Effects of water-miscible ionic liquids on cell growth and nitro reduction using *Clostridium sporogenes*

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Water miscible ionic liquids had various effects on the physiology of *Clostridium sporogenes* when tested as additives in culture media or reaction media for reduction of nitrobenzene. 2-Hydroxy ethyl trimethyl-ammonium dimethyl phosphate ([EtOHNMe$_3$][Me$_2$PO$_4$]) and *N,N*-dimethylethanolammonium acetate (DMEAA) increased the growth rate of *C. sporogenes* by as much as 28%, suggesting that they were either metabolised or increased the availability of nutrients. By contrast, 1-butyl-3-methylimidazolium tetrafluoroborate ([BMim][BF$_4$]) and AMMOENG™ 100 inhibited growth. Although 1-ethyl-3-methyl imidazolium ethylsulfate ([EMim][EtSO$_4$]; 2% w/v) inhibited growth by 58%, it was sufficiently non-toxic to allow efficient reduction of nitrobenzene using harvested cells, providing aniline yields up to 79%. The high product yield with reactions in [EMim][EtSO$_4$] represented a significant improvement over conventional solvents, since the yield was only 8% in aqueous ethanol (4% v/v) and 45% in a biphasic heptane/aqueous system (phase ratio 0.33). The ionic liquid appeared to suppress unproductive substrate consumption by an unknown mechanism

Introduction

Ionic liquids are salts in which the crystal packing is so defective that they are liquid at room temperature.[1,2] Since they are composed of ions, their behaviour is different from conventional organic solvents, and this has lead to intense interest in this unique class of compounds. Most importantly, the polarity and solvent miscibility are adjustable over the whole range required for chemical processing, through modification of the composite cation and anion structure. This provides unprecedented opportunities to tune the solvent to match the needs of the process with exquisite precision. In addition to this, most ionic liquids have near-zero vapour pressure and excellent thermal stability, and biodegradable and non-toxic ionic liquids can be designed.[3–11] This means that ionic liquids offer the prospect of cleaner, greener chemical manufacturing. These 'futuristic' solvents are now finding uses in a wide range of chemical and biocatalytic processes.[1,2,12,13]

Ionic liquids are particularly attractive for biocatalysis because there is such a restricted range of conventional solvents that is suitable for use in biotransformations.[14] There are already numerous examples of improved enzyme biocatalysis in ionic liquids, resulting from the ability to tune the solvent structure for optimal solvation of substrate and product whilst also retaining enzyme activity. Additional benefits include improved enantioselectivities, higher yields, improved enzyme stability and stimulated activity in comparison to organic solvents.[2,9]

In contrast, there have been rather few reports on the use of ionic liquids for whole cell biotransformations,[15–23] even though ionic liquids may offer considerable advantages over conventional solvents. In particular, conventional solvents tend to be toxic to whole microbial cells, and there is a real need for better solvents.[14,24–26] Indeed, there have been some notable successes in using ionic liquids for whole cell biotransformations[16,17,21,22] and some preliminary rules for solvent selection are beginning to emerge.[27,28] However, most of the studies have focused on water-immiscible ionic liquids. Whilst two-liquid phase reaction systems are extremely useful for biotransformations involving non-polar toxic substrates, there are numerous examples of biotransformations where substrate delivery depends simply on solubilisation of relatively non-toxic substrates, and there is no need to resort to the complexity of multi-phase processing.[24,25] In such cases, water-miscible, conventional solvents[29–31] or detergents[32] are frequently used for substrate delivery. However, these materials can be extremely toxic to cells unless used at low concentrations,[25,26,33] and this restricts the substrate concentrations that can be achieved in the process. Therefore, water miscible ionic liquids may provide an alternative to deliver poorly water soluble substrates in whole cell biotransformations.[15]

In this study, we tested the effect of water-miscible ionic liquids on *Clostridium sporogenes*, a proteolytic spore-forming anaerobe. This organism is an extremely versatile biocatalyst for transformation of nitrogen-containing precursors to chiral and achiral amines,[34–37] which together comprise the single most useful group of precursors for manufacturing pharmaceuticals.[38,39] Nitro reduction is a particularly attractive target, due to the

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ease with which the substrates can be prepared. *C. sporogenes* is known to reduce *p*-nitrobenzoate and 2-nitroethanol.[34,35] Although these substrates are water soluble, the most useful substrates are much less soluble and this would restrict productivity in the biotransformations. Therefore, we studied the use of water-miscible ionic liquids to deliver water-insoluble substrates. We used nitrobenzene as a simple example of a poorly water soluble substrate to provide a suitable model for initial feasibility studies.

We wish to report that 1-ethyl-3-methyl imidazolium ethylsulfate **5** ([EMim][$EtSO_4$]; Fig. 1) provided improved reduction of nitrobenzene to aniline using *Clostridium sporogenes* compared with the conventional solvents, heptane and ethanol. In addition, we report that selection of water-miscible ionic liquids for biotransformations requires care, due to unpredictable effects on cell physiology and the potential for reactions with microbial culture media.

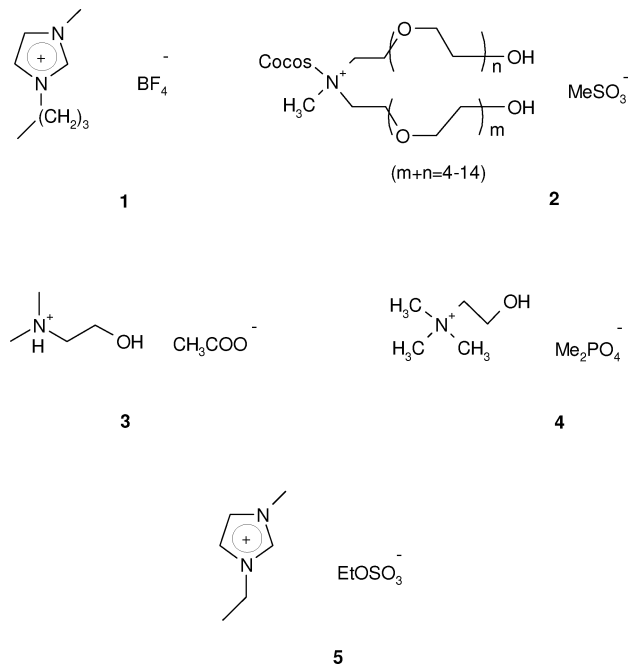

Fig. 1 Ionic liquids tested for biocompatibility with *Clostridium sporogenes.*

Results and discussion

Reaction optimisation

Reduction of nitrobenzene has not been determined using *Clostridium sporogenes*, and therefore, we started by confirming that nitrobenzene was reduced to aniline as expected. Nitrobenzene is rather insoluble in water (1.9 g/L at 20 °C; 0.015 mM), whereas the reduced product, aniline, is water soluble (36 g/L at 20 °C; 2.57 mM).[40] Therefore, we used a two phase heptane/water reaction system for initial tests, in which the Log D heptane/water for nitrobenzene was 1.7.

C. sporogenes was grown on phenylalanine, and then harvested and washed anaerobically. The cell suspensions were then tested anaerobically for reduction of nitrobenzene (3.5 mM in heptane). The reduction was successful when H_2 was used as the electron donor, providing aniline in 45% yield. We found that H_2 was the best electron donor, since yields with fructose, starch and glucose were 13, 16 and 29% lower, respectively, than with H_2 (Table 1). Therefore, H_2 was chosen as the electron donor for further experimental studies. Nevertheless, it should be noted that glucose and starch can be produced from plant biomass, and are relatively cheap and safe to use. Therefore, the carbohydrates may be better candidates than H_2 for future industrial use, since product yields were only slightly lower.

Table 1 Effect of electron donors on the reduction of nitrobenzene by *Clostridium sporogenes*

Electron donor	Aniline (mM)	Yield (%)
H_2	1.44 ± 0.25	41
Fructose	1.36 ± 0.16	39
Starch	1.23 ± 0.02	35
Glucose	0.95 ± 0.14	32

Harvested cells (92 $g_{dry\ wt}$ L^{-1}) were incubated with nitrobenzene with the electron donors shown. The data are the mean of duplicate reactions and the standard deviations are shown. The experiment was repeated on 3 occasions and representative data are shown.

Nitrobenzene was consumed completely with each of the electron donors, even though the product yield was less than 45% (Table 1). This could be attributed to formation of side products, since the aqueous phase of the reaction mixtures contained either phenol or an unidentified product. The product ratios varied when the experiment was repeated with different cell preparations, although the aniline yields were similar. However, the side products were not characterised further because we obtained higher yields in later experiments (see below). We also checked for product consumption by incubating *C. sporogenes* with aniline (2 mM) for 24 h under H_2. Less than 2 (± 0.5)% of the aniline was consumed, suggesting that the low yields were not due to product consumption.

Effect of ionic liquids on growth of *C. sporogenes*

The next step was to investigate the biocompatibility of ionic liquids to determine their suitability for use as a replacement for heptane in the biotransformation. Since water-miscible ionic liquids have rarely been used in whole cell biotransformations,[15] we decided to test diverse structures. We tested for biocompatibility by measuring the growth rate of *C. sporogenes* on phenylalanine under anaerobic conditions in the presence and absence of various concentrations of ionic liquids (Table 2). Growth of *C. sporogenes* was also monitored in the presence of ethanol, as an example of a conventional, water miscible organic solvent.

Ethanol was only slightly inhibitory, even at 2% (v/v). By contrast, 1-butyl-3-methylimidazolium tetrafluoroborate **1**([BMim][BF_4]) allowed growth at low concentrations, but inhibited growth completely when added to 0.5% (w/v), indicating that *C. sporogenes* is more sensitive to this ionic liquid than *E. coli.*[41] However, the sensitivity may not be due to the ionic liquid *per se*, since [BMim][BF_4] hydrolyses in water, forming HF.[42] Indeed, we found that the pH fell from 7.0 to 5.9 in uninoculated medium containing 2% [BMim][BF_4] after 24 h, whereas the pH did not change significantly in the absence of

Table 2 Growth of *Clostridium sporogenes* in the presence of ionic liquids and ethanol

Ionic liquids		Growth rate[a] (%) at different concentrations of ionic liquids (% w/v)				
		0.10	0.25	0.50	1.0	2.0
[BMim][BF_4]	**1**	91 ± 2	59 ± 3	0 ± 0	0 ± 0	19 ± 0
AMMOENG™ 100	**2**	9 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
DMEAA	**3**	117 ± 3	85 ± 2	98 ± 6	83 ± 5	59 ± 3
[$EtOHNMe_3$][Me_2PO_4,]	**4**	98 ± 6	128 ± 15	102 ± 6	123 ± 12	91 ± 2
[EMim][$EtSO_4$]	**5**	79 ± 2	81 ± 2	89 ± 11	77 ± 2	42 ± 5
Ethanol (% v/v)		92 ± 6	98 ± 10	96 ± 6	87 ± 7	76 ± 5
No solvent (control)			100 ± 9			

[a] *C. sporogenes* was grown anaerobically in the presence of ionic liquids or ethanol at the concentrations shown. The control contained water in place of solvent. The growth rates (shown as a percentage relative to the control experiments) are the average of 4 cultures and the standard deviations are shown.

ionic liquid. This suggests that the observed toxicity may be associated with HF formation.

Next, we tested AMMOENG™ 100 **2** as an example of an ethylene glycol functionalized ammonium salt. There was almost no growth even at 0.1% (w/v) ionic liquid, but AMMOENG™ 100 caused immediate precipitation of the culture medium. This suggested that the apparent growth inhibition was due to a decreased availability of essential nutrients. We assume that the ionic liquid was reacting with ionic substances in the culture medium and forming an insoluble precipitate. The precipitate dissolved again after incubation for 24 h, but there was still no growth. We have encountered precipitation with other ionic liquids using different media (data not shown), so it seems that this is a fairly general problem. However, it is dependent on the type of culture medium and the type of ionic liquid.

Two of the ionic liquids stimulated growth, causing an increased growth rate and a shorter lag phase. Thus, *N,N*-dimethylethanolammonium acetate **3** (DMEAA) increased the growth rate by 17% when provided at 0.1% (v/v), but caused slight inhibition at higher concentrations. 2-Hydroxy ethyl trimethylammonium dimethyl phosphate **4** ([$EtOHNMe_3$][Me_2PO_4]) also increased the growth rate (up to 28%), and growth stimulation continued at concentrations up to 1% before slight inhibition was observed. We were surprised to see such a large increase in growth rate, since the cells were growing in a complex culture medium, containing amino acids, peptone and yeast extract which has already been optimised to support rapid growth of *C. sporogenes.*[34,35,43] It is possible that the cells were able to use the ionic liquids as an additional nutrient source, but it is difficult to explain why these xenobiotics would support more rapid growth than naturally-occurring amino acids and protein hydrolysates, which are the natural substrates for this proteolytic organism.[44] Therefore, it is also possible that the ionic liquids increased the availability of other nutrients in the medium. However, phenylalanine has a solubility of 20 g/L in water,[40] but the solubility only increased slightly, to approximately 23–24 g/L in 4% DMEAA or [$EtOHNMe_3$][Me_2PO_4]. Therefore, it seems more likely that the growth stimulation may have been due to solubilisation of other nutrients or utilisation of the ionic liquids as nutrients. To distinguish between these possibilities, it would be necessary to test for growth in minimal medium with the ionic liquids as the sole carbon and energy source. However, such studies would be fraught with difficulty because *C. sporogenes* has an absolute requirement for supplementary amino acids in the culture medium[45] and it would not be possible to distinguish between growth on the amino acids and the ionic liquids. Whatever the explanation, the uncertainty about the effects of DMEAA and [$EtOHNMe_3$][Me_2PO_4] on *C. sporogenes* makes these ionic liquids unsuitable for use in biotransformations.

1-Ethyl-3-methyl imidazolium ethylsulfate **5** ([EMim][$EtSO_4$]) caused growth inhibition (23%) at all concentrations up to 1% (w/v), with further inhibition (58%) as the concentration was increased to 2%. This inhibition was relatively modest compared with [BMim][BF_4] and AMMOENG™ 100. Therefore, it was possible that this ionic liquid might be useful for biotransformations, because we have shown that biotransformations can be less sensitive to inhibition by ionic liquids than growth.[22] Therefore, we selected [EMim][$EtSO_4$] for use in nitrobenzene reduction.

Nitrobenzene reduction in ionic liquid solutions

We found that nitro reduction was more efficient in the ionic liquid solutions than in conventional solvents. Thus, washed, harvested *C. sporogenes* cells were able to reduce nitrobenzene in 4% [EMim][$EtSO_4$], forming aniline in 45% yield (Fig. 2). By contrast, the average yield was only 8% in 4% ethanol. Therefore, the water-miscible ionic liquid provided better biocatalyst performance than the conventional solvent. When ethanol or [EMim][$EtSO_4$] were used, the substrate was still consumed completely, as with heptane, but side products were not formed. This suggests that the formation of the side product was associated specifically with use of heptane as solvent.

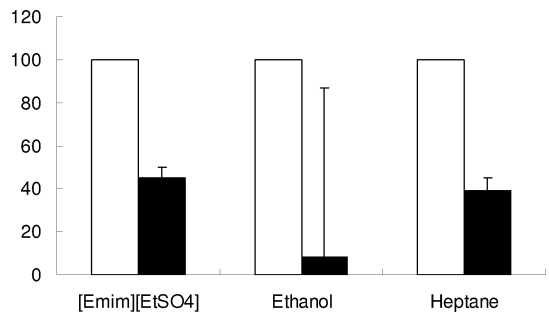

Fig. 2 Reduction of nitrobenzene with *Clostridium sporogenes* in different solvent systems. Nitrobenzene was reduced using harvested cells (24 $g_{dry\ wt}$ L^{-1}) with H_2 as the electron donor using the solvent systems shown and the substrate consumed (■) and product formed (□) expressed as percentages. Data are a mean of duplicate reactions and error values are the standard deviations.

When nitro reduction was tested in heptane, the aniline yield was only 39%, compared with 45% in Table 1. However, the cell concentration (24 $g_{dry\ wt}$ L^{-1}) was much lower in this experiment compared with the experiment reported in Table 1 (92 $g_{dry\ wt}$ L^{-1}). This suggested that the product yield might be related to cell concentration. Therefore, we tested nitrobenzene reduction in 4% [EMim][$EtSO_4$] using an increased cell concentration (92 $g_{dry\ wt}$ L^{-1}). In this case, the aniline concentration increased to 2.76 ± 0.18 mM, and the yield was 79%. Therefore, unproductive substrate conversion had decreased, and more of the substrate

was converted to the desired product. Most importantly, the yield was 43% higher than in heptane under equivalent reaction conditions.

Interpreting the results is complex. All three solvent systems provided effective solubilisation of the substrate, nitrobenzene. Therefore, it seems that the superior performance of the ionic liquid may be due to suppression of unproductive substrate conversion. As noted above, this ionic liquid inhibited growth by up to 58%, so it is fairly toxic. This suggests that it may inhibit metabolic processes that are associated with unproductive substrate conversion. However, further speculation about the mechanism would be premature at this stage, and further studies will be needed to investigate the biological basis of this phenomenon.

Experimental

Chemicals

Ionic liquids were obtained as follows: [BMim][BF_4] from Fluka; [EMim][$EtSO_4$], AMMOENG™ 100, and [EtOHNMe$_3$][Me_2PO_4] from Solvent Innovation; DMEAA from Bioniqs Limited. All other chemicals were obtained from Sigma-Aldrich at the highest purity unless otherwise stated.

Measurement of growth rates

Clostridium sporogenes was maintained and cultured in an anaerobic cabinet as described previously.[37] Ionic liquids were deoxygenated by purging with N_2.[46] The ionic liquids and ethanol were mixed with deoxygenated, sterile distilled water in the anaerobic cabinet and then added to the culture medium. AMMOENG™ 100 was hygroscopic and solidified immediately on contact with air, and was dissolved by mixing with water at 50 °C and cooling anaerobically. The cultures were inoculated (4% v/v) from pre-cultures that had been grown overnight to an OD_{660} of 3. There was no growth in uninoculated controls, showing that the solvents were self sterile. The cultures were mixed anaerobically on a IKA Vibrax® shaker at 120 rpm at 30 °C. Samples were taken aseptically, the OD_{660} (N_t) was measured and the growth rates (μ) were calculated from data collected during the exponential growth phase using the following equation: $\ln N_t/N_o = \mu t$.

Biotransformations

Cells were grown and harvested anaerobically as described previously.[37] The cells were resuspended in 50 mM potassium phosphate buffer pH 7.0 to approximately 10% of the original culture volume and used as the biocatalyst. Nitrobenzene was dissolved in the solvent, deoxygenated, and then transferred to the anaerobic cabinet. Solutions in heptane were added to a phase ratio of 0.33, with nitrobenzene at 3.5 mM across both phases. For the water miscible systems, nitrobenzene was dissolved in ethanol or [EMim][$EtSO_4$], deoxygenated, transferred to the anaerobic cabinet, mixed with an equal volume of anaerobic water and then added to the reaction mixture to 3.5 mM nitrobenzene and 4% solvent. For reactions with H_2 as electron donor, the H_2 was passed over the headspace of the reaction vessel for 5 min before sealing the reaction vessels and inverting them to minimise gas loss. For reactions with fructose, glucose or starch, the electron donor was added as an anaerobic aqueous solution (1 mL; final concentration 0.45% w/v) and the headspace of the reaction vessel was flushed with N_2. In all cases, the total volume of reaction mixtures was 12 mL and the cell concentrations (stated in the text) were adjusted to the same concentration across the whole reaction mixture by diluting with buffer as necessary. The reaction mixtures were incubated in Supelco® bottles with polypropylene screw tops and PTFE septum seals.

Analytical methods

The cells were removed from all samples by centrifugation at 4000 rpm for 20 min in an Eppendorf 5210R bench centrifuge (UK). The heptane phases from biphasic reactions were separated before centrifuging the aqueous phase, and the heptane phase and aqueous supernatant were analysed directly without further extraction. The supernatants containing water miscible solvents were extracted once with 3 mL ethyl acetate for samples containing [EMim][$EtSO_4$] or dichloromethane for samples containing ethanol. Samples were analysed using a Thermo Finnigan ISD gas chromatograph coupled to an ion trap mass spectrometer detector. Samples (1 µL, split ratio 33:1) were analysed on a ZB-5MS capillary column (0.25 mm × 30 m, 0.25 µm nominal film thickness; Phenomenex, USA) with He as the carrier gas (1 mL/min). The column was held at 80 °C for 2 min, and the temperature was increased by 15 °C min^{-1} to 190 °C, and then maintained for 1 min. Mass spectra were acquired using positive ion electron impact ionization at 70 eV with the source temperature at 200 °C. Mass spectra were scanned from 50–350 mass units at a rate of 3 scans/s. Aniline and nitrobenzene concentrations were determined using calibration curves made with authentic standards.

Conclusion

Water-miscible ionic liquids have a variety of different effects on the physiology of *Clostridium sporogenes*. Thus, DMEAA and [EtOHNMe$_3$][Me_2PO_4] caused large increases in the growth rate of *C. sporogenes*, by an unknown mechanism. We also found that AMMOENG™ 100 can react with culture media. Whilst this causes growth inhibition, it is not because the ionic liquid is toxic but because the reaction reduces the availability of essential nutrients. Therefore, great care has to be exercised when selecting ionic liquids for use with whole microbial cells. It is becoming evident that simple tests for cell viability are insufficient,[15,17,21] and tests for growth, with suitable controls, are needed to rule out effects on cell physiology or reactions with growth media. In addition, the lessons learned in this study suggest that care may also be needed to ensure that the ionic liquid does not react with components of reaction media for biotransformations, although there were no such problems with the media used in this study.

The unexpected effects on cell physiology also extended to nitro reduction using *C. sporogenes*. [EMim][$EtSO_4$] was as an effective additive to increase the solubility of nitrobenzene in aqueous reaction mixtures. However, the benefits extended beyond improved substrate delivery. Remarkably, the ionic liquid increased the yield of the amine product, compared with

reactions run in aqueous ethanol or a biphasic heptane/water system. The yield increase appeared to be due to suppression of unproductive substrate consumption by an unknown mechanism, so that more of the substrate was converted to product. [EMim][$EtSO_4$] inhibited growth rather strongly, and it is possible that the target for growth inhibition was associated with the side reactions on the biotransformation substrate, so that both growth and side reactions were inhibited. This unexpected outcome has considerable practical benefit.

Our finding that a growth inhibitory ionic liquid can provide benefits for nitro reduction shows that it is not necessary to use completely non-toxic ionic liquids for biotransformations. Indeed, this is consistent with our earlier finding that moderately toxic ionic liquids are also suitable for oxygenase-catalysed whole cell biotransformations.[22] Therefore, we recommend that both non-inhibitory and partially inhibitory ionic liquids should be screened for use in biotransformations.

Overall, we conclude that water-miscible ionic liquids may have considerable potential as additives in culture media and reaction media to obtain improved growth and biotransformation using *C. sporogenes*. Further work is now needed to determine the generality of such phenomena amongst microbial biocatalysts.

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