Using a biphasic ionic liquid/water reaction system to improve oxygenase-catalysed biotransformation with whole cells

Robert J. Cornmell,[a] Catherine L. Winder,[b] Stephanie Schuler,[a] Royston Goodacre[b] and Gill Stephens*[a]

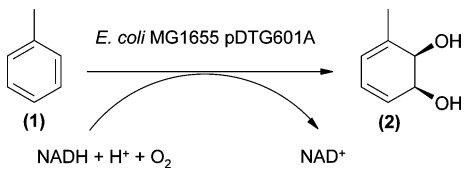
Received 29th October 2007, Accepted 17th March 2008
First published as an Advance Article on the web 11th April 2008
DOI: 10.1039/b716602h

Whole cells are usually used for oxygenase-catalysed biotransformations to ensure efficient cofactor recycling and to avoid problems with purification and stability of these complex, multi-subunit enzymes. Productivity in oxygenase-catalysed biotransformations is frequently restricted by toxicity of the substrates to the cells, but attempting to solve this problem using two-liquid phase reaction systems with conventional solvents provides only modest improvements in productivity. Therefore, we developed new, two phase systems using water-immiscible ionic liquids. Even though [$NMeOct_3$][NTf_2] and [$P_{6,6,6,14}$][NTf_2], inhibited growth of *Escherichia coli* by 39% and 23% respectively, both ionic liquids could be used to improve toluene dioxygenase-catalysed conversion of toluene to toluene *cis*-glycol using recombinant *E. coli* cells. The two-liquid phase reaction system improved resistance to toluene toxicity by 8-fold, and product concentrations increased by 2.5-fold in shake flask cultures. Product concentrations and specific product yields were improved by 200% and 238% respectively in bioreactors with an unrestricted oxygen supply.

Introduction

We wish to report the first oxygenase-catalysed biotransformation using whole cells in a biphasic ionic liquid/water reaction system. The use of ionic liquids for biotransformations with isolated enzymes is becoming well established because of their versatility and their improved safety and environmental credentials compared with conventional molecular solvents.[1,2] By contrast, the use of whole cell biocatalysts in ionic liquids is much less well developed, and has been restricted so far to hydrolyses and a range of reductions.[3–8] Herein we report the application of ionic liquids to obtain increased product concentrations using toluene dioxygenase expressed in a recombinant *Escherichia coli* strain.

Like many other oxygenase-catalysed biotransformations, the use of toluene dioxygenase to produce synthetically important *cis* diols[9–12] (Scheme 1) is hindered by low productivities due to inhibition of the whole cell biocatalyst by substrate toxicity.[13–15]



Scheme 1 Toluene dioxygenase-catalysed dihydroxylation of toluene to toluene *cis*-glycol.

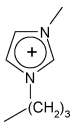
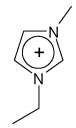
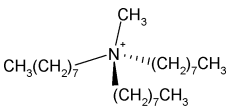
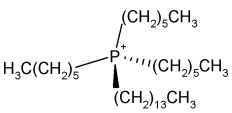
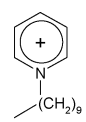
[a]*School of Chemical Engineering and Analytical Science, Manchester Interdisciplinary Biocentre, University of Manchester, 131 Princess Street, M1 7DN, Manchester, UK. E-mail: gill.stephens@manchester.ac.uk; Fax: +44 161 3068918; Tel: +44 161 3064377*
[b]*School of Chemistry, Manchester Interdisciplinary Biocentre, University of Manchester, 131 Princess Street, M1 7DN, Manchester, UK*

Two-liquid phase reaction systems should provide a simple method to overcome the inhibition.[16–21] These systems involve the use of a nontoxic, water-immiscible organic solvent as a second liquid phase in the reaction mixture. The solvent dissolves the substrate preferentially, and thus maintains a sub-inhibitory concentration of the substrate in the aqueous phase. The solvent phase also acts as a reservoir for the substrate, because the substrate equilibrates between the two phases as the cells convert it to product in the aqueous phase. Unfortunately, wide application of this technology has been hindered by the extremely restricted range of biocompatible molecular solvents.[17,20,22] Thus, optimum biocatalyst performance is only obtained when there is a serendipitous match between the physical properties of the substrate and one of the biocompatible organic solvents.[17,22] No such match is available for the toluene dioxygenase reaction; for example, using tetradecane as the water-immiscible phase provides only modest increases in product concentration using recombinant *E. coli* strains as the biocatalyst (1.2–1.4-fold).[23,24] Therefore, it is clear that a wider range of biocompatible, water-immiscible solvents is needed, and hydrophobic ionic liquids were obvious candidates for testing.

Results and discussion

The first step was to identify biocompatible ionic liquids to test in the biotransformation. For redox biotransformations using whole cell biocatalysts, the required level of biocompatibility is to maintain cell viability and metabolic activity in the presence of the solvent, so that the cells can recycle the cofactor. Conventional growth rate and viable count measurements provide a reliable and comprehensive measure of cellular integrity and metabolic potential, whereas ionic liquid-induced changes in cell

Table 1 Effect of ionic liquids on growth of *E. coli*

Cation	Anion	
	$[Cl]^-$	$[NTf_2]^-$
 **[BMim]$^+$**	Water miscible (nd)	Toxic ($\mu = 0\ h^{-1}$)
 **[EMim]$^+$**	Water miscible (nd)	Toxic ($\mu = 0\ h^{-1}$)
 **[OMim]$^+$**	Toxic ($\mu = 0\ h^{-1}$)	na
 **[$NMeOct_3$]$^+$**	Toxic ($\mu = 0\ h^{-1}$)	Biocompatible ($\mu = 0.219 \pm 0.01\ h^{-1}$)
 **[$P_{6,6,6,14}$]$^+$**	Toxic ($\mu = 0\ h^{-1}$)	Biocompatible ($\mu = 0.276 \pm 0.006\ h^{-1}$)
 **[NDecPy]$^+$**	na	Toxic ($\mu = 0\ h^{-1}$)
Tetradecane (phase ratio 0.23)	$\mu = 0.336\ h^{-1}$ (± 0.006)	
No solvent (control)	$\mu = 0.357\ h^{-1}$	

E. coli MG1655 was grown in the presence of ionic liquids with the cations and anions shown at phase ratios from 0.0025 to 0.4 (solvent volume/total volume). "Biocompatible, $\mu = x\ h^{-1}$" gives the growth rate (μ) at a phase ratio of 0.23; "Toxic, $\mu = 0\ h^{-1}$" indicates that there was no growth at any concentration tested; nd, not determined for the reason stated; na, not available. The growth rates (μ) without solvent and with tetradecane were also measured as controls. Data are the average for 5 replicates. The percentage inhibition was calculated from eqn (1).

$$\%\,\text{inhibition} = \frac{[\mu_{\text{control}} - \mu_{\text{solvent}}]100}{\mu_{\text{control}}} \quad (1)$$

morphology complicate quantitative measurements of viability using vital stains. Therefore, we measured growth rates for *E. coli* MG1655 using high-throughput nephelometry in the presence of various water-immiscible ionic liquids (Table 1). Control cultures were grown in the absence of solvent, and with tetradecane. We used the host strain without the recombinant plasmid to examine the effect of the ionic liquids alone, since this would avoid physiological complications caused by maintenance of the high copy number plasmid, over expression of the biocatalyst genes and the biotransformation.

In the absence of biotransformation, tetradecane inhibited growth by only 5.9% compared with growth in the absence of solvent (Table 1) when added to a phase ratio of 0.23 (solvent volume/total volume). $[P_{6,6,6,14}][NTf_2]$ was more inhibitory than tetradecane but was, nevertheless, the least inhibitory of the ionic liquids tested, with 23% inhibition at a phase ratio of

0.23. [$NMeOct_3$][NTf_2] inhibited growth by 39% at a phase ratio of 0.23. Thus, both of these ionic liquids were sufficiently biocompatible to allow growth at reasonable rates. By contrast, none of the other water-immiscible ionic liquids tested allowed growth. Thus, changing the anion to chloride resulted in complete growth inhibition even when the phase ratio of [$P_{6,6,6,14}$][Cl] and [$NMeOct_3$][Cl] was decreased to 0.0025. [OMim][Cl] was also inhibitory. The homologues with shorter side chains are water miscible, and, therefore, were not tested. We did not test the [PF_6] anion, since it hydrolyses in water to form HF.[25,26]

Since [$NMeOct_3$][NTf_2] and [$P_{6,6,6,14}$][NTf_2] were found to be biocompatible, we tested a wider range of [NTf_2] salts for biocompatibility, but [BMim][NTf_2], [EMim][NTf_2] and [NDecPy][NTf_2] inhibited growth completely at phase ratios between 0.0025 and 0.4. This demonstrates that both the cation and anion determine toxicity in a rather unpredictable manner. Furthermore, the sensitivity to ionic liquids varies between different species, since [BMim][NTf_2] is suitable for use with *Lactobacillus kefir*,[3] but not *E. coli.*

Since [$NMeOct_3$][NTf_2] and [$P_{6,6,6,14}$][NTf_2] were sufficiently biocompatible to allow growth of *E. coli* MG1655, we tested their effect on the toluene dioxygenase-catalysed biotransformation of toluene **1** to toluene *cis*-glycol **2** (Scheme 1) using growing cultures of the recombinant *E. coli* strain, MG1655 pDTG601A. The substrate **1** dissolved preferentially in the ionic liquids, and we found that the K_D values were 0.68 and 0.60 in the [$NMeOct_3$][NTf_2]/media and [$P_{6,6,6,14}$][NTf_2]/media systems, respectively. In contrast, **2** partitioned completely into the aqueous phase when solutions of **2** (5 mM and 10 mM) were equilibrated with [$NMeOct_3$][NTf_2] and [$P_{6,6,6,14}$][NTf_2] (phase ratio 0.23).

We determined that the optimum initial toluene concentration for the biotransformation in single phase aqueous cultures was 3.76 mmol l^{-1} at 50 ml scale. When the biotransformation was tested using [$NMeOct_3$][NTf_2] and [$P_{6,6,6,14}$][NTf_2] under the same conditions as a benchmark, the reaction rate and final product concentrations were unaffected by the presence of either ionic liquid (Fig. 1). The overall chemical yield of product in each reaction system remained unchanged at 87–90% (± 4%) after 20 h. As reported previously,[23] a small quantity of 3-methylcatechol (0.5–0.62 mM) was also formed.

Although product formation was unaffected by the ionic liquids, viable counts were slightly lower (Fig. 1). Furthermore, the growth rate of the cells during the biotransformation was 0.25 h^{-1} and 0.21 h^{-1} in the presence of [$P_{6,6,6,14}$][NTf_2] and [$NMeOct_3$][NTf_2] respectively, compared to 0.37 h^{-1} for control biotransformations without solvent. Therefore, cell growth was more sensitive to inhibition by the ionic liquids than the biotransformation. This is unsurprising because growth depends on a great many cellular reactions that are potentially prone to inhibition, whereas the biotransformation presents far fewer targets for inhibition, since it depends only on the dioxygenase and a few supporting reactions.

The toxicity of the ionic liquids was intensified during the biotransformation. Thus, [$P_{6,6,6,14}$][NTf_2] inhibited growth by 32% during the biotransformation, compared with 23% in the absence of toluene and the recombinant plasmid (Table 1). Similarly, [$NMeOct_3$][NTf_2] inhibited growth by 43% during the biotransformation, compared with 39% in the absence of

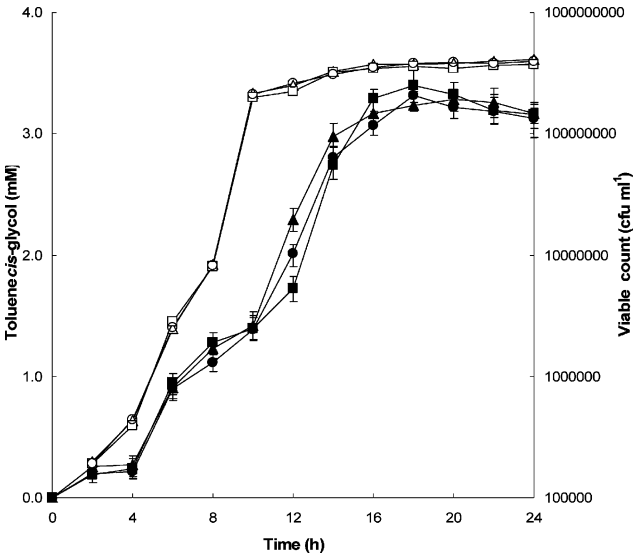


Fig. 1 Effect of ionic liquids on biotransformation of toluene at the optimum concentration (3.76 mmol l^{-1}) by *E. coli* MG1655(pDTG601A). Concentrations of toluene *cis*-glycol (closed symbols) and viable counts (open symbols) were measured for cultures grown without additions (▲,△) or in the presence of [$P_{6,6,6,14}$][NTf_2] (●,○) or [$NMeOct_3$][NTf_2] (■,□). Data points are the average of 3 replicates and error bars show the maximum variance from the mean.

toluene. On the other hand, the growth rate under biotransformation conditions in the absence of solvent was 3.6% higher than in control cultures without biotransformation. Therefore, the presence of substrate, product, recombinant plasmid and inducer intensify the growth inhibition by the ionic liquids but do not affect the biotransformation.

Since [$NMeOct_3$][NTf_2] and [$P_{6,6,6,14}$][NTf_2] did not have any adverse effects on the biotransformation, they were good candidates for further development of the two-liquid phase reaction system with ionic liquids. Therefore, we tested the ability of the ionic liquids to protect the cells from toluene toxicity, by checking for growth over a range of toluene concentrations. In cultures grown without solvent, the maximum toluene concentration tolerated by the biocatalyst was 9.4 mmol l^{-1}, since growth was inhibited completely at higher concentrations. There was still significant inhibition with 9.4 mmol l^{-1} toluene, since the final biomass concentration was only 59% of the concentration in the cultures grown with the optimum toluene concentration (3.76 mmol l^{-1}).

In the two-liquid phase system with either [$NMeOct_3$][NTf_2] or [$P_{6,6,6,14}$][NTf_2] (phase ratio 0.23), the maximum toluene concentration tolerated was 75.2 mmol l^{-1}. This was an 8-fold increase compared with the control cultures without ionic liquid. Therefore, the ionic liquids were extremely effective in protecting the cells from toluene toxicity.

The protection against toluene toxicity made it possible to run the biotransformation with a greatly increased toluene concentration. This was extremely beneficial, since it resulted in a 2.5-fold increase in toluene *cis*-glycol concentration (to *ca.* 20 mM) in the presence of the ionic liquids compared with the single phase system (Table 2). This is much better than the improvement available using recombinant *E. coli* with tetradecane as the solvent, which only gives a maximum of

Table 2 Effect of ionic liquids on biotransformation of toluene and on the maximum toluene concentration tolerated by the cells

Reaction system	Substrate **1** concentration/mmol l^{-1}	Product **2** concentration/mM	Product yield **2** (%)	Specific yield of **2**/mmol g^{-1} dry weight
Aqueous	9.4	8.31 (± 0.36)	88.4 (± 3.8)	0.83 (± 0.04)
$[P_{6,6,6,14}][NTf_2]$	75.2	20.7 (± 0.13)	27.5 (± 0.17)	2.68 (± 0.02)
$[NMeOct_3][NTf_2]$	75.2	20.3 (± 0.46)	27.0 (± 0.6)	2.89 (± 0.07)

E. coli MG1655 pDTG601A was grown in the presence and absence of ionic liquids (3 replicates), with the maximum concentration of toluene tolerated in the reaction system under test conditions. Product concentration, chemical yield and specific yield were measured after 24 h.

1.2- to 1.4-fold increase depending on the culture system.[23,24] Most importantly, the product concentration with the ionic liquids was 10.5-fold higher than in the two phase system with tetradecane.[24]

There was no evident difference between the performance of the two ionic liquids in the 50 ml scale biotransformations, even though $[NMeOct_3][NTf_2]$ inhibited growth more than $[P_{6,6,6,14}][NTf_2]$. This further emphasises that the biotransformation is not affected by the growth inhibition caused by the ionic liquid. It is also important to note that there was no emulsion formation in any of the cultures grown with the ionic liquids, whereas there are serious problems with emulsification when tetradecane is used, due to release of surface active materials by the cells.[27–30]

The only problem was that the conversion of toluene to toluene *cis*-glycol was incomplete (27% yield). However, the oxygen supply was restricted to the air available in the culture headspace because the culture vessels had to be sealed to prevent loss of substrate. This suggested that the incomplete conversion could be due to oxygen limitation (it should be noted that this is unrelated to the low solubility of oxygen in ionic liquids,[31] since the cells were in the aqueous phase and oxygen is reasonably soluble in water). This hypothesis was tested by using a bioreactor for the biotransformation (at 1.25 L scale) so that an unrestricted oxygen supply could be provided. Thus, the dissolved oxygen concentration was maintained above 5% by automatic control of the stirrer speed.

Initially, we grew triplicate control cultures in the bioreactor in the absence of the ionic liquids, using a feed of liquid toluene. *E. coli* is extremely sensitive to liquid toluene, and this meant that growth and product formation varied significantly between the replicate control cultures (Fig. 2). Thus, the final toluene *cis*-glycol concentration was 26 mM, 19 mM and 9.6 mM in the three replicate biotransformations, and the specific product yields were 5.5, 3.2 and 1.69 mmol $g^{-1}_{\text{dry weight cells}}$ respectively. In each case, product formation stopped when the cells had stopped growing and had begun to lyse. We suggest that the variability was due to exposure of the cells to droplets of liquid toluene, which is known to be extremely growth inhibitory compared with dissolved toluene.[32]

The next step was to repeat the biotransformations with ionic liquid. $[P_{6,6,6,14}][NTf_2]$ (phase ratio 0.23) was chosen as the water-immiscible phase because it was less inhibitory than $[NMeOct_3][NTf_2]$, and because it is cheaper and more readily available in bulk. Nevertheless, the number of replicate cultures had to be limited to two due to the cost of these experiments (£1500 for the ionic liquid alone) It should be noted that industrial implementation would increase demand and drive down the cost, whilst further cost reductions can be achieved by recycling the solvent, as for any other solvent in an industrial process.

Using the ionic liquid allowed the use of significantly higher feed rates of liquid toluene (Fig. 2). In theory, the maximum feed rate should be 8 times higher than the cells would tolerate in the absence of the ionic liquid, since the toluene concentration tolerated in flask cultures increased 8-fold in the presence of ionic liquid. However, we started the toluene feed more cautiously than this, at the same rate as the control, and then increased the feed rate as quickly as possible thereafter, by checking that there was no growth inhibition, cell lysis or inhibition of product formation after each increase in rate. Therefore, it was possible to adjust the toluene feed rate by trial and error to give a substantial increase over the rate tolerated by control cultures. This resulted in a significant increase in mean product concentration (55.5 ± 22.5 mM) compared with the flask cultures (20.7 ± 0.13 mM), indicating that productivity was indeed limited by the oxygen supply in the flasks. Like the cultures without ionic liquids, the biotransformations stopped when the cells stopped growing and the viable count had begun to fall. We assumed that the death of the cells had caused irreversible loss of activity.

The final product yields per unit biomass were very similar in the replicate cultures, at 12.5 mmol $g^{-1}_{\text{dry weight cells}}$ and 10.9 mmol $g^{-1}_{\text{dry weight cells}}$ for replicate 1 and 2, respectively. However, the final toluene *cis*-glycol concentration in replicate 1 was 2.3-times higher (78 mM) than in replicate 2 (33 mM). By contrast, product formation was very reproducible in the flask cultures. In the flasks, the toluene was dissolved directly in the ionic liquid at the start of the experiment, whereas liquid toluene was fed continuously to the two-phase medium in the bioreactor. This meant that each droplet of liquid toluene would have to equilibrate between the two liquid phases, and the cells would be exposed periodically to high levels of liquid toluene in the aqueous phase on a random basis. We suggest that this would be sufficient to cause the observed variations in cell growth and product formation in the bioreactor.

Despite the variations in product concentration, it was evident that the average specific product yield in the presence of $[P_{6,6,6,14}][NTf_2]$ in the bioreactor was significantly

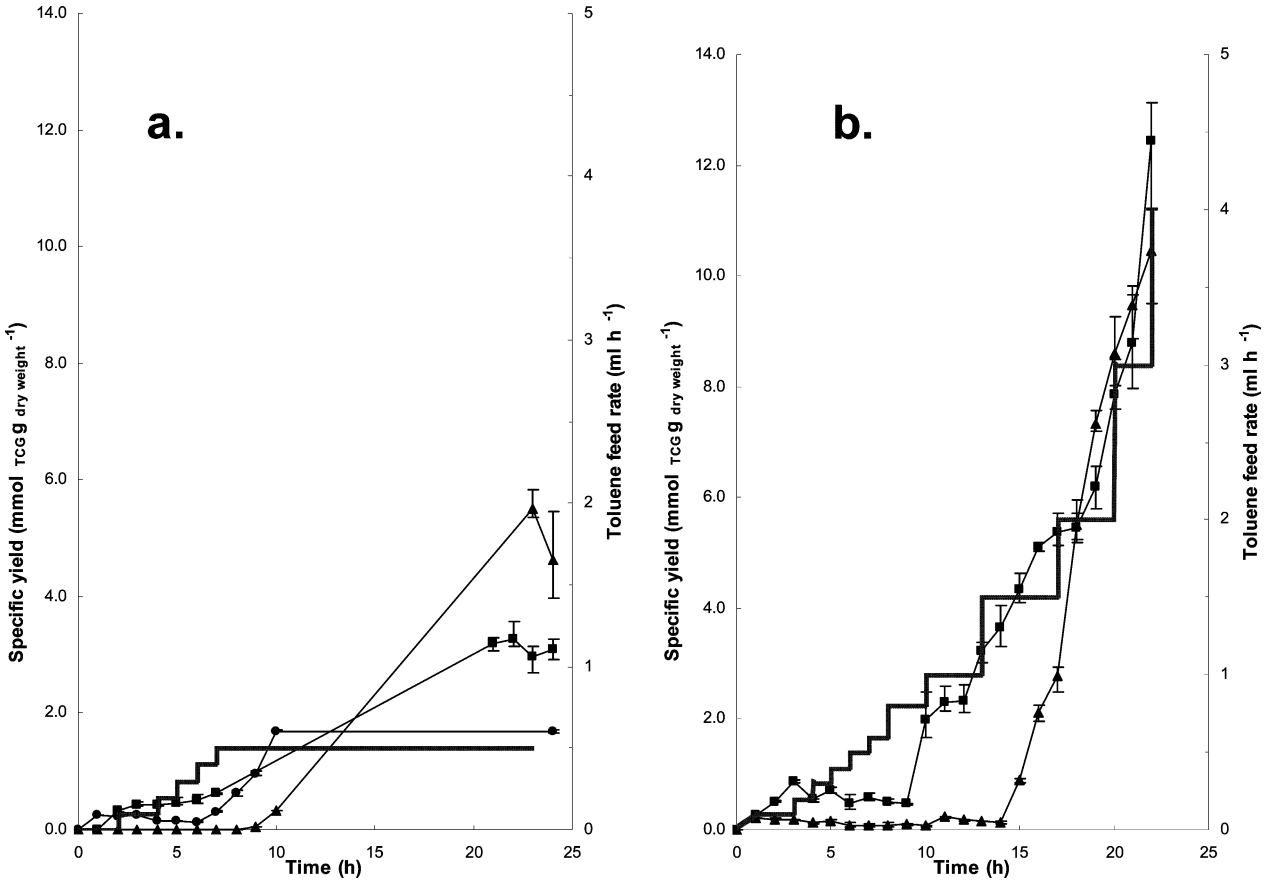


Fig. 2 Effect of $[P_{6,6,6,14}][NTf_2]$ on toluene *cis*-glycol production in bioreactors using *E. coli* MG1655(pDTG601A). *E. coli* MG1655 pDTG601A was grown at 1.25 L scale in bioreactor cultures with a feed of liquid toluene in the absence (a; 3 replicate cultures, ●,▲,■) and presence of $[P_{6,6,6,14}][NTf_2]$ at a phase ratio of 0.23 (b; 2 replicate cultures, ●,▲). Specific yields of toluene *cis*-glycol are expressed as $mmol_{TCG}\ g^{-1}{}_{dry\ weight}$, and are the average of 3 replicate analyses. Error bars show the maximum variance from the mean. Toluene feed rates (thick continuous line) are provided in the right hand axis.

better (11.7 mmol $g^{-1}{}_{dry\ weight}$) than in the flask cultures (2.68 mmol $g^{-1}{}_{dry\ weight}$) and in the monophasic biotransformations in the bioreactor (3.46 mmol $g^{-1}{}_{dry\ weight}$). It should be noted that the chemical yields cannot be calculated accurately, because the reactor was sparged with air and this would strip the volatile substrate, toluene, out of the reactor at a significant rate. However, an approximate calculation of the *minimum* yield can be based on the total volume of toluene added during the fermentation (270 mmol). Thus, the apparent minimum product yields were 36% and 15%, calculated as $mmol_{TCG}/mmol_{toluene\ added} \times 100$. The stripping of toluene by the air flow would mean that the *actual* yields should be signifcantly higher than this.

Further work is now needed to optimise the regimes for growth and toluene feeding to obtain reproducible biotransformation in the presence of ionic liquids in bioreactors, although the current cost of the ionic liquid takes this beyond the scope of academic research.

Experimental

Chemicals: Authentic toluene *cis*-glycol samples were kindly provided by Chris Allen (Queen's University, Belfast). Ionic liquids were obtained as follows: [OMim][Cl] and [NDecPy][Cl] from Ken Seddon (QUILL, Queen's University, Belfast); $[NMeOct_3][NTf_2]$, from Solvent Innovation; $[BMim][NTf_2]$, $[EMim][PF_6]$, $[EMim][NTf_2]$, $[NMeOct_3][Cl]$, $[P_{6,6,6,14}][Cl]$ and $[P_{6,6,6,14}][NTf_2]$ from Sigma-Aldrich. Other chemicals were from Sigma-Aldrich unless stated otherwise.

Measurement of growth rates: Growth rates of *E. coli* K12 strain MG1655[33] were measured in MSX medium[27] in microcultures (200 µl including solvent) using a Bioscreen C incubator/plate reader (Thermo Labsystems, Franklin, MA). Biotransformation of toluene was tested using *E. coli* MG1655 transformed[34] with pDTG601A[35] (provided by Rebecca Parales, University of California, Davis).

Biotransformations: Cultures for small scale biotransformations (50 ml)[27] were grown at 37 °C and 200 rpm with toluene in the presence or absence of solvents at a phase ratio of 0.23. Isopropyl-β-D-thiogalactoside (IPTG; 1 mM) was added after 5 h. All solvents were self sterile, since there was no microbial growth when 1 ml solvent was incubated in sterile medium for 30 h at 37 °C. The phase ratio was kept constant by taking samples from both the aqueous and solvent phases in the required volume ratio.

Larger scale biotransformations were performed in a Pierre Guerin bioreactor (Mauze, France) fitted with a 2 L vessel and operated at a working volume of 1.25L. Double strength

MS medium with ampicillin (100 μg ml^{-1}) was inoculated with 750 μl of a culture which had been grown in MSX medium[27] with 100 μg ml^{-1} ampicillin for 18 h at 37 °C. The culture was grown for approximately 15 h at 37 °C, pH 6.8 ± 0.1 (controlled by automatic addition of 1 M HCl or 1 M NaOH), and a dissolved oxygen concentration above 5%, maintained by automatic control of stirrer speed (minimum 200 rpm) with an air flow of 25 l h^{-1}. Foaming was controlled by manual addition of the minimum volume of polypropylene glycol (P2000, 3% v/v) needed to break any foam, as soon as it began to form. When the OD_{680nm} reached 2.0, the pH was increased to 7.2 to prevent decomposition of toluene *cis*-glycol, and IPTG (1 mM) was added 30 min later, together with a feed of liquid toluene, added using a syringe pump (model 100 series, KD Scientific, USA). For cultures grown without ionic liquid, the toluene feed rate was maintained at 0.1 ml h^{-1} for 2 h, and then increased every hour at a rate of 0.1 ml h^{-1} to 0.5 ml h^{-1}, and then maintained at this rate for a further 17 h. Overall, this meant that 90 mmol toluene were fed to the cultures. A feed of glucose salts solution[15,27]was started after 2 h, when the glucose in the culture medium had been depleted, and was fed at a rate sufficient to continue exponential growth of the bacteria, whilst also providing an electron donor for the biotransformation. The final culture volume was 1.25 L, after addition of the feeds and removal of samples. For the two phase reactions, the initial culture volume was 963 mL. $[P_{6,6,6,14}][NTf_2]$ (287 mL) was pumped into the bioreactor when the OD was 2, so that the total culture volume was 1.25 L. The cells were allowed to adapt for 2 h before beginning the biotransformation as described above. The toluene feed was begun at 0.1 ml h^{-1} for 2 h, and was increased hourly by 0.1 ml h^{-1} until a rate of 0.8 ml h^{-1} was established. After 10 h, the feed rate was increased to 1 ml h^{-1}, then further increased to 1.5 ml h^{-1} at 13 h; at 17 h, the feed was increased to 2 ml h^{-1} and then to 3 ml h^{-1} at 20 h. A final toluene feed rate of 4 ml h^{-1} was established at 22 h and held for 2 h. Overall, 270 mmol toluene were fed to the cultures. As before, the glucose feed was started after 2 h, but further aliquots of ionic liquid were added to compensate for increased culture volume due to glucose feeding, and thus keep the phase ratio as constant as possible. It was unnecessary to remove ionic liquid from the cultures to compensate for sampling, because the cultures were completely mixed, and all samples contained both the ionic liquid and aqueous phases in the correct phase ratio. The phases were allowed to separate before chemical analysis of the aqueous phase. The volume of the culture was 1.25 L at the end of the fermentation, including the ionic liquid.

Analytical methods: Product concentrations were determined by HPLC as described previously[27] except that a Spherisorb ODS (2) stainless steel analytical column (25 cm × 4.6 mm, 5 μm particle size) was used. Viable counts were measured by plating serial dilutions of cultures in physiological saline (0.9% NaCl) on LB agar containing ampicillin (100 μg ml^{-1}).

Conclusion

The water-immiscible ionic liquids, $[NMeOct_3][NTf_2]$ and $[P_{6,6,6,14}][NTf_2]$, are biocompatible for *E. coli*, and have been used successfully to improve productivity and yield in the toluene dioxygenase-catalysed conversion of toluene to toluene *cis*-glycol using a recombinant *E. coli* strain. To our knowledge, this is the first demonstration that ionic liquids can be used to improve an oxygenase-catalysed biotransformation using whole cells. The ionic liquids protect the biocatalyst from substrate toxicity by altering the availability of toluene in the aqueous phase. Therefore, using ionic liquids allows the formation of significantly higher product concentrations than in aqueous reaction systems or the conventional two-liquid phase system containing tetradecane. This alone is sufficient motive for further development of ionic liquid based reaction systems for redox biotransformations. The additional benefits accruing from lack of solvent emulsification, improved safety and lower environmental impact will push whole cell biocatalysis in ionic liquids to a much higher position on the research agenda.

Acknowledgements

This work was supported by the Engineering and Biological Systems committee of the UK Biotechnology and Biological Sciences Research Council. We thank Ken Seddon (QUILL, Queen's University, Belfast) for providing ionic liquids and for numerous helpful discussions. We are also grateful to Chris Allen (Queen's University, Belfast) for providing analytical standards and Rebecca Parales (University of California, Davis) for providing the plasmid, pDTG601A.

Notes and references

1 S. Park and R. J. Kazlauskas, *Curr. Opin. Biotechnol.*, 2003, **14**, 432–437.
2 F. van Rantwijk, R. M. Lau and R. A. Sheldon, *Trends Biotechnol.*, 2003, **21**, 131–138.
3 H. Pfruender, M. Amidjojo, U. Kragl and D. Weuster-Botz, *Angew. Chem., Int. Ed.*, 2004, **43**, 4529–4531.
4 S. G. Cull, J. D. Holbrey, V. Vargas-Mora, K. R. Seddon and G. J. Lye, *Biotechnol. Bioeng.*, 2000, **69**, 227–233.
5 M. D. Baumann, A. J. Daugulis and P. G. Jessop, *Appl. Microbiol. Biotechnol.*, 2005, **67**, 131–137.
6 M. Okochi, I. Nakagawa, T. Kobayashi, S. Hayashi, S. Furusaki and H. Honda, *J. Biotechnol.*, 2007, **128**, 376–382.
7 Y. Fatima, H. Kansal, P. Soni and U. C. Banerjee, *Process Biochem.*, 2007, **42**, 1412–1418.
8 S. Brautigam, S. Bringer-Meyer and D. Weuster-Botz, *Tetrahedron: Asymm.*, 2007, **18**, 1883–1887.
9 T. Hudlicky, H. Luna, J. D. Price and F. Rulin, *J. Org. Chem.*, 1990, **55**, 4683–4687.
10 T. Hudlicky, D. Gonzalez and D. T. Gibson, *Aldrichim. Acta*, 1999, **32**, 35–62.
11 D. R. Boyd, N. D. Sharma and C. C. R. Allen, *Curr. Opin. Biotechnol.*, 2001, **12**, 564–573.
12 D. R. Boyd and G. N. Sheldrake, *Nat. Prod. Rep.*, 1998, **15**, 309–324.
13 D. R. Boyd, N. D. Sharma, B. Byrne, M. V. Hand, J. F. Malone, G. N. Sheldrake, J. Blacker and H. Dalton, *J. Chem. Soc., Perkin Trans. 1*, 1998, 1935–1943.
14 D. R. Boyd, N. D. Sharma, N. I. Bowers, J. Duffy, J. S. Harrison and H. Dalton, *J. Chem. Soc., Perkin Trans. 1*, 2000, **9**, 1345–1350.
15 R. O. Jenkins, G. M. Stephens and H. Dalton, *Biotechnol. Bioeng.*, 1987, **29**, 873–883.
16 K. Jablczynski, W. Wieckowski and A. Klein, *Z. Anorg. Allg. Chem*, 1925, **143**, 343.
17 C. Laane, S. Boeren, K. Vos and C. Veeger, *Biotechnol. Bioeng.*, 1987, **30**, 81–87.
18 M. D. Lilly, *Philos. Trans. R. Soc. London, Ser. B*, 1983, **300**, 391–398.
19 G. J. Lye and J. M. Woodley, in *Multiphase Bioreactor Design*, ed. J. M. S. Cabral, M. Mota and J. Tramper, Taylor and Francis, London, Editon edn, 2001, p. 115.
20 G. J. Salter and D. B. Kell, *Crit. Rev. Biotechnol.*, 1995, **15**, 139–177.

21 M. Vermue, J. Sikkema, A. Verheul, R. Bakker and J. Tramper, *Biotechnol. Bioeng.*, 1993, **42**, 747–758.
22 A. J. J. Straathof, *Biotechnol. Prog.*, 2003, **19**, 755–762.
23 P. Phumathon and G. M. Stephens, *Enzyme Microb. Technol.*, 1999, **25**, 810–819.
24 J. T. Tsai, L. P. Wahbi, G. A. Dervakos and G. M. Stephens, *Biotechnol. Lett.*, 1996, **18**, 241–244.
25 K. R. Seddon, A. Stark and M. J. Torres, *Pure Appl. Chem.*, 2000, **72**, 2275–2287.
26 A. E. Visser, R. P. Swatloski, W. M. Reichert, S. T. Griffin and R. D. Rogers, *Ind. Eng. Chem. Res.*, 2000, **39**, 3596–3604.
27 L. P. Wahbi, D. Gokhale, S. Minter and G. M. Stephens, *Enzyme Microb. Technol.*, 1996, **19**, 297–306.
28 H. Baldascini and D. B. Janssen, *Enzyme Microb. Technol.*, 2005, **36**, 285–293.
29 A. Schmid, A. Kollmer and B. Witholt, *Enzyme Microb. Technol.*, 1998, **22**, 487–493.
30 J. Kieboom, J. J. Dennis, J. A. M. de Bont and G. J. Zylstra, *J. Biol. Chem.*, 1998, **273**, 85–91.
31 J. L. Anthony, E. J. Maginn and J. F. Brennecke, *J. Phys. Chem. B*, 2002, **106**, 7315–7320.
32 C. J. Hack, J. M. Woodley, M. D. Lilly and J. M. Liddell, *Enzyme Microb. Technol.*, 2000, **26**, 530–536.
33 F. R. Blattner, G. Plunkett, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. ColladoVides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau and Y. Shao, *Science*, 1997, **277**, 1453–1462.
34 D. Hanahan, *J. Mol. Biol.*, 1983, **166**, 557–580.
35 G. J. Zylstra and D. T. Gibson, *J. Biol. Chem.*, 1989, **264**, 14940–14946.