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Enhancement of whole cell dioxygenase biotransformations of haloarenes by toxic ionic liquids†

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Accessing chirally pure *cis*-diols from arenes using micro-organisms over-expressing toluene dioxygenase (TDO) is now well established, but the conversions remain low for the more toxic and volatile substrates. For such arenes, improved production has already been achieved in the presence of hydrophobic non-toxic ionic liquids (ILs) acting in the form of a reservoir for the arene substrate. Yet, the costs associated with such ILs require extensive process development to render them viable. Herein, we show that optimization of the hydrophobic IL's cationic moiety and of the IL's concentration are key to enhanced conversion yielding between a 2–5 fold yield increase in the conversion of four haloarenes (Ph–X; X = F, Cl, Br, I). Additionally, we report that hydrophilic imidazolium-based ILs offer opportunities to achieve similarly high yielding biotransformations, with further improved reaction rates (<6 h), and this at very low ILs' concentrations (0.0015 V_{IL}/V_{aq}). We also demonstrate that the increased biotransformations are due to these ILs being inhibitors of cellular respiration processes and thus favoring the shunting of NADH and O_2 towards the overexpressed biocatalytic process.

Introduction

The use of whole cell biocatalysis to obtain chirally-defined fine chemicals and the development of "greener" chemical processes is becoming common practice for the chemical industry.[1,2] For instance, the versatile production of chirally pure *cis*-diols from arenes can be achieved in variable yields using organisms such as *Pseudomonas putida* UV4.[3] Such compounds, which cannot be obtained effectively *via* any other sources, are ideal materials to be used in fine chemical and chiral ligand syntheses.[4–6] While successful, improvements in the biotransformation conditions and related processes are always being sought. To this end, organic solvents or mixed/biphasic aqueous solutions/organic media whereby improved conversions/yields can be achieved, have been extensively studied. These have been developed to overcome limitations due to low substrate solubility in aqueous solutions or to minimise product inhibition.[7] For example, reactions performed in mono- or biphasic conditions for isolated enzymes or whole cell systems have been developed to release the substrate to the biocatalyst at concentrations remaining below the inhibitory concentration, thereby improving regio-selectivity and/or overall yields.[8] As such, the development of biocatalytic processes in alternative media has been of growing interest even though the rationale underpinning the observed effects remains somewhat uncharted.[9–12]

Previous studies, including the pioneering work of Cull *et al.*[13] have shown that ILs, including hydrophobic ILs, can be used to enhance biotransformations with a variety of whole cell biocatalysts.[8,14,15] The primary mechanism of enhancement with hydrophobic substrates is thought to involve increasing substrate availability and, therefore, mass transfer to the enzyme, whilst decreasing free substrate overall concentration to limit cell toxicity. However, other studies have shown that ILs can be strong inhibitors for whole cell metabolic processes. The limited rationale for such broad ranging behaviour[8] has thus limited their widespread application.[2,15]

In an attempt to examine how hydrophobic ionic liquids can be used as suitable agents in the biphasic whole-cell dihydroxylation of halobenzenes by *P. putida* UV4, conditions were identified whereby improved yields and reaction rates were easily achieved, but for which the biocatalyst was compromised by these same conditions. The aims were therefore to establish how the ILs could be used to enhance biotransformation rates *via* a two phase system while improving the mass transfer, and establish the mechanism(s) which was responsible for the increases in rate observed. This allowed for routes, *via* inhibition of cellular biochemistry leading to an indirect enhancement of enzyme activity, to be probed. Therefore, for the first

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time, the nature of the IL and its effect on the biocatalyst has been related to the overall biotransformation process.

Results and discussion

Impact of hydrophobic ILs on *cis*-dihydroxylation biotransformations

The biocompatibility of secondary phases is an important criterion in the choice of the solvent for the whole cell biotransformation processes.[16–18] Therefore, an understanding of the toxicity of the ILs for the biotransforming cell lines is crucial. Pfruender *et al.* have shown that the biocompatibility and partition coefficient of four ILs based on the $[PF_6]^-$ and $[NTf_2]^-$ anions,[17,18] including $[C_4mim][NTf_2]$, had a major positive impact on the yield of conversion of 4-chloroacetophenone to (*R*)-1-(4-chlorophenyl)ethanol by *Lactobacillus kefir* compared with the addition of organic solvents. The improved yields were proposed to be due to the non-destructive effect of the ILs on the cell membrane when compared with the organic solvents. Additional studies led to the proposal that the formation of small droplets of ILs containing the arene substrate were generated and improved the arene's dispersion in solution thus overcoming the high viscosity associated with the ILs used. In comparison, Stephens and co-workers showed that the same non-water miscible IL ($[C_4mim][NTf_2]$) was toxic to the *E. coli* strain MG1655 pDTG601A over-expressing TDO and, therefore, was unsuitable for the oxygenase-based biotransformations catalysed by that clone, while tetraalkyl phosphonium and tetraalkyl ammonium $[NTf_2]^-$ based ILs provided 2.5 fold enhancement for the biotransformations.[8]

In order to establish how selected ILs enhance the conversion of arenes to *cis*-diols by another ring-hydroxylating dioxygenase expressing organism, the TDO expressing bacterium *P. putida* UV4 was chosen. This organism has been shown to have a wide range of activity towards diverse arene substrates and is, therefore, a useful industrial catalyst.[19] Investigative whole cell biotransformations were first conducted with chlorobenzene (Ph–X, where X = Cl), as the biotransformation of chlorobenzene to *cis*-1,2-dihydroxy 3-chlorobenzene has been demonstrated to be a very high yielding biotransformation with this enzyme system.[19,20]

The initial rates of the biotransformation of chlorobenzene to *cis*-1,2-dihydroxy 3-chlorobenzene with bacterium *P. putida* UV4 and the maximum conversion yields for the product were quantified by ^{1}H-NMR. In these studies, a range of ionic liquids employed under biphasic conditions was examined and these ILs were: 1-butyl,3-methylimidazolium-bis(trifluoromethanesulfonyl)imide ($[C_4mim][NTf_2]$), 1-hexyl,3-methylimidazolium-bis(trifluoromethanesulfonyl)imide ($[C_6mim][NTf_2]$), 1-methyl,3-octylimidazolium-bis(trifluoromethanesulfonyl)imide ($[C_8mim][NTf_2]$), 1-decyl,3-methylimidazolium-bis(trifluoro-methanesulfonyl)imide ($[C_{10}mim][NTf_2]$), *N*-butyl,*N*-methylmethylimidazolium-bis(trifluoromethanesulfonyl)imide $[C_4dmim][NTf_2]$, 1-hexyl,2,3-dimethylimidazolium-bis(tri-fluoromethanesulfonyl)imide ($[C_6dmim][NTf_2]$), 1-octyl,2,3-dimethylimidazolium-bis(trifluoromethanesulfonyl)imide ($[C_8dmim][NTf_2]$), 1-decyl,2,3-dimethylimidazolium-bis(tri-fluoromethanesulfonyl)imide ($[C_{10}dmim][NTf_2]$); *N*-butyl,*N*-methylpyrrolidinium-bis(trifluoromethanesulfonyl)imide ($[C_4mpyrrol][NTf_2]$), *N*-octyl,*N*-methylpyrrolidinium-bis(tri-fluoromethanesulfonyl)imide ($[C_8mpyrrol][NTf_2]$), trihexyl-tetradecyl-phosphonium-bis(trifluoromethanesulfonyl)imide ($[P_{66614}][NTf_2]$); 3-hexoxycarbonylmethyl-1,2-dimethyl-imidazolium-bis(trifluoromethanesulfonyl)imide, $[C_6H_{13}OCOCH_2dmim][(CF_3SO_2)_2N]$; 3-octoxycarbonylmethyl-1,2-dimethylimidazolium-bis(trifluoromethanesulfonyl)imide, $[C_8H_{17}OCOCH_2dmim][(CF_3SO_2)_2N]$, *N*-hexoxycarbonyl-methyl-*N*-methylpyrrolidinium-bis(trifluoromethanesulfonyl)imide, $[C_6H_{13}OCOCH_2mpyrrol][(CF_3SO_2)_2N]$; *N*-octoxycarbonyl-methyl-*N*-methylpyrrolidinium-bis(trifluoromethanesulfonyl)imide, $[C_8H_{17}OCOCH_2mpyrrol][(CF_3SO_2)_2N]$, 3-(*N*-butyl-*N*-methylcarbamoylmethyl)-1,2-dimethylimidazolium-bis(trifluoromethanesulfonyl)imide, $[C_4H_9CH_3NCOCH_2dmim][(CF_3SO_2)_2N]$ and *N*′-(*N*-butyl-*N*-methylcarbamoylmethyl)-*N*′-methylpyrrolidinium-bis(trifluoromethanesulfonyl)imide $[C_4H_9CH_3NCOCH_2mpyrrol][(CF_3SO_2)_2N]$. The volume ratio of the IL:water in the two phase system ranged from 0.0015 to 0.02 (V_{IL}/V_{aq}) ratio.[21]

In the first instance, the initial reaction rates of chlorobenzene by *P. putida* UV4 in the presence (0.02 (V_{IL}/V_{aq})) or absence of ILs were recorded and the amounts of biotransformed product were measured after 6 h of reaction (Table 1). Under these conditions, only $[C_8dmim][NTf_2]$ was found to result in improved yields for the *P. putida* UV4 catalysed biotransformation compared with the non-IL based system. For the imidazolium-based ILs incorporating a side chain below eight carbons, the biotransformation rate was reduced which may be due to the IL toxicity (*e.g.* $[C_4(d)mim][NTf_2]$) or inhibition (*e.g.* $[C_6(d)mim][NTf_2]$) of the enzyme. At 0.02 (V_{IL}/V_{aq}), $[C_4mpyrrol][NTf_2]$ and the $[C_8mpyrrol][NTf_2]$ demonstrated mainly an inhibitory effect on the biotransformation process (Table 2). Finally, the effect of the $[P_{66614}][NTf_2]$ ILs on the initial rates and overall yields were minimal when compared with the control (Table S2 ESI†). These results are in agreement with Stephens *et al.*[8] where the C4 alkyl chain containing ILs proved unsuitable media to conduct biotransformations. The difference in biotransformation profiles, in particular with regards to "toxicity" *vs.* "inhibition", led to investigating the direct effects of the ILs on the biocatalyst and this as a function of the IL's concentration and of the IL's cation.

Clearly, ILs incorporating short chain lengths had a detrimental effect on the overall biotransformation process (Tables 1 and 2). However, the imidazolium-based ILs which incorporated longer side-chains ($n > 6$), exhibited faster initial reaction rates and 3-fold increase in yields after 2 h when compared with the control experiment where no IL was present.

The effect of the volume ratio can be best visualised in Fig. 1, which shows the reaction-time profile for the conversion of chlorobenzene in the biphasic system using $[C_8dmim][NTf_2]$ present in volume ranging between 0 and 0.02 (V_{IL}/V_{aq}) ratio. From this trend, it is clear that minimal amounts of IL are required to achieve a sizable enhancement in the biotransformation with 0.0015 (V_{IL}/V_{aq}) showing the most significant increase observed.

Table 1 Biphasic biotransformation of chlorobenzene in the presence of imidazolium-IL with *P. putida* UV4 (2.0% (V_{IL}/V_{aq}) IL + substrate) for OD_{600} = 0.9 or 0.35 g L^{-1} dcw[a]

	Control	[C_4dmim][NTf_2]	[C_6dmim][NTf_2]	[C_8dmim][NTf_2]	[C_{10}dmim][NTf_2]	[C_4mim][NTf_2]	[C_6mim][NTf_2]	[C_8mim][NTf_2]	[C_{10}mim][NTf_2]
Conversion @ 6 h [%]	36.8 (±0.6)	n.r.	2.3	47.8 (±0.2)	25.1 (±1.0)	n.r.	2.1 (±0.0)	37.0 (±1.7)	21.7 (±0.7)
Initial reaction rate [mM h^{-1}]	0.47 (±0.01)	n.r.	n.q.	2.1 (±0.1)	0.92 (±0.30)	n.r.	n.q.	1.97 (±0.09)	0.612 (±0.19)

[a] n.r. = no reaction; n.q. = not quantifiable; calculated values show mean of duplicate samples.

Interestingly, when the same volume ratio (0.0015) was used employing [C_8mpyrrol][NTf_2] a similar enhancement in the biotransformation activity was observed as found with [C_8dmim][NTf_2] (Fig. 2). In contrast, little effect was observed on the initial rates and overall bioconversion using the phosphonium based ILs at these low concentrations. This is in stark contrast to the work of Cornmell *et al.* who reported >200% bioconversion enhancement using an *E. coli* cloned TDO for this phosphonium IL.[8] However, the concentration of ILs used in the present work is very different from that employed in other studies with dioxygenases, where a phase ratio greater than 0.20 was used. The different behaviour may, therefore, be linked with improved mass transfer at high IL loading.

To further examine whether other structural aspects of the imidazolium and pyrrolidinium cationic part of the ILs which impacts on their polarity and solubility, play a role in the observed results, a range of ester and amide modified imidazolium and pyrrolidinium-bis(trifluoromethanesulfonyl)imide based ILs were examined at 0.0015 (V_{IL}/V_{aq}). The ester containing ILs included [$C_6H_{13}OCOCH_2$dmim][NTf_2], [$C_8H_{17}OCOCH_2$dmim][NTf_2], [$C_6H_{13}OCOCH_2$mpyrrol][NTf_2], and [$C_8H_{17}OCOCH_2$mpyrrol][NTf_2]. The presence of an ester side chains on the imidazolium cation resulted in an increase in rate and activity similar to that found for [C_8dmim][NTf_2] (see Table S6†) over the same period, with sustained activity with the longer chain, albeit slightly lower than that of [C_8dmim][NTf_2]. Surprisingly, the modified pyrrolidinium based ILs displayed no effect on the biocatalytic process. The homologous amide chains containing ILs ([$C_4H_9CH_3NCOCH_2$dmim][NTf_2] and [$C_4H_9CH_3NCOCH_2$mpyrrol][NTf_2]) were also compared with [C_8dmim][NTf_2] in the biotransformation of chlorobenzene by *P. putida* UV4 (Fig. 3).

Unlike ester side chains, the amide side chain on the imidazolium or pyrrolidinium cations showed little effect on the biotransformation rates. However, this functionalization, despite being of similar chain length to the C_6 alkyl parent, did not result in the inhibition of biocatalyst unlike that found in the case of [C_6dmim][NTf_2], for example. In this case, a linear increase to high conversion over the first six hours of biotransformation was observed for [$C_4H_9CH_3NCOCH_2$dmim][NTf_2] (Fig. 3). This may be due to the fact that the delivery of substrate in the aqueous solution may have been limited by the high viscosity of the amide functionalised IL which was greater that of [C_8dmim][NTf_2]. Alternatively, this IL might be working *via* a different mechanism from the [C_8dmim][NTf_2] and the [C_8mpyrrol][NTf_2] ILs.

Impact of hydrophobic ILs on *cis*-dihydroxylation biotransformations of Ph–X

To examine whether the biotransformation optimised for chlorobenzene could also be applied with similar enhanced conversions to the other haloarenes, these conditions were applied to fluoro-, bromo- and iodo-benzene in the presence of [C_8dmim][NTf_2] and [C_8mim][NTf_2] ILs at 0.0015 (V_{IL}/V_{aq}) and compared with the reaction in the absence of the IL.[22,23] Table 3 summarises the results of these biotranformations. For all

Table 2 Biphasic biotransformation of chlorobenzene in the presence of phosphonium and pyrrolidinium based-IL with *P. putida* UV4 (2.0% (V_{IL}/V_{aq}) IL + substrate) for OD_{600} = 0.9 or 0.35 g L^{-1} dcw[a]

	Control	$[P_{66614}][NTf_2]$	$[C_4pyrrol][NTf_2]$	$[C_8pyrrol][NTf_2]$
Conversion @ 6 h [%]	36.8 (±0.6)	35.5 (±0.7)	n.r.	15.4 (±0.0)
Initial reaction rate [mM h^{-1}]	0.47 (±0.01)	0.54 (±0.3)	n.r.	0.56 (±0.0)

[a] n.r. = no reaction; calculated values show mean of duplicate samples.

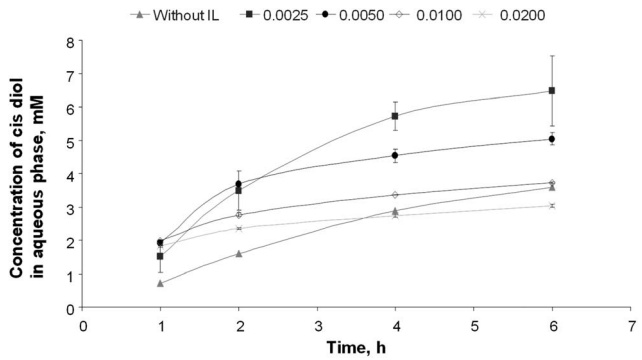


Fig. 1 Effect of volume ratio (V_{IL}/V_{aq}) on the biotransformation yield using $[C_8dmim][NTf_2]$ phase while maintaining a constant number of moles of chlorobenzene substrate, set at 0.98 mmol.

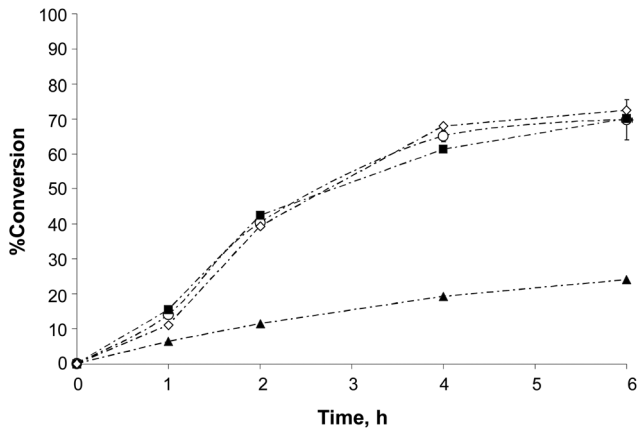


Fig. 2 Biotransformation of chlorobenzene by *P. putida* UV4 with 0.0015 (V_{IL}/V_{aq}) $[C_8mim][NTf_2]$, $[C_8dmim][NTf_2]$ and $[C_8mpyrrol][NTf_2]$. Conversion of 0.98 mmol chlorobenzene in a buffer (pH 7.2), (▲) without IL and biphasic system volumetric ratio 0.0015 (V_{IL}/V_{aq}) IL, (○) $[C_8mim][NTf_2]$, (■) $[C_8dmim][NTf_2]$ and (◇) $[C_8mpyrrol][NTf_2]$. OD_{600} = 1.8 or 0.70 g L^{-1} dcw – data points show mean of duplicate samples.

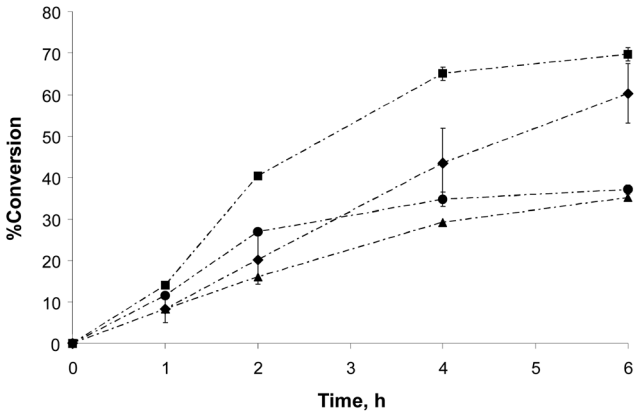


Fig. 3 Biotransformation of chlorobenzene by *P. putida* UV4 with novel-amide-IL phases (imidazolium and pyrrolidinium cations). Conversion of 0.98 mmol chlorobenzene in buffer (pH 7.2), (▲) without IL and biphasic system volumetric ratio 0.0015 (V_{IL}/V_{aq}), (■) $[C_8dmim][NTf_2]$, (◆) $[C_4H_9CH_3NCOCH_2dmim][NTf_2]$ and (●) $[C_4H_9CH_3NCOCH_2mpyrrol][NTf_2]$. OD_{600} = 1.8 or 0.70 g L^{-1} dcw – data points show mean of duplicate samples.

substrates, the presence of the ILs enhances the overall yields and rates of the biotransformations with a large increase found for fluorobenzene. This is important as the high volatility of fluorobenzene compared with that of the other halogenated arenes is a major limiting factor for its implementation in bioprocesses. Here the effect of $[C_8(d)mim][NTf_2]$ might indicate improved mass transfer for the fluorobenzene through its retention in the growth medium.

To establish whether the improve biotransformation is due to an improved mass transfer and substrate availability, the solubility of the arenes in the hydrophobic ILs (Fig. 4a) and their release into the growth medium over time (*e.g.* for iodobenzene, Fig. 4b) were determined. The ILs demonstrated an ability to host effectively the haloarenes and deliver them to the aqueous phase in a controlled manner over a 4 h period (Fig. 4b).

The solubility of all the arenes in ILs increased as a function of the chain length of the cation and its increased lipophilicity. The overall mass transfer coefficient was then determined for each of the arenes in the $[C_8(d)mim][NTf_2]$ (Fig. 5). The data clearly showed that the arene that was most effectively released from the ILs was, as expected, the fluorobenzene and that increased lipophilicity of the arene resulted in slower release rates. Importantly, the overall mass transfer of chlorobenzene from $[C_ndmim][NTf_2]$ ILs (n= 6, 8 and 10) to the growth medium remained constant at $\sim 4 \times 10^{-5}$ s^{-1} and was found to decrease only slightly from 6×10^{-5} s^{-1} to 4×10^{-5} s^{-1} on increasing the chain length (n = 6, 8, 10) for the $[C_nmim][NTf_2]$ ILs.

Impact of hydrophilic ILs on *cis*-dihydroxylation biotransformations

One proposed mechanism for the IL effect on the biotransformation of arenes using *P. putida* UV4 is that it is due to a measurable storage/release process whereby the controlled

Table 3 Biotransformation of haloarenes by *P. putida* UV4 with imidazolium hydrophobic-IL phases. Conversion of 0.98 mmol halobenzene in buffer (pH 7.2), OD_{600} = 1.8 or 0.70 g L^{-1} dcw – data points show mean of duplicate samples

	Fluorobenzene	Chlorobenzene	Bromobenzene	Iodobenzene
Without IL	5.1% ± 0.3%	33.4% ± 0.4%	36% ± 0.9%	41.9% ± 2.4%
[C_8dmim][NTf_2]	18.3% ± 1.8%	68.0% ± 1.7%	83.5% ± 1.2%	81.2% ± 3.4%
[C_8mim][NTf_2]	25.6% ± 0.4%	70.0% ± 1.6%	84.3% ± 2%	77.6% ± 1%
% Improvement	502	210	230	190

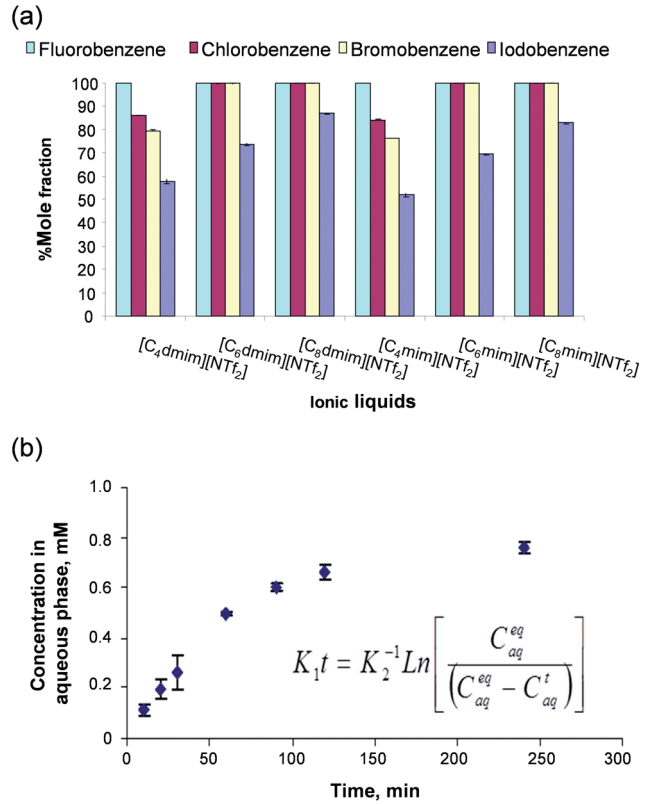


Fig. 4 (a) Solubility of haloarenes in hydrophobic ILs as a function of the imidazolium side chain length; %mole fraction of substrate to IL at equilibrium after 4 h; (b) delivery of iodoarenes into the growth medium from a saturated [C_8dmim][NTf_2]–iodobenzene solution. 0.0025 (V_{IL}/V_{aq}) system shaken at 150 rpm at 30 °C over 240 min.

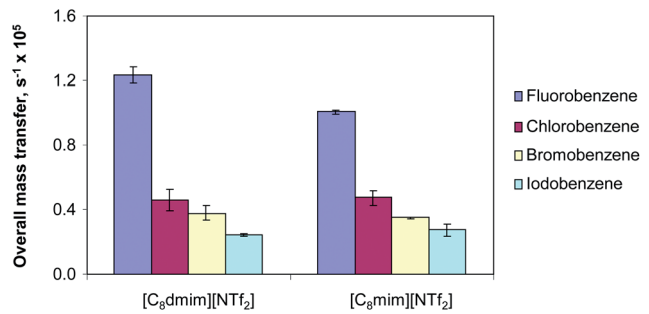


Fig. 5 Overall mass transfer coefficient of haloarenes in hydrophobic [C_8(d)mim][NTf_2] ILs.

mass transfer of the arenes into the growth medium is responsible for the observed enhancement. Yet, the extent of the enhancement on the biotransformations for each of these arenes does not correlate with simple improved mass transfer or substrate availability as the overall mass transfer coefficient remains fairly constant for three of the four haloarenes studied in [$C_n$mim][NTf_2] and [$C_n$dmim][NTf_2] (n= 6 and 8) ILs, while the overall yields and rate differ as a function of ILs.

Additionally, the presence of a methyl group at the C(2) position improves the biotransformation more effectively than the ILs with a H in the C(2) position on the imidazolium cation despite the fact that these ILs are also more toxic to the cells (Fig. 6). The increased solubility of the hydrophobic-IL in water, which was found to be higher for [C_8mim][NTf_2] than [C_8dmim]-[NTf_2] (Table S3†) is therefore unlikely to be solely responsible for the "activation through inhibition" of the biocatalysts or improved mass transfer. In contrast, [C_8mpyrrol][NTf_2], displaying further reduced aqueous solubility in water (Table S4†) when compared with [C_8mim][NTf_2] and [C_8dmim][NTf_2], displayed an inhibitory influence on the biocatalyst at high concentration and enhanced biocatalysis both in terms of rate and over yields when present at low concentration. Additionally, the C4 and C6 alkyl chain containing ILs, whilst having greater solubility in water, thus improving mass transfer and decreased cellular toxicity compared to the longer alkyl chain containing ILs, completely suppress the biocatalytic conversion (Tables 1 and 2). These latter ILs are likely, therefore, to inhibit enzyme(s)

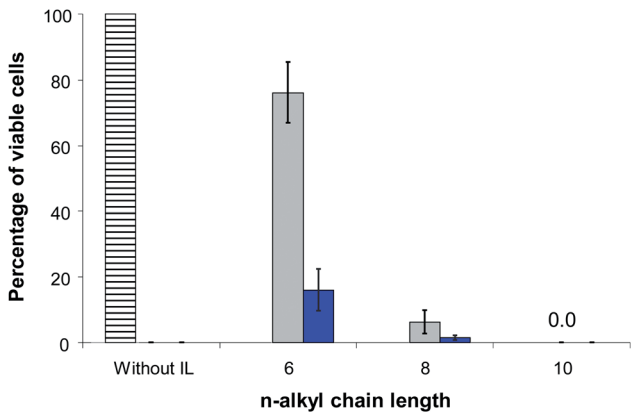


Fig. 6 Viable cell count of *P. putida* UV4 in the presence of 0.02 (V_{IL}/V_{aq}) [C(n)mim][NTf_2] (grey), [C(n)dmim][NTf_2] (blue) and control without IL (Horizontal line). Cells re-suspended in carbon source free buffer solution (pH 7.2) and exposed to the IL for 3 h. OD_{600} = 2 or 0.78 g L^{-1} dcw – data points show mean of triplicate samples with range.

of the dihydroxylation pathway which are not critical to cell survival. With increased chain length, the [C_8mpyrrol][NTf_2] inhibits the biotransformation process when present at high concentration. However at low concentrations, it appears to enable biotransformation *via* a mechanism similar to that of the C8-imidazolium-based ILs. Clearly, mass transfer and solubility of ILs in the aqueous phase are not the sole parameters responsible for the enhanced biocatalysis in the presence of the octyl-based ILs.

Additionally, two hydrophilic ILs ([C_8dmim]Br and [C_8mpyrrol]Br) displayed similar effects on biotransformations to that of the corresponding-bis(trifluoromethanesulfonyl) imide based ILs at low V_{IL}/V_{aq}. This indicates that the enhancing behaviour of the ILs is likely to stem from the cationic component (Fig. S3†). As these ILs are fully soluble, any effects due to the biphasic nature of the system with the growth medium, for example maintaining a reservoir of the substrate, which is present with the bis(trifluoromethanesulfonyl)imide ILs cannot be used to explain the effect.

Therefore, an examination of the cation on cellular biochemistry was undertaken in order to understand their mechanism of action. In this case, the ILs may enhance the biotransformation through inhibition of respiratory processes. To test this idea further, the effect of the ILs on the growth of the cells and on the oxygen dependent respiration was studied.

Synergy between ILs toxicity and whole cell *cis*-dihydroxylation using *P. putida* UV4

Possible inhibition or toxicity effects of the ILs were initially evaluated through viable cell count experiments (Fig. 6). When examined for their toxicity towards the biocatalyst, the ILs incorporating longer side chains were found to inhibit growth. This was shown clearly for [C_8mim][NTf_2], [C_{10}mim][NTf_2], [C_6dmim][NTf_2], [C_8dmim][NTf_2] and [C_{10}dmim][NTf_2]. The trend of growth inhibition observed with an increase of the alkyl chain length was in agreement with the results reported by Docherty *et al.*[24] Herein, the toxicity of the alkyl chains increased from hexyl to octyl to decyl on the imidazolium cation. This has also been observed for pyridinium and imidazolium based ILs containing octyl chains which have been reported to be more toxic than those possessing butyl and hexyl side chains.[25] Whilst the octylimidazolium based ILs showed high levels of toxicity to the cells, importantly, this IL also showed significant enhancement to whole cell biotransformations.

Bernot *et al.*[25] have proposed the possibility of lipophilic interactions of ILs with the organisms' cell membrane. It was, therefore, unclear whether the effect of the IL phase on the bacteria was due to the molecular toxicity associated with the aqueous solubility of the ILs or the phase toxicity associated with the biphasic surface, or a combination of both. The surprising increase in the conversion rate associated with a dramatic decrease in cell availability could potentially be explained by a cell lysis resulting in the immediate removal of mass transfer restrictions leading to a rate increase. Yet, even at low IL concentrations, molecular toxicity remained a critical parameter, whilst retention of microbe cellular integrity was observed even at high IL concentration (Fig. 7). Additionally, cell lysis would lead to a dilution effect with the multi-component dioxygenase complex of several orders of magnitude, as demonstrated in various studies where enzyme purification of dioxygenases is made difficult by this very effect – activity is so low that radiochemical based assays are needed to measure the activity of dioxygenases in cell-free extracts.[22]

To further test our hypothesis, the effect of the cation on oxygen respiration in resting *P. putida* UV4 cells was probed. Cells were presented to an oxygen electrode in the presence or absence of the IL cation (Fig. 3a–c in ESI†). In these experiments, the data clearly indicates that the imidazolium cation affects cellular respiration in a very similar fashion to establish inhibitors of oxidative phosphorylation, *i.e.* cyanide and azide, see ESI† for details. As the concentration of these compounds is increased oxygen turn-over is clearly inhibited in the presence of an electron donor (*e.g.* glucose). Cyanide and azide are known to inhibit directly the Cytochrome C component of the respiratory chain. Table 4 compares the kinetic parameters of the two established inhibitors with the hydrophilic ILs examined. These data suggest that the ILs are at least as effective as azide as respiratory inhibitors, under the same conditions. The C8-pyrrolidinium was included in this study as a negative control as it would not be expected to inhibit respiration from earlier data presented, herein.

In addition to establishing the inhibitory properties of [C_8dmim]Br and comparing them with that of [C_8mpyrrol]Br, their rate and overall yield enhancement was compared with the biotransformations conducted in the presence of respiratory

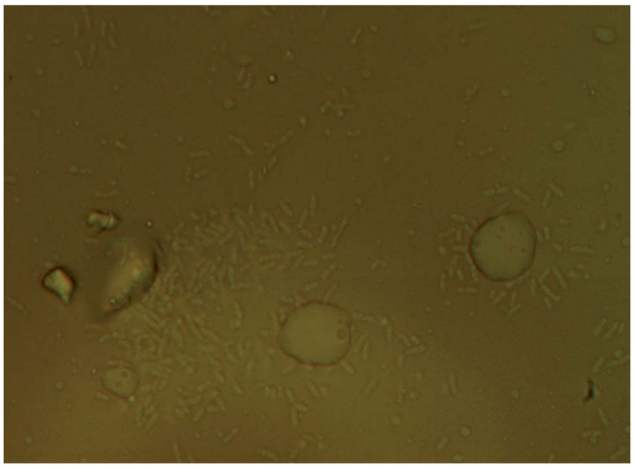
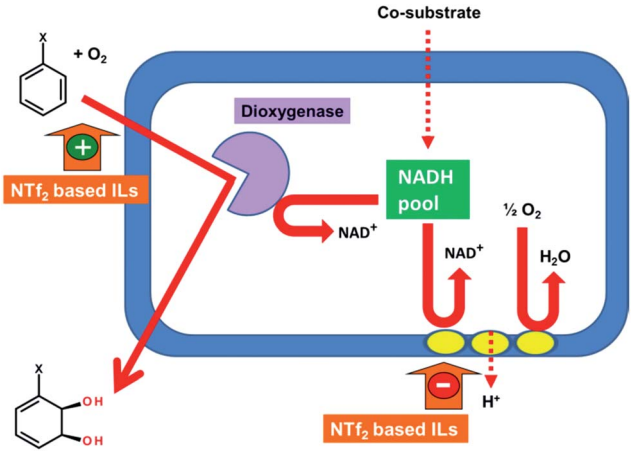


Fig. 7 Visible light microscopy image (×1000 magnification) showing *P. putida* UV4 cells incubated in the presence of the [C_8dmim][NTf_2] IL at 0.02 (V_{IL}/V_{aq}). Cells appear to be unaffected by the presence of the IL with no obvious damage to the cellular envelope. Similar analysis was also performed for [C_6dmim][NTf_2] and [C_{10}dmim][NTf_2]. Critically, the paradoxical observations shown herein can be explained from one key hypothesis that the oxidative phosphorylation in the bacterial cell is specifically inhibited by the imidazolium-based ionic liquids containing octyl groups. This would mean that the presence of ILs inhibited growth but at the same time enhanced the availability to the dioxygenase enzymes of both NADH/NAD(P)H electron donors and oxygen-obviously a key component for dihydroxylation. This effect is summarised in Scheme 1.



Scheme 1 A model for indirect enhancement of biocatalysis rates using dioxygenase enzymes in *P. putida* UV4. Diagram showing the proposed mechanism of action of the ILs on dioxygenase-mediated biotransformations: (A) – ILs may have a positive effect on biotransformation yields by enhancement of substrate uptake *via* diffusion to the enzyme active site across the cell membrane; (B) – our data also suggest that the inhibition of oxidative phosphorylation specifically by the ILs also enhances biotransformation by increasing the pool of NADH equivalents available for the dioxygenase activity provided by the co-substrate, and also reducing cellular demand for oxygen.

Table 4 Inhibition coefficient of cell respiration for *P. putida* UV4. The data show the apparent K_i obtained for the inhibition of the electron chain transport of *P. putida* UV4 by [C_8dmim]Br, KCN and NaN_3. Calculated values show mean of duplicate samples. See Fig. E.1 to E.4 in ESI

Inhibitors of *Pseudomonas putida* UV4	$K_{i(app)}$ – mmol per g per dry cell
Sodium azide	6.15 ± 0.02
Potassium cyanide	4.85 ± 0.50
[C_8dmim]Br	5.99 ± 0.43
[C_8mpyrrol]Br	15.25 ± 2.1

inhibitors. This study aimed to establish whether the known respiratory inhibitors, *i.e.* cyanide and azide, had the same effect as the imidazolium based ILs on the biotransformations (Fig. 8). The results demonstrated that the use of NaN_3 and KCN resulted in improved yields for *P. putida* UV4 catalysed biotransformations in a similar fashion to that observed for [C_8dmim]Br. These data confirmed that there is very little difference on dioxygenase biotransformations between the cytotoxic ILs and the known respiratory inhibitors.

The higher increase in yields observed in the presence of KCN than for [C_8dmim]Br may, therefore, be due to the fact that faster inhibition of cell respiration is achieved with cyanide than with [C_8dmim]Br, allowing a faster initial conversion of chlorobenzene to its *cis*-diol product. Nonetheless, the cation of specific ILs can be utilised to enhance biotransformation through inhibition of cell respiration as demonstrated in Fig. 8.

In addition, simultaneous addition of [C_8dmim]Br (2.4 mM) and KCN (0.5 mM) to the reaction media yielded synergistic enhancements, with for instance 89% overall conversion of chlorobenzene to the *cis*-diols after 8 hours (Table 5). An optimal conversion of chlorobenzene by *P. putida* UV4 was achieved in 99% in a shake flask experiment, indicating that the application of this technique to a fermenter process with controlled O_2 concentration could possibly increase the production of *cis*-diols quite dramatically, both in term of overall yields and time of reaction. The cumulative effect noted during the co-inhibition experiment would suggest that the ILs cations inhibit a different complex than the cyanide and azide anions. Unfortunately, while the data clearly indicate an effect of these ILs on the electron chain transport pathway, the experiments do not provide information on the specific enzymatic step or steps which the cations are able to compromise.

In summary, the concept that the use of ILs in biotransformations is to be limited by their property as reservoir of substrate and this as long as they remain innocuous to the biocatalyst has been challenged. While hydrophobic ILs were able to release in the growth medium a range of haloarenes in a controlled manner and therefore allow for an effective mass transfer between two phases, the outcome of this property did not differ from that of using the parent hydrophilic ILs acting as a biocatalyst enhancer, through inhibition. Here, the low molar concentration of imidazolium bromide salts (*i.e.* hydrophilic IL) used to achieve high conversion diminishes the need for its recycling, a key aspect to most IL-based processes, where the ILs are used in much greater quantities. Finally, it has been demonstrated that the inhibition of oxygen respiration during biotransformation leads to an improvement in the conversion of substrate in dioxygenase enzymatic reactions. The effect of this inhibition on rates and overall yields is of particular importance to biotransformation reactions where the biotransformed product is unstable over time, and can potentially be useful to any biotransformation processes where increased NADH availability to the intracellular medium results in increased enzymatic reaction rates. Nonetheless, it is still unclear where the IL-cation interacts within the electron transfer chain. Further experiments are required with pure enzymes from the electron transport chain to determine the mechanism of inhibition of these cations.

Experimental

The general synthetic procedures and characterisations of the ionic liquids used in this work can be found in the ESI.† All the biotransformation processes were conducted under biphasic conditions.

General biotransformation monitoring procedure

The experiments involved time-course studies of biotransformations with chlorobenzene. The residual substrate concentration of chlorobenzene has not been shown. The duplicate time-course biotransformation studies were measured by HPLC reverse phase. The aliquot of a single date point was sampled at each time interval. In each experiment 0.5 cm^3 of the biotransformation mixture was sampled and centrifuged for 2 min at high speed in an Eppendorf Microcentrifuge 5415 D. The

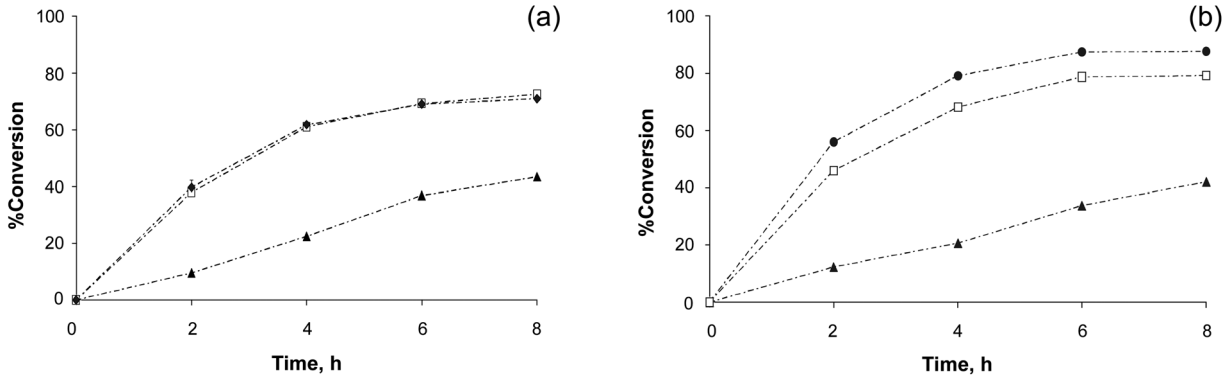


Fig. 8 Comparison between [C_8dmim]Br and (a) sodium azide and (b) potassium cyanide on the biotransformation of 0.98 mmol of chlorobenzene (pH 7.2) by *P. putida* UV4, (▲) without inhibitor, (□) 2.4 mM [C_8dmim]Br, (a) (◆) 4 mM sodium azide and (b) (●) 3.0 mM potassium cyanide. OD_{600} = 1.8 or 0.70 g L^{-1} dcw. Data points show mean of duplicate samples.

Table 5 Conversion of chlorobenzene with addition of potassium cyanide to an initial concentration of 2.4 mM [C_8dmim]Br with *P. putida* UV4. OD_{600} = 1.8 or 0.70 g L^{-1} dcw. Calculated values show mean of duplicate samples

[C_8dmim]Br – mM	Potassium cyanide – mM	Conversion of chlorobenzene after 8 h – %	Initial reaction rate – mM h^{-1}
2.4	0	72.1 ± 4.5	1.63 ± 0.00
2.4	0.25	85.9 ± 0.6	2.64 ± 0.18
2.4	0.5	89.1 ± 0.5	2.85 ± 0.11
2.4	1	81.4 ± 3.7	2.85 ± 0.10

centrifuged supernatant was diluted by half with ethanol and centrifuged at high speed to remove any insoluble material before HPLC reverse phase quantification. A standard sample of the biotransformation product chlorobenzene *cis*-diol was kindly provided by Professor D. R. Boyd and Dr N. D. Sharma of the School of Chemistry and Chemical Engineering, Queen's University Belfast. The metabolites obtained throughout the experiments reported in this work were identified by comparison of HPLC retention times and UV spectra with the standard compounds. HP1100 HPLC System (Agilent Technologies) was used with a C8 column (Supercosil 150 × 4.6 mm, 5 μm) and UV detection to separate metabolite from substrate and ionic liquids. Chlorobenzene biotransformations were analysed using a binary gradient solvent system (acetonitrile–H_2O, 1 cm^3 min^{-1} flow rate). The column was first equilibrated at 10% CH_3CN–H_2O, the run started with a linear gradient and increased to 40% CH_3CN over three minutes and followed by a second linear gradient which increased to 60% CH_3CN over the next two minutes. A third gradient increase from 60% to 100% CH_3CN was applied over 5 min, followed immediately with a linear gradient decrease from 100% to 10% CH_3CN during 3 min. An equilibration time of two minutes at 10% CH_3CN was finally applied to stabilise the C8 column for the next run. In these biotransformations only one metabolite was formed and the same HPLC method was applied.

Conclusions

We have demonstrated that under non-mass transfer conditions, cytotoxic ILs can enhance biotransformation rates with an oxygenase dependent biocatalysis system and suggested that the $[C_8dmim]^+$ cation could inhibit a different protein than that inhibited by cyanide or azide. We have presented strong evidence to support our hypothesis proposed where the cations of hydrophobic-ILs containing an octyl chain improve the biotransformation of enzymes dependent on O_2 and NADH in whole cell bio-catalyst. This mechanism of action could be highly complementary to other modes of biotransformation process optimisation. For example, it has been widely accepted that non-toxic hydrophobic IL (*e.g.* tetraalkyl phosphonium-bis(trifluoromethanesulfonyl)imide) can be used to enhance biotransformation processes. If this approach was combined with a low level of an appropriate respiratory inhibitor, *e.g.* cyanide or azide, in the same reaction vessel, one could anticipate a synergistic enhancement effect. Further studies could help validate this counter-intuitive possibility.

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