4. Enzyme screening

A total of 66 commercially available keto-reductase preparations were used at 2 g L^{-1} in 100 mM potassium phosphate dibasic buffer containing 1.2 molar equivalents of NADH or NADPH based on the dependency of the enzyme. Substrate (10 g L^{-1} final concentration) was delivered into the reaction in 10% (v/v) toluene. After 5 h incubation at 30 °C reactions were sampled into acetonitrile (to a 1/20 dilution) for reverse phase HPLC analysis, then dried down under nitrogen and resuspended in methanol for chiral HPLC analysis.

2.5. ADH RE and GDH 103 activity assays

For determination of ADH RE residual activity two solutions were made up: solution A containing 2.5 μL p-Cl acetophenone in 7 mL 200 mM potassium phosphate dibasic buffer (pH ~7.2) and solution B containing 24.5 mg NADH in 7 mL buffer. 175 μL and 35 μL respectively of these solutions were added into a single well of a 96 well microtitre plate. 10 μL of sample was added to a separate well and the assay was initiated by the addition of 190 μL of the mixture of solutions A and B from the first well to the sample well. Absorbance at 340 nm was recorded every 20 s over 2 min and plotted. The slope of this line compared to a standard of known enzyme concentration gave the effective enzyme concentration of the sample. For GDH 103 a single reaction mixture was required containing 24.5 mg NAD and 71.9 mg of glucose in 7 mL buffer. The samples were analysed as for ADH RE.

2.6. Enzyme half-life studies

Enzyme (1 mg) was dissolved in 900 μL of 100 mM potassium phosphate dibasic buffer (pH 6.8) in a HPLC vial and 100 μL of appropriate co-solvent was added. These were shaken at 1400 rpm and 30 °C on a thermomixer (Eppendorf) and samples periodically taken and assayed for enzyme activity as described in Section 2.5. Half-life was determined as the time

taken for the activity to reach half that of the original activity.

2.7. Bioconversion studies

The initial screening of bioconversions involving a range of co-solvents was carried out at 10 mL reaction volume in jacketed, cone shaped pH stats (Mettler Toledo). 500 mg substrate was added directly to the vessel and the appropriate co-solvent added. They were then mixed to dissolve the substrate at 30 °C. Mixing of each vessel was by a magnetic stirrer bar ($d = 10$ mm) at high speed. After 1 h, 7 mL buffer was added with glucose (for co-factor recycling) pre-dissolved to give a final overall concentration of 1.5 molar equivalents of substrate. NAD was dissolved in 1 mL buffer to give a final NAD concentration of 1 g L^{-1}, and was added to the vessel. The pH was then corrected to 6.8 ± 0.1. Once the pH was appropriately adjusted, 1 mL further of buffer was added containing ADH RE and GDH 103 to final concentrations of 5 g L^{-1} and 1 g L^{-1} respectively. Samples of 50 μL were regularly taken to determine conversion by dissolving them into 950 μL acetonitrile in a HPLC vial. These were then further diluted 1/10 into acetonitrile and assayed by reverse phase HPLC as described in Section 2.8.1.

Subsequent studies of bioconversion kinetics were performed in miniature Multimax™ reactors (Mettler Toledo, Columbia, MS, USA) of 30 mL reaction volume fitted with overhead Rushton turbine impellers ($d_i = 24$ mm). The vessel was heated by a circulating water jacket and temperature and pH were continuously monitored. Reactions were performed as in the pH stat experiments with adjustments made for the additional volume. In both configurations pH was controlled by the addition of 2.0N sodium hydroxide. Initial rates of reaction were calculated based on base addition rate at the start of the reaction recorded by the online monitoring systems of both reactor configurations.

2.8. Solute analysis

2.8.1. Reverse phase HPLC analysis

An Agilent series 1100 HPLC system equipped with a Zorbax SB-C18 column (4.6 mm × 50 mm) (Mac-Mod Analytical, Chadds Ford, PA, USA) was employed for the separation of the substrates and products. In the case of 6-Br-β-tetralone and 6-Br-β-tetralol separation was by isocratic elution with a mobile phase of 50% (v/v) acetonitrile and acidified water (0.1% (v/v) phosphoric acid) at a flow rate of 1.0 mL min^{-1}. Detection was by UV detector at 220 nm. The assay runtime was 4 min with substrate eluting at 2.2 min and product at 1.6 min. In the case of 4′-Br-2,2,2-trifluoroacetophenone and 4′-Br-2,2,2-trifluoroacetophenyl alcohol separation was by isocratic elution by a mobile phase of 40% (v/v) acetonitrile and acidified water (0.1% (v/v) phosphoric acid) at a flowrate of 1.5 mL min^{-1}. Detection was by UV detector at 265 nm. The assay runtime was 5 min with substrate eluting at 1.3 min and product at 2.6 min.

2.8.2. Normal phase chiral assay

An Agilent series 1100 HPLC system equipped with a Chiralcel OD-H column (Daicel Chemical Industries Limited, USA) was employed for the separation of the two enantiomers of 6-Br-β-tetralol using a mobile phase of hexane with 2% (v/v) IPA modifier pumped isocratically at a flowrate of 1.75 mL min^{-1} and detection at 210 nm. Runtime was 20 min with substrate eluting at 9.8 min, product (*S*) enantiomer at 12.2 min and (*R*) enantiomer at 16.1 min.

2.8.3. Supercritical fluid chromatography (SFC)

A SFC system equipped with a Chiralcel AD-H column was employed for the separation of the two enantiomers of 4′-Br-2,2,2-trifluoroacetophenyl alcohol. The mobile phase employed comprised of supercritical CO_2 modified with 4% (v/v) methanol for 4 min, which is then increased by gradient to 40% (v/v) methanol at a rate of 2% min^{-1} with a 3 min hold at 40% (v/v) methanol (total run time 25 min). The assay was run at 35 °C and 200 bar pressure with a mobile phase flowrate of 1.5 mL min^{-1}. Detection was by UV detection at 215 nm with the (*S*) enantiomer eluting at 10.5 and the (*R*) enantiomer at 12.2 min.

3. Results and discussion

3.1. Reduction of 4′-Br-2,2,2-trifluoroacetophenone

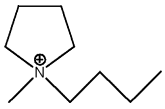
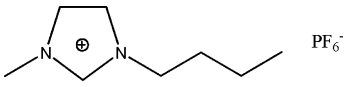
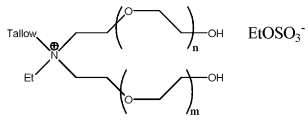
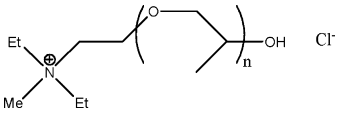
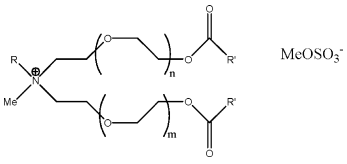
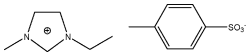
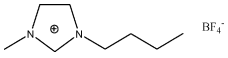
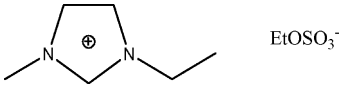
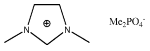
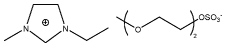
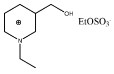
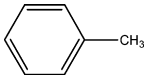
3.1.1. Biocatalyst identification

Enzymes suitable for the reduction of 4′-Br-2,2,2-trifluoroacetophenone to (*R*) 4′-Br-2,2,2-trifluoroacetophenyl alcohol (Fig. 1) were initially identified by screening the substrate against a commercially available ketone reduction library containing 66 enzymes and catalysts yielding both product enantiomers were found. The alcohol dehydrogenase isolated from *Rhodococcus erythropolis* (ADH RE) gave the desired (*R*) enantiomer with an enantiomeric excess (ee) > 99% while the ketoreductase KRED 101 gave the (*S*) enantiomer (data not shown). Both of these enzymes used a second enzyme, glucose dehydrogenase 103 (GDH 103), to regenerate the cofactor (NADH).

3.1.2. Bioconversions

All bioconversions were investigated at an industrially relevant initial substrate concentration of 50 g L^{-1}. For bioconversions in buffer (Fig. 3(a)) or with 10% (v/v) of miscible organic co-solvent such as methanol, DMSO or THF only 10% (w/w) conversion was achieved. This was not due to the low solubility of the substrate as the solubility in buffer alone is >6 g L^{-1}. Rather, the poor conversion was due to rapid deactivation of both enzymes as shown by the activity profiles for ADH RE and GDH 103 in Fig. 3(b) and (c) respectively. Separate enzyme stability studies in buffer with 10 g L^{-1} substrate (the aqueous solubility limit) showed an enzyme half-life of <2 h which is severely reduced compared to enzyme stability measured in buffer only where the half-life is 78 h for ADH RE and 128 h for GDH 103 (Table 1). Biphasic systems with organic solvents gave some improvement to conversion as for example up to 20% w/w conversion in the presence of 10% (v/v) toluene (Fig. 3(a)). The toluene acted as a reservoir for the substrate so the aqueous substrate concentration the enzymes were exposed to was lowered to around 4 g L^{-1}. As shown in Fig. 3(b) and (c)

Table 1
Half-life of alcohol dehydrogenase isolated from *Rhodococcus erythropolis* (ADH RE) and glucose dehydrogenase 103 (GDH 103) in a range of co-solvents (10% (v/v)) at 30 °C and pH 7

Co-Solvent	Structure	Half-life, $t_{1/2}$ (h)	
		ADH RE	GDH103
None		78	128
[BMP][NTf2]		266	>300
[Bmim][PF_6]		135	220
AmmoEng^{TM} 102		12	>300
AmmoEng^{TM} 110		77	>300
AmmoEng^{TM} 120		40	170
[Emim][TOS]		82	41
[Bmim][BF_4]		45	239
EcoEng^{TM} 212		147	>300
EcoEng^{TM} 1111P		182	108
EcoEng^{TM} 21M		22	97
[EMP][ES]		144	27
Toluene		10	5
THF		<3	25
DMSO		231	34

"None" represents 100% (v/v) 100 mM potassium phosphate dibasic buffer. Experiments were performed as described in Section 2.6.

the presence of 10% (v/v) toluene reduced the deactivation on the enzyme such that low level enzyme activity was detectable after 24 h.

Bioconversions in the presence of ionic liquid such as the immiscible [BMP][NTf2] showed rapid reaction rates with complete conversion of an initial 50 g L^{-1} substrate concentration in less than 10 h (Fig. 3(a)). The use of AmmoEng^{TM} 102 also led to improved reactions. The calculated initial rate of reaction with no co-solvent was around 3 g(prod) L^{-1} h^{-1} which almost doubled in the presence of 10% (v/v) AmmoEng^{TM} 102 to 5.5 g(prod) L^{-1} h^{-1} and doubled again to 12.3 g(prod) L^{-1} h^{-1} in the presence of 10% (v/v) [BMP][NTf2]. In the presence of a number of ionic liquids, especially [BMP][NTf2], the ADH RE half-life was markedly improved as shown in Table 1, and its residual activity was less affected by the presence of substrate as shown in Fig. 3(b). A total of four of the ionic liquids tested

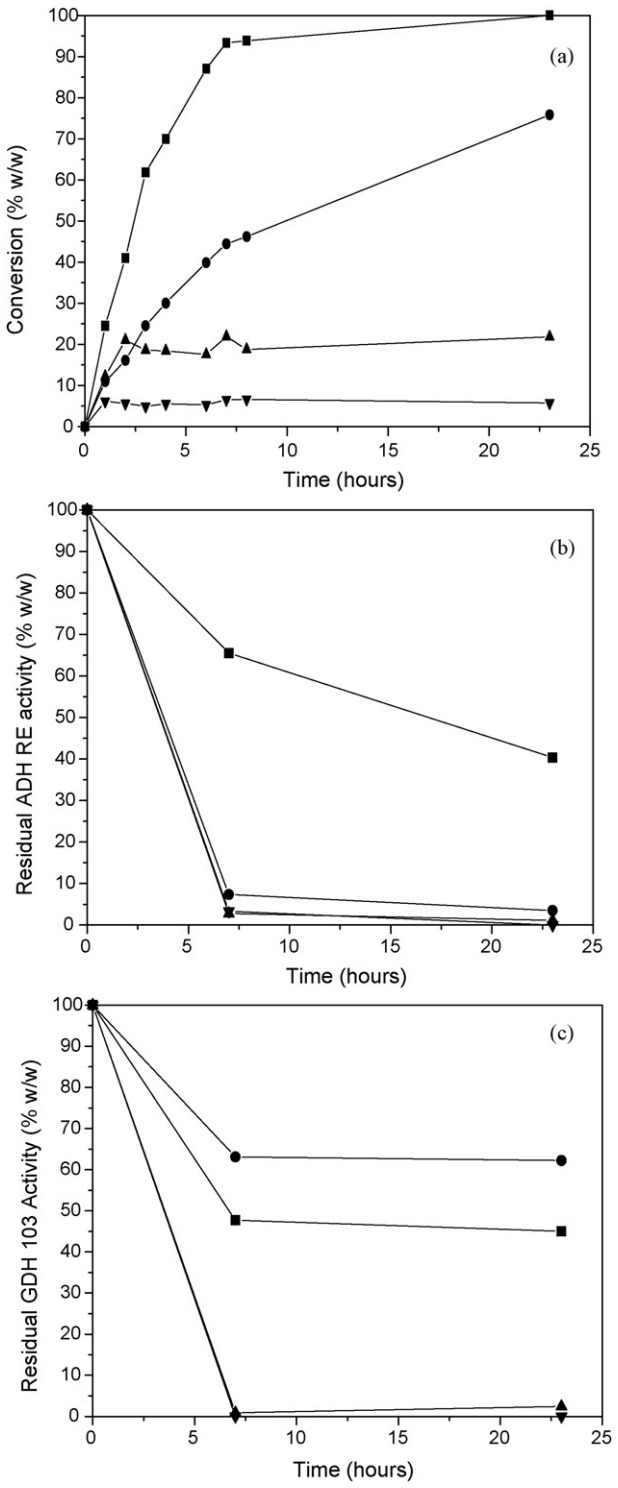


Fig. 3. Comparison of the conversion kinetics of 50 g L^{-1} 4′-Br-2,2,2-trifluoroacetophenone to (*R*)-4′-2,2,2-trifluoroacetophenyl alcohol by ADH RE in different co-solvent systems: (a) conversion kinetics, (b) residual ADH RE activity and (c) residual GDH 103 activity. Experiments performed in the presence of 10% (v/v) co-solvent: (■) [BMP][NTf2], (●) AmmoEng™ 102, (▲) toluene and (▼) with no co-solvent (buffer only). Reactions were carried out in Multimax™ miniature reactors with 30 mL working volume at 30 °C and pH 6.8 ± 0.1 as described in Section 2.7.

gave conversion improvements over the best organic co-solvent tested and three of these significantly protected the enzyme from the deactivation effects of the substrate (Table 2).

ADH RE appeared stable at 30 °C in the presence of up to 20% (v/v) DMSO for 48 h, with only 30% loss of activity. In contrast, toluene was much more inhibitory with less than 5% residual activity after 48 h in the presence of 10% (v/v) co-solvent. This is in contrast to enzyme in 100% buffer (pH 7.0) where 65% original activity was retained after 48 h. Seven out of the eleven ionic liquids screened (Table 1) offered an advantage toward the stability of both ADH RE and GDH 103 compared to the organic solvents tested when no substrate is present. In particular the stability of ADH RE was improved in the presence of the immiscible ionic liquids [BMP][NTf2] and [Bmim][PF_6] where 80% and 78% original activity was maintained respectively over 64 h compared to only 57% activity retention in buffer. This is in contrast to AmmoEng™ 102 and EcoEng™ 21 M where >95% enzyme activity was lost after just 15 h. The increase in enzyme stability in the presence of [BMP][NTf2] however is not sufficient to account for the vast increases in initial activity observed in Fig. 3(a), suggesting a further effect of improved mass transfer of substrate from the ionic liquid phase to the aqueous phase might be in operation.

3.1.3. *Effect of enzyme concentration*

Experiments in which the initial ADH RE concentration was reduced from 1 g L^{-1} to 0.5 g L^{-1} (GDH 103 concentration was reduced to 0.5 g L^{-1}) still allowed for 100% (w/w) conversion of 50 g L^{-1} 4′-Br-2,2,2-trifluoroacetophenone to (*R*)-4′-Br-2,2,2-trifluoroacetophenyl alcohol within 24 h. The use of 0.1 g L^{-1} ADH RE, although found to be feasible, resulted in too low a rate of reaction to give full conversion within the lifetime of the enzyme in the system. In this case enzyme concentration became the limiting factor as evidenced by the linear conversion rates through the majority of the reaction course (data not shown).

3.1.4. *Ionic liquid re-use*

In order to investigate ionic liquid re-use a 0.25 g L^{-1} ADH RE, 50 g L^{-1} initial substrate concentration and 15% (v/v) [BMP][NTf2] experiment was performed at 30 °C in a pH stat (as described in Section 2.6) for ∼28 h, converting ∼95% (w/w) 4′-Br-2,2,2-trifluoroacetophenone to alcohol. In order to isolate the product and unreacted substrate, this mixture was taken, centrifuged, and the aqueous layer removed. The product and remaining substrate was then extracted from the ionic liquid layer by 4 washes with diethyl ether until >99% (w/w) of product was recovered (as determined by reverse phase HPLC). It was apparent by eye that some of the ionic liquid had been absorbed into the diethyl ether layer so fresh ionic liquid was added (∼0.25 mL giving a total ionic liquid volume of 1.5 mL) to replenish the system. A second bioconversion in which the recovered ionic liquid was used with fresh enzyme was then performed under the same reaction conditions and compared to a simultaneously run fresh ionic liquid experiment. The recovered ionic liquid profile mirrored the fresh ionic liquid profile very well, but the rate always remained slightly lower than in the fresh ionic liquid case. In the case of the recovered ionic liquid

Table 2
Summary of initial reaction rates, conversion, residual ADH RE activity and substrate solubility in the ionic liquid bioconversion screen for the reduction of 50 g L^{-1} 4′-Br-2,2,2-trifluoroacetophenone to (*R*)-4′-Br-2,2,2-trifluoroacetophenyl alcohol (Fig. 1)

Co-solvent	Initial rate (g(prod) L^{-1} h^{-1})	Conversion (% w/w)	Residual ADH activity (% w/w)	Substrate solubility (g L^{-1})
None	3.1	5.6	0	6.5
[BMP][NTf2]	12	100	40	5.1
[Bmim][PF_6]	11	99.6	47	3.8
AmmoEng™ 102	5.5	75.8	4	14
[Emim][TOS]	2.5	46.1	46	8.2
[Bmim][BF_4]	0.1	0	0	6.3
EcoEng™ 1111P	0.1	0.8	0	11
Toluene	6.1	20.8	1	8.2

All experiments were performed in the presence of 10% (v/v) co-solvent as described in Section 2.7. In the case of miscible co-solvents substrate solubility refers to the equilibrium saturation of substrate in the co-solvent mixture. In the case of immiscible co-solvents substrate solubility refers to the equilibrium saturation concentration of substrate in the aqueous phase.

85% (w/w) conversion was achieved compared to 92% (w/w) in the case of fresh ionic liquid.

3.1.5. Product recovery and overall yield

The isolated product from the original reaction with [BMP][NTf2] was recovered by rotary vacuum evaporation of the diethyl ether leaving a viscous oil identified as (*R*)-4′-Br-2,2,2-trifluoroacetophenyl alcohol by reverse phase HPLC (Section 2.8.1) and SFC analysis (Section 2.8.3). The overall yield of recovered product was 85% (w/w), based on complete conversion of the added substrate, with an ee of >99% in favour of the desired (*R*) enantiomer.

3.2. Reduction of 6-Br-β-tetralone

3.2.1. Biocatalyst identification

A second ketone reduction of pharmaceutical importance is the asymmetric reduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol, which is a key intermediate in the synthesis of the anti arrhythmia drug candidate MK-0699 [29]. A library of 66 commercially available enzyme preparations for ketone reductions was tested against this substrate in an automated screen (Section 2.4) and a selection of the results can be seen in Table 3. It can be seen that the enantiomer of interest can be synthesized with excellent selectivity by 4 of the enzymes each with 100% ee. In contrast, the (*R*) enantiomer does not appear to be so readily formed. In considering the screening data, the enantio-selectivity of the enzyme is deemed more critical than the conversion as the latter can be more readily manipulated through altering some of the engineering parameters when performing the conversion. It was found that the alcohol dehydrogenase isolated from *Rhodococcus erythropolis* was an effective catalyst for the reduction of interest (Fig. 2), with a >99% enantiomeric excess of the desired (*S*) enantiomer. Consequently both the ionic liquid library and the organic solvents selected were screened for enzyme stability at a range of co-solvent volume fractions (data not shown).

3.2.2. Bioconversions

Bioconversions in the presence of all the ionic liquids were subsequently performed in pH stats (working volume 10 mL), again at an initial substrate concentration of 50 g L^{-1}, to determine which co-solvent would yield the best conversion and rate. These reactors were mixed by a magnetic flea and visual observation of the reactions indicated effective mixing of the two phase systems was achieved with good dispersion of the ionic liquid phase into the bulk fluid. A summary of the data for selected co-solvents is presented in Table 4.

The immiscible ionic liquids [BMP][NTf2] and [Bmim][PF_6] performed the best of all co-solvent systems analyzed. The enzyme stability in the presence of these co-solvents is good with

Table 3
Summary of conversions and product ee of enzymes identified from a screen of 66 ketoreductase preparations for the reduction of 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol (Fig. 2)

Enzyme	Conversion (% w/w)	Product ee (%)
KRED1	100.0	24.4
KRED4	25.9	54.7
KRED7	22.7	27.9
KRED8	98.9	−37.1
KRED9	42.3	13.3
KRED10	30.9	55.1
KRED11	98.5	29.4
KRED26	100.0	100
KRED27	100.0	−71.8
KRED28	100.0	−71.5
KRED29	69.6	−43.2
KRED30	92.3	6.4
KRED31	47.7	35.6
KRED exp-A1A	25.7	41.9
KRED exp-A1B	100.0	−43.2
KRED exp-A1C	100.0	−45.1
KRED exp-A1D	100.0	−40.8
KRED exp-A1E	74.1	−18.4
KRED exp-A1I	33.3	30.3
KRED exp-A1J	83.8	9.3
KRED exp-A1L	68.7	78.3
KRED exp-A1P	99.1	100
KRED exp-A1T	73.8	−30.0
KRED exp-A1U	50.0	93.5
KRED exp-A1V	36.3	33.3
ADH RE	99.5	100
KRED NADH 102	100.0	100

Product ee was calculated for the (*S*) enantiomer. Enzyme nomenclature as supplied by the manufacturer (http://www.biocatalytics.com/kred.html). Experiments were performed as described in Section 2.3.

Table 4
Summary of initial reaction rates, conversion, residual ADH RE activity and substrate solubility in the ionic liquid bioconversion screen for the reduction of 50 g L^{-1} 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol (Fig. 2)

Co-solvent	Initial rate (g(prod) L^{-1} h^{-1})	Conversion (% w/w)	Residual ADH activity (% w/w)	Substrate solubility (g L^{-1})
None	3.1	100	74	0.1
[BMP][NTf2]	14	100	43	0.2
[Bmim][PF_6]	14	98.5	28	0.2
AmmoEng^{TM} 102	12	100	9.4	6.0
[Emim][TOS]	6.5	89.0	51	3.1
[Bmim][BF_4]	9	99.5	38	1.2
EcoEng^{TM} 1111P	1.2	38.5	66	1.3
Toluene	5.8	100	41	1.0
DMSO	2.3	58.4	42	0.5
THF	2	24.9	0.8	0.3
DMF	3.6	95.8	7.6	0.6

All experiments were performed in the presence of 10% (v/v) co-solvent as described in Section 2.7. In the case of miscible co-solvents substrate solubility refers to the equilibrium saturation of substrate in the co-solvent mixture. In the case of immiscible co-solvents substrate solubility refers to the equilibrium saturation concentration of substrate in the aqueous phase.

residual ADH RE activities after 24 h of 43% and 28% (w/w) respectively (Table 4). The steady state aqueous solubility of the substrate from [BMP][NTf2] and [Bmim][PF_6] to the aqueous phase was measured at 0.21 g L^{-1} and 0.18 g L^{-1}, respectively which is much lower than for the miscible co-solvent systems. However, the complete conversions and high initial rates of reaction achieved in the presence of these co-solvents suggest enhanced mass transfer rates of substrate from these ionic liquids. The use of the immiscible 10% (v/v) toluene as co-solvent significantly out performed the other organic co-solvent systems in terms of conversion (Table 4, Fig. 4), and the residual ADH RE activity was similar to [BMP][NTf2]. In addition to this, the solubility of the substrate after equilibration in the aqueous phase is 1 g L^{-1} suggesting the rate of reaction should be greater than that for [BMP][NTf2]. However the rate is almost one third that of [BMP][NTf2] indicating solute mass transfer is significantly slower from the toluene phase.

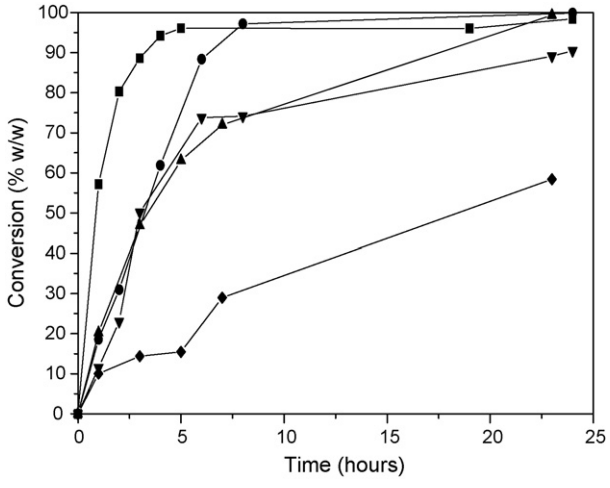


Fig. 4. Comparison of the conversion kinetics of 50 g L^{-1} 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol by ADH RE in the presence of 10% (v/v) co-solvent: (■) [BMP][NTf2], (●) AmmoEng^{TM} 102, (▲) toluene (♦) DMSO and (▼) with no co-solvent (buffer only). Reactions were carried out in Multimax^{TM} miniature reactors with 30 mL working volume at 30 °C and pH 6.8 ± 0.1 as described in Section 2.7.

Despite a very low aqueous solubility of tetralone (<1 g L^{-1}) the reaction carried out with no co-solvent (and an added initial amount of substrate equivalent to 50 g L^{-1}) goes to completion within 24 h at a rate of 10.1 g(prod)L^{-1} h^{-1} (Fig. 4). The residual ADH RE concentration after this time was 74% (w/w) (Table 4). The reaction begins as a two-phase system of aqueous buffer and solid substrate (as the buffer is unable to dissolve the substrate) but as product is synthesised an immiscible oil phase is formed. It is suggested that the dispersed oil and solid phase enhance the mass transfer rate of substrate into the aqueous phase yielding the full conversion observed in 24 h. The presence of dispersed solid phases in particular is known to enhance gas–liquid mass transfer coefficients in bioreactors [33]. The initial rate of reaction in the presence of [BMP][NTf2] is still greater than with the buffer alone (which have comparable aqueous solubilities) suggesting that the mass transfer improvements of the [BMP][NTf2] to aqueous phase are greater than the effects of the mass transfer enhancements conferred by the product oil phase.

In the case of miscible ionic liquid co-solvents, the 10% (v/v) AmmoEng^{TM} 102 co-solvent system yielded 100% (w/w) conversion with an initial rate of 12 g(prod) L^{-1} h^{-1} (Table 4, Fig. 4). Residual ADH RE activity was only 10% of the initial value as the enzyme has been shown to have a very short half-life in the presence of this co-solvent (Table 1). However the solubility of the substrate in the co-solvent mixture is boosted to 6 g L^{-1} facilitating more rapid reactions.

In summary, while there does appear to be a correlation between bioconversion efficiency and parameters such as enzyme activity, aqueous substrate solubility and solute mass transfer efficiency there is still a need to carry out screening of reaction conditions. Based on the data available, reliably predicting which conditions will give the highest rates and yields is not currently feasible. For example, the ionic liquid [Emim][TOS] provides an aqueous solubility of 3.1 g L^{-1} (Table 4) and ADH RE half-life is around 80 h (Table 1) in the presence of 10% (v/v) co-solvent, but conversion is limited to <90% (w/w) at a much lower initial rate of 6.5 g(prod) L^{-1} h^{-1} when compared to [BMP][NTf2].

3.2.3. Effect of ionic liquid volume fraction

For immiscible co-solvents the volume fraction of the dispersed phase will determine the total amount of substrate that can be dissolved in the system and the interfacial area available for mass transfer. In the case of [BMP][NTf2], the best ionic liquid found in Section 3.2.2, there appears to be no readily discernable difference in rate or conversion between 10% and 20% (v/v) volume fractions while at 50% (v/v) the measured rate is approximately 60% that of the lower volume fractions (Fig. 5). This is in agreement with the model of Greiner and co-workers who found increasing volume fraction beyond 25% led to a decrease in rate and overall conversion [34]. Normally mass transfer would be expected to be improved with the increased co-solvent concentration. Interestingly the enzyme stability data shows that there is no significant difference in the rate of ADH RE or GDH 103 degradation between the three systems (Fig. 5(b) and (c)). This apparent disparity in reaction rate at high volume fraction may be due to the influence of the viscosity of the ionic liquid as the viscosity of the pure ionic liquid is two orders of magnitude greater than that of water (9×10^{-2} Pa S). All three ionic liquid concentrations were operated at the same stirrer speed (1000 rpm) yet the mass averaged viscosity varied 2–3-fold across the experiment resulting in a 50% change in the Reynolds number. The Reynolds number is an engineering parameter indicating the degree of turbulence within a system and is based on the ratio of inertial forces to viscous forces due to agitation. It is defined as $Re = \rho N d_i^2/\mu$ where ρ is the mass averaged system density, N is the impeller speed, d_i is the impeller diameter and μ the mass averaged system viscosity.

The effect of Reynolds number on the conversion was further examined at Reynolds numbers of 450 and 900 as determined by minimum and maximum impeller speeds achievable on the lab scale equipment. Fig. 6 shows the conversion kinetics for two 10% (v/v) [BMP][NTf2] co-solvent systems, one at a Reynolds number of 450, the other at 900, and a 20% (v/v) [BMP][NTf2] co-solvent system with a Reynolds number of 450. As expected, at a fixed co-solvent fraction and in a heterogeneous reaction system that is mass transfer limited doubling the Reynolds number doubles the initial rate of reaction. Likewise at a fixed Reynolds number doubling the co-solvent fraction leads to a corresponding increase in the measured rate of reaction due to an increase in the area available for solute mass transfer. These results confirm that mass transfer is the limiting factor for the ionic liquid bioconversions studied in this work.

3.2.4. Effect of enzyme concentration

Due to the high cost of enzyme preparations, industrial enzymatic processes are rarely run such that mass transfer is the limiting factor. Enzyme concentration is a more restrictive constraint [9] and so was also examined. At a fixed Reynolds number of 450 and 10% (v/v) [BMP][NTf2] co-solvent, various initial ADH RE concentrations (0.5–5.0 g L^{-1}) were used to determine the enzyme concentration at which mass transfer becomes rate limiting (GDH 103 concentration was maintained at 0.5 g L^{-1} for all experiments). As expected increasing initial enzyme concentration increases the rate of reaction and the time to complete conversion is reduced. The results indicated that solute mass

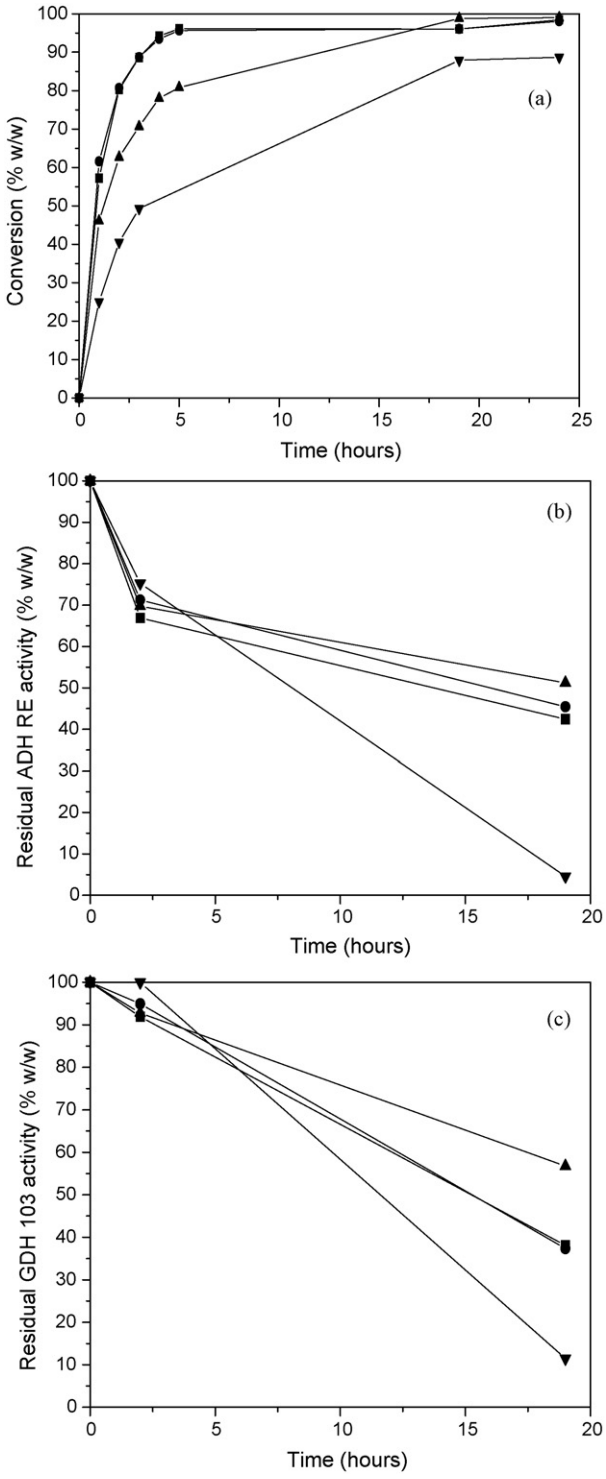
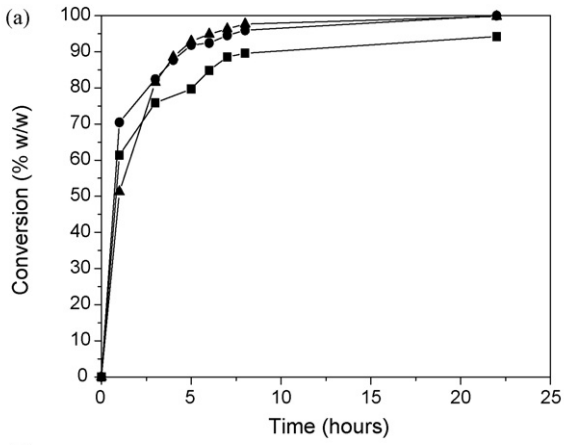


Fig. 5. Effect of co-solvent volume fraction on the conversion of 50 g L^{-1} 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol by ADH RE in a range of co-solvents: (a) conversion kinetics, (b) residual ADH RE activity and (c) residual GDH 103 activity. Experiments performed in the presence of: (■) 10% (v/v) [BMP][NTf2], (●) 20% (v/v) [BMP][NTf2], (▲) 50% (v/v) [BMP][NTf2] and (▼) 10% (v/v) toluene. Reactions were carried out in Multimax™ miniature reactors as described in Section 2.7.



(b)

Re	450	900	450
[BMP][NTf2] (% v/v)	10	10	20
Initial rate ($g(prod)L^{-1}hr^{-1}$)	6.8	14.1	14.3

Fig. 6. Effect of impeller Reynolds number and dispersed phase volume fraction on initial rate of reaction for conversion of 50 g L^{-1} 6-Br-β-tetralone to (*S*)-6-Br-β-tetralol by ADH RE. (a) Conversion kinetics of: (■) 10% (v/v) [BMP][NTf2], *Re* = 450, (●) 10% (v/v) [BMP][NTf2], *Re* = 900, (▲) 20% (v/v) [BMP][NTf2], *Re* = 450. (b) Calculated initial rates of reaction. Reactions were carried out in Multimax™ miniature reactors as described in Section 2.7.

transfer first becomes rate limiting at an initial ADH RE concentration of 2 g L^{-1} (data not shown).

3.2.5. Product recovery and overall yield

A study of the partition coefficient for 6-Br-β-tetralone between [BMP][NTf2] and various organic solvents showed that toluene gave the best extraction of product from the ionic liquid. A simple isolation procedure was feasible whereby the product in the immiscible ionic liquid can be separated from the aqueous phase by centrifugation followed by three half-volume extractions with toluene from the ionic liquid (~60% (w/w) average extraction efficiency). The product was then isolated from the toluene by vacuum evaporation of the solvent yielding 88% (w/w) product, based on complete conversion of added substrate, with an enantiomeric excess of >99% in favour of the desired (*S*) enantiomer.

3.3. Possible mechanisms of enzyme stabilization

Use of an immiscible ionic liquid [BMP][NTf2], has been found to lead to a dramatic improvement in reaction performance for both of the bioconversions studied in this work. This increase in performance is a combination of increased half-life of both ADH RE and GDH 103 co-factor recycling enzymes (Table 1) and other effects dominated by mass transfer (Section 3.2.3). The increased enzyme half-life in the presence of hydrophobic co-solvents is attributed to a number of possible mechanisms. Hydrophilic co-solvents have been found to strip enzymes of "internally bound" water and soluble components of solvents interact electrostatically to adversely affect enzyme activity contrary to the action of hydrophobic co-solvents such as [BMP][NTf2] which can promote enzyme stability [19]. A more compact enzyme conformation resulting from changes of α-helix structures to β-sheet within the protein has also been observed with hydrophobic ionic liquids [35] and could act to protect the enzyme within a co-solvent system. It has been postulated that changes in the hydrogen bonding environment in ionic liquids may be responsible for disrupting the α-helix or β-sheet motifs within the enzyme leading to observed shifts in enzyme stability [36]. For the reduction of 4′-Br-2,2,2-trifluoroacetphenone, [BMP][NTf2] acted to decrease the deactivation effect of the substrate on the ADH RE. Aside from deactivation, inhibition of the enzyme may have been taking place in the presence of the organic solvents as has previously been observed [37]. Potential changes in the redox environment within a co-solvent system may also have an effect on the dynamics of the co-factor binding and transfer within the system [38,39], as well as on the binding and transfer of substrate resulting in the kinds of changes in initial rate of reaction observed in this work.

4. Conclusions

The asymmetric reduction of industrially relevant quantities of prochiral ketones to their corresponding alcohols in the presence of ionic liquid as a co-solvent and with coupled enzyme regeneration of co-factor has been demonstrated. In the reactions studied here the ionic liquid was found not to alter the enantiomeric selectivity of the enzyme. Use of an immiscible ionic liquid, in particular [BMP][NTf2], led to a dramatic improvement in reaction performance (Fig. 3(a)), resulting in a previously infeasible reaction becoming potentially industrially viable. Recovery of product from the ionic liquids has also been shown to be viable, with little loss in yield and purity.

Half-life increases to >250 h for ADH RE and to >300 h for GDH 103 at 30 °C in the presence of 10% (v/v) [BMP][NTf2] have been shown. Previous work on ADH from *Lactobacillus brevis* showed increases of half-life to 50 h under similar conditions in the presence of [Bmim][$(CF_3SO_2)_2N$] [27]. Rates of reaction have also been vastly improved with >95% (w/w) conversion of 50 g L^{-1} substrate achieved in less than 8 h.

Based on the current results and the few examples in the literatures [20–26], there does not at present appear to be a rational way to select ionic liquids for biocatalytic processes based upon their structure or functional groups. Screening of a limited range of ionic liquids has been shown to be effective in a number of cases [40–42]. A representative library of ionic liquids can be readily formed for most applications. Of the eleven ionic liquids screened here for the 6-Br-β-tetralone conversion, five gave rates and conversion equivalent or better than those of the best organic solvent screened. In the case of the 4′-Br-2,2,2-trifluoracetophenone reduction four ionic liquids gave improvements over the best organic co-solvent but critically they all facilitated much greater conversion over organic solvents and buffer. Important factors in ionic liquid selection appear to be solubility of substrate in the aqueous phase of het-

erogeneous phase systems, mass transfer rates of substrate from immiscible ionic liquids into the aqueous phase and biocatalyst stability in the aqueous/ co-solvent mixture. Any strategy for the high throughput screening of ionic liquids for biocatalytic applications should centre upon these factors.

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