Green Chemistry

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Cite this: *Green Chem.*, 2011, **13**, 1843

www.rsc.org/greenchem

PAPER

Screening ionic liquids for use in biotransformations with whole microbial cells†

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Received 20th September 2010, Accepted 20th April 2011
DOI: 10.1039/c0gc00579g

A wide range of ionic liquids, both water miscible and water immiscible, containing a diverse set of cations and anions, were screened for toxicity towards *Escherichia coli* K-12, using both Agar Diffusion tests and growth inhibition tests in liquid cultures. The data provide preliminary rules to enable the design of non-toxic ionic liquids for use in biocatalytic processes.

Introduction

Biocatalytic processing provides an increasingly attractive option for highly selective, atom efficient production of high value chemicals in short reaction sequences.[1] Furthermore, microbial fermentations enable production of biofuels and value-added chemicals from renewable feedstocks, using natural or engineered metabolic pathways.[2] Unfortunately, biotransformation products and/or substrates are frequently toxic towards microbial biocatalysts, and this may restrict productivity, making the processes uneconomic. The problem can often be solved by using a biphasic biotransformation process, where the toxic material is extracted *in situ* into a water-immiscible solvent, to minimise contact with the cells in the aqueous phase.[3] Unfortunately, polar conventional solvents are, themselves, toxic.[4] Therefore, *in situ* extraction is only possible when there is a serendipitous match between the physical properties of the material to be extracted and the available, non-polar biocompatible solvents.[3] For this reason, there is considerable interest in using ionic liquids as alternative extraction media in whole cell biotransformations.

There have been some notable successes. For example, biphasic systems containing water-immiscible ionic liquids provided significant improvements in productivity compared with conventional solvents for redox biotransformations using whole cells.[5] Even water-miscible ionic liquids can provide significant increases in product yields compared with conventional solvents when used as additives.[6] Therefore, ionic liquids are extremely promising co-solvents for use in whole cell biotransformations.

To date, relatively few ionic liquids have been tested for use in microbial processes. The beauty of ionic liquids is the ability to vary the structure and, thus, tune the physical properties of the solvent to match the specific requirements of the process (*e.g.* efficient extraction of a biotransformation product). Therefore, it would be desirable to identify a wider range of biocompatible types.

We studied the toxicity of over ninety ionic liquids from diverse structural classes towards *Escherichia coli*. *E. coli* is a good choice because it is used very frequently as a host strain for expression of industrial enzymes and for metabolic engineering. Therefore, our results can be implemented rapidly in industrial biocatalysis. We screened ionic liquids based on imidazolium, pyridinium, quaternary ammonium, alkanolammonium, and quaternary phosphonium cations (Table 1), combined with a diverse range of anions. Whole cell biotransformations depend on having live, functional cells to ensure efficient cofactor recycling, enzyme functionality, substrate uptake, *etc*. The simplest, fastest indicator of cell functionality is to measure growth of the cells. This also ensures that the results are general, and can be applied to any biocatalytic reaction. Therefore, we used our high throughput, agar diffusion test to test for growth inhibition,[7] backed up with high throughput measurement of growth rates in the presence of the ionic liquids.[5b] Most importantly, we have extended the screening method to allow testing of water-immiscible ionic liquids, thus enabling selection of solvents for biphasic biotransformations. Therefore, preliminary rules are now available to guide the selection of non-toxic ionic liquids for use in whole cell biotransformations, together with strategies to design a wider range of new, biocompatible ionic liquids.

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† Electronic supplementary information (ESI) available: Synthesis and characterisation of ionic liquids. See DOI: 10.1039/c0gc00579g

Table 1 Cations used in this study

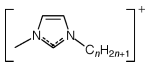
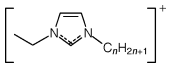
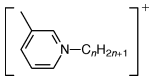
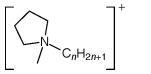
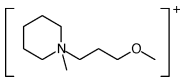
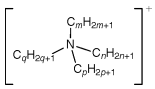
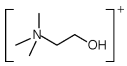
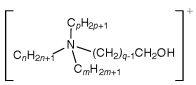
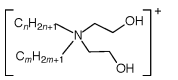
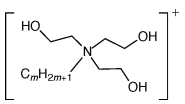
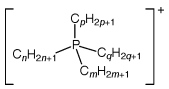
Name or class	Abbreviation	Structure
1-alkyl-3-methylimidazolium	$[C_n mim]^+$	
1-alkyl-3-ethylimidazolium	$[C_n eim]^+$	
1-alkyl-3-methylpyridinium	$[C_n m_\beta py]^+$	
1-alkyl-1-methylpyrrolidinium	$[C_n mpyrr]^+$	
1-methyl-1-(3-methoxypropyl)-piperidinium	$[C_{3O1}mpip]^+$	
tetraalkylammonium (linear chains)	$[N_{m\,n\,p\,q}]^+$	
trimethyl(2-hydroxyethyl)-ammonium(cholinium)	$[N_{1\,1\,1\,2OH}]^+$	
singly ω-hydroxylated tetraalkylammonium	$[N_{m\,n\,p\,qOH}]^+$	
bis(2-hydroxyethyl)-dialkylammonium	$[N_{m\,n(2OH)2}]^+$	
tris(2-hydroxyethyl)-alkylammonium	$[N_{m(2OH)3}]^+$	
tetraalkylphosphonium (linear chains)	$[P_{m\,n\,p\,q}]^+$	

Table 2 Non-sulfate anions used in this study

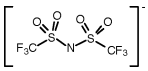
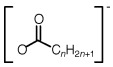
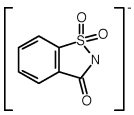
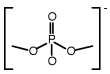
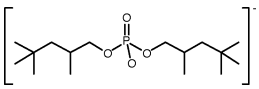
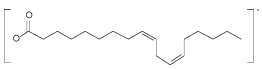
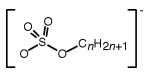
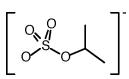
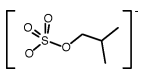
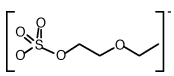
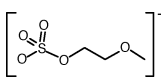
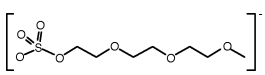
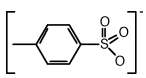
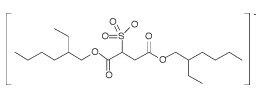
Name or class	Abbreviation	Structure
bis{(trifluoromethyl)-sulfonyl}amide-(bistriflamide)	$[NTf_2]^-$	
2-hydroxypropanoate((±)-lactate)	[lactate]$^-$	
linear alkanoate	$[C_n COO]^-$	
saccharinate	[sacch]$^-$	
dimethyl phosphate	$[(C_1O)_2PO_2]^-$	
bis(2,4,4-trimethylpentyl)-phosphinate-(diisooctylphosphinate)	$[(^iC_8O)_2PO_2]^-$	
cis,*cis*-9,12-octadecadienoate-(linoleate)	[linoleate]$^-$	

Table 3 Sulfate and sulfonate anions used in this study

Name or class	Abbreviation	Structure
alkyl sulfate	$[C_n OSO_3]^-$	
isopropyl sulfate	$[^iC_3OSO_3]^-$	
isobutyl sulfate	$[^iC_4OSO_3]^-$	
2-ethoxyethyl sulfate	$[C_2OC_2OSO_3]^-$	
2-methoxyethyl sulfate	$[C_1OC_2OSO_3]^-$	
2-[2-(2-methoxy)-ethoxy]ethoxyethyl sulfate	$[C_1(OC_2)_3OSO_3]^-$	
4-methoxyphenyl sulfonate (tosylate)	[tosylate]$^-$	
1,4-bis(2-ethylhexoxy)-1,4-dioxo-butane-2-sulfonate (docusate)	[AOT]$^-$	

Experimental

Microorganism and growth

E. coli MG1655[8] was obtained from Simon Andrews (Reading University, UK) and was maintained on LB agar. Inocula were grown from a single colony in either LB or MSX medium[9] at 37 °C with shaking at 200 rpm. Agar No. 2 was purchased from Lab M, and yeast extract was purchased from Foremedium. All other chemicals and media components were purchased from Sigma–Aldrich.

High throughput agar diffusion test for ionic liquid toxicity

The agar diffusion test developed previously[7,10] was used to screen ionic liquids (Tables 1–3), except that the lawn of *E. coli* MG1655 was prepared by spreading a sample from an overnight culture (100 µl) on an LB agar plate. The ionic liquids were added to sterile, pre-weighed filter paper discs as before, except that the discs were 6 mm in diameter and were transferred onto LB agar plates. In some cases, ionic solids were tested to obtain further insight into the structure-toxicity relationship. These salts were weighed, and sterile distilled water was added drop-wise until the

salt dissolved. An aliquot (5 μl) of the salt solution was added to the filter paper disc. The weights absorbed were in the range 0.004–0.0125 g of dissolved ionic solids added to the filter paper. All plates were incubated overnight in a static incubator at 37 °C, and the radius of the inhibition zone around the filter paper was recorded as before.[7] Each ionic liquid was tested in triplicate and inhibition zones quoted are the average of the three replicates, unless otherwise stated.

Ionic liquid toxicity testing in liquid media

Growth rates were measured in LB and MSX media in microculture (200 μl) in a Bioscreen C incubator/plate reader (Thermo Labsystems, Franklin, MA) in the presence and absence of ionic liquids. It should be noted that the platereader used previously was unsuitable for use with water-immiscible materials.[7] To decrease the viscosity, water miscible ionic liquids were weighed and mixed with sterile, distilled water to produce a stock solution (95% w/v). Viscous ionic liquids or ionic liquids with unusual phase behaviour (*e.g.* with the 1,4-bis(2-ethylhexoxy)-1,4-dioxobutane-2-sulfonate $[AOT]^-$ anion) were also prepared in the same way, but in this case a suspension was made. The stock solutions were vortexed before adding an aliquot (4 μl) to the microcultures to a final concentration of 2% (v/v). Non-viscous water-immiscible ionic liquids (*e.g.* $[NTf_2]^-$ salts) were added directly to the cultures by volumetric measurement to produce a biphasic system (2% v/v). The cultures were inoculated with a sample (10 μl) from an overnight culture of *E. coli* MG1655 grown in the same medium. The plates were incubated for 24 h at 37 °C with continuous, intensive shaking.

The OD measurement for each well was recorded automatically every 10 min, using a wideband filter. Growth curves were produced using Microsoft Excel (Microsoft Office 2003). Specific growth rates (μ, h^{-1}) were calculated by selecting two points at time t_1 and t_2 in the exponential growth phase and applying the equation

$$\mu = \frac{\ln(OD_2/OD_1)}{(t_2 - t_1)}$$

Specific growth rates are the mean of three replicates, unless otherwise stated, and are reported as a percentage of the growth rate in control cultures grown without ionic liquids.

Syntheses of ionic liquids

The methods used to synthesise the ionic liquids are described in the ESI.†

Results

More than ninety ionic liquids were screened for biocompatibility with *E. coli* MG1655, using both the agar diffusion test and measurements of growth rates in the presence and absence of ionic liquids. Growth curves were generated in both LB and MSX culture media, which contain entirely different chemical components. Thus, LB contains yeast extract, peptone and glucose, whereas MSX is a defined salt solution, with glucose as the sole carbon and energy source. This made it possible to control for potential interactions between the ionic liquids and medium components[6] or potential changes in ionic liquid toxicity with different physiological states of the cells. The effect of the ionic liquids on pH was checked in cultures and uninoculated controls using universal indicator (20 μl) added before and after growth. In general, effects on pH were small (<0.5 pH units) except where stated in the text. The results are presented according to the anions tested.

Table 4 Effect of imidazolium halides on growth of *E. coli*

Ionic liquid	Inhibition zone/cm	μ/% in MSX	μ/% in LB
$[C_2mim]Cl^S$	0	*n.d.*	*n.d.*
$[C_4mim]Cl^S$	0	97 ± 0.72	45 ± 1.36
$[C_6mim]Cl^L$	0.5 ± 0.1	0	0
$[C_8mim]Cl^L$	0.8 ± 0.1	0	0
$[C_{10}mim]Cl^L$	1.1 ± 0.06[1]	0	0
$[C_2mim]Br^S$	0	90 ± 1.38*	73 ± 0.82
$[C_4mim]Br^L$	0	56 ± 5.56*	77 ± 4.75
$[C_6mim]Br^L$	0.6 ± 0.1	0	0
$[C_8mim]Br^L$	0.9 ± 0.15	0	0
$[C_4mim]I^L$	0.37 ± 0.06	0	60 ± 15.66
$[C_6mim]I^L$	1.0 ± 0.1	0	0
$[C_8mim]I^S$	*n.d.*	0	0

Inhibition zones were measured using the agar diffusion test, and growth rates (μ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. *n.d.* – data not available. Data are the means of 3 replicates and the standard deviations are shown. All of the ionic liquids were water miscible. [S] – Solid, [L] – Liquid. [1] – White precipitate around filter paper disc. * – only two replicates available.

Halides

Initially, a range of 1-alkyl-3-methylimidazolium halide ionic liquids was screened for toxicity, aiming to benchmark the behaviour of *E. coli* against previous studies with other living organisms (Table 4). The $[C_2mim]^+$ and $[C_4mim]^+$ chlorides and bromides did not produce inhibition zones in the agar diffusion test, but inhibition zones were produced with increasing radii when the alkyl chain was increased to hexyl or octyl. The specific growth rates varied between the LB and MSX media, suggesting possible interactions between the ionic liquid and the medium, or medium-dependent variations in cell physiology, which changed the susceptibility to ionic liquid toxicity. However, the overall trend was that growth was inhibited progressively as the length of the 1-alkyl chain increased. Similar relationships between increasing length of the alkyl chain/lipophilicity of the imidazolium cation and the increasing toxicity of the ionic liquid have been widely reported,[11] suggesting that the response of *E. coli* MG1655 to imidazolium halides is similar to other living organisms.

We also wish to note that the iodides were more toxic than the chlorides and bromides, possibly because iodide is the most easily oxidised. The large errors associated with the iodide salts may have been due to the black colouration caused by photosensitised oxidation of the iodide anion.

A range of other halide salts was also tested, in which the imidazolium cation was replaced with pyrrolidinium, piperidinium and tetraalkylammonium cations (Table 5). As with mammalian cells,[11h,12] it was found that the toxicity of pyrrolidinium and piperidinium halides towards *E. coli* increased with increasing alkyl chain length. Similarly,[12] there was a reasonable correlation

Table 5 Effect of pyrrolidinium, piperidinium and quaternary ammonium halides on growth of *E. coli*

Ionic liquid	Inhibition zone/cm	μ/% in MSX	μ/% in LB
$[C_4mpyrr]Cl$	0.08 ± 0.03	91 ± 6.60	99 ± 2.28
$[C_4mpyrr]Br$	0	0	53± 1.36
$[C_6mpyrr]Br$	0.16 ± 0.06	0	0
$[C_4mpip]Br$	0.05	83 ± 5.69	70 ± 3.35
$[C_6mpip]Br$	0.2 ± 0.06	0	0
$[C_8mpip]Br$	0.5 ± 0.06	0	0
$[N_{1124}]Br$	0.13 ± 0.06	88 ± 3.73	94 ± 2.49
$[N_{1148}]Br$	0.9 ± 0.12	0	0
$[N_{1148}]I^{I}$	0.6 ± 0.12	0	0
$[N_{1888}]Cl^{L,I}$	0.3 ± 0.06	0	0
$[N_{1888}]Br^{L,I}$	0.3 ± 0.06	0	0
$[N_{1143OH}]Cl$	0	110 ± 0.53	95 ± 1.41
$[N_{24(2OH)2}]Br^{L}$	0.1 ± 0.1	108 ± 7.90	122 ± 2.57
$[N_{1123OH}]Br$	0	104 ± 1.71	96 ± 0.74

Inhibition zones were measured using the agar diffusion test, and growth rates (μ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. Data are the means of 3 replicates and the standard deviations are shown. All were water miscible solids except ^{L} – liquid, ^{I} – water immiscible.

Table 6 Effect of saccharinate and alkanoic acid salts on growth of *E. coli*

Ionic liquid	Inhibition zone/cm	μ/% in MSX	μ/% in LB
$[C_6mim][sacch]^{S}$	0.5 ± 0.06	0	0
$[C_8mim][sacch]$	0.7 ± 0.06	0	0
$[N_{1148}][sacch]^{I}$	0.8 ± 0.06	0	0
$[N_{1888}][sacch]^{I}$	0.07 ± 0.06	0	*n.d.*
$[C_4mim][C_1COO]$	0.06 ± 0.05	27 ± 6.07	64 ± 4.25
$[C_8mpip][C_1COO]^{S}$	0.9 ± 0.06	0	0
$[N_{1112OH}][C_1COO]$	0	93 ± 1.02	92 ± 0.63
$[N_{1112OH}][C_2COO]^{S}$	0	77 ± 6.56*	64 ± 2.82
$[N_{1112OH}][C_5COO]^{S}$	0	50 ± 2.81*	23 ± 2.83
$[C_2mim][lactate]$	0.33 ± 0.06[1]	0	0
$[C_4mim][lactate]$	0.33 ± 0.12	0	0
$[C_4mim][lactate]^{2}$	0	67 ± 13.87	86 ± 5.84
$[C_4mpyrr][lactate]$	0.1 ± 0.12	*n.d.*	*n.d.*

Inhibition zones were measured using the agar diffusion test, and growth rates (μ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. *n.d.* – data not available. Data are the means of 3 replicates and the standard deviations are shown. All were water miscible liquids, except ^{I} – water immiscible or ^{S} – solid. [1] – halo effect, [2] – silver-free synthesis. * Only 2 replicates used.

between the agar diffusion results for the imidazolium and the corresponding pyrrolidinium and piperidinium ionic liquid with the same alkyl chain length.

As in earlier studies,[11h,13] increasing the alkyl chain length of tetraalkylammonium cations produced toxic ionic liquids. Thus, $[N_{1124}]Br$ produced less than 12% inhibition of the growth rate (although there was an inhibition zone on agar diffusion tests) whereas the halides of $[N_{1148}]^+$ and $[N_{1888}]^+$ were toxic.

When one or more of the alkyl chains was replaced with an alkanol group, the ionic liquids were much less toxic. However, it should be noted that the majority of the quaternary ammonium salts contained relatively long chain substituents (*e.g.* octyl), whereas the ethanolamine and propanolamine salts contained shorter alkyl substituents, which made them less toxic in any case (see above). Introduction of single hydroxyl groups into quaternary ammonium cations also decreases inhibition of the enzyme, acetylcholinesterase, by one order of magnitude[14] and decreases cytotoxicity.[11h]

In general, the initial data correlate well with literature already published, thus providing a good indication that the agar diffusion method can be used to screen a range of structurally diverse ionic liquids. However, it is worth noting that there were some discrepancies between the agar diffusion tests and the growth rate studies. Thus, inhibition zones were observed for $[C_4mpyrr]Cl$ and $[C_4mpip]Br$, whereas these ionic liquids had little effect on growth rates (<30% inhibition). Similarly, $[C_4mpyrr]Br$ did not produce an inhibition zone, even though growth was inhibited completely in MSX, with 47% inhibition in LB. Similar discrepancies were also observed for $[N_{1124}]Br$ and $[N_{24(2OH)2}]Br$, which suggests that the agar diffusion test may be less reliable for quaternary ammonium halides than for imidazolium halides.

The acquired data suggest a number of possibilities for further investigation. Of the cyclic and acyclic quaternary ammonium halides tested, those with short alkyl chains were preferable, and introducing a hydroxyl group into one or more of the alkyl substituents tended to produce non-toxic salts. Furthermore, both bromide and chloride anions produced reasonably biocompatible ionic liquids when combined with short chain cations, whereas the iodides were invariably toxic. It should be noted that the quaternary ammonium and alkanolamine salts form an extremely large group, and there is considerable scope to discover new variants of the non-toxic structures.

Saccharinates

Saccharin-based ionic liquids have been suggested as a good starting point to produce non-toxic ionic liquids, as saccharin (sodium saccharinate) is non-toxic and has already been approved for human consumption and is often used as a non-nutritive sweetener.[15] Therefore, we tested a range of imidazolium and quaternary ammonium saccharinates for toxicity. Unfortunately, none allowed growth of *E. coli* (Table 6). It should be noted however, that the cations tested were also toxic when combined with halide anions (Table 4 and 5). Thus, our findings confirm that the toxicity of ionic liquid is dominated by the toxicity of the most toxic component, in this case the cation.[16]

An additional point of note is the apparent alteration of pH produced by the saccharinate ionic liquids. The saccharinate ionic liquids produced a drop in the pH value from pH 7.5 to pH 5.0 after the addition of the ionic liquid to the medium. Even with increased buffering capacity in the medium,[22] the saccharinate ionic liquids were the only ones to produce such a large shift in the pH value, and this, rather than inherent toxicity, may account for the observed growth inhibition.

Alkanoates and lactates

Recently, there has also been a lot of interest in producing ionic liquids from naturally-occurring anions (*e.g.* alkanoates, lactate)[17] and cations (*e.g.* cholinium, $[N_{1112OH}]$),[16a] since the component ions are well known to be biodegradable. However,

the group 1 and 2 metal salts of alkanoates and lactate are widely used as food preservatives (as are the free acids), and are known to be toxic towards *E. coli*.[18] Therefore, we wished to analyse their effects on *E. coli* when combined with other cations, to make ionic liquids.

In initial tests, we found that the imidazolium and pyrrolidinium lactates tested were toxic. The lactate-containing ionic liquids originally tested were synthesised from silver salts and concern was raised that the toxicity of the ionic liquids may be the result of silver contamination rather than their inherent toxicity. To test this, [C_4mim][lactate] was synthesised using a silver-free synthesis, and compared with the same ionic liquid made from the silver salt (Table 6). The silver-free ionic liquid was much less toxic, confirming that it is important to use a silver-free synthesis to produce lactate-based ionic liquids. Indeed it emphasises that knowledge of the samples' synthetic history is important in reporting toxicological data, and this is not always available for commercially aquired samples.

Although ethanoic acid and its corresponding sodium salt are known to be toxic for *E. coli*,[18a] cholinium ethanoate was non-toxic. By contrast, [C_4mim][C_1COO] caused significant growth inhibition (Table 6), but growth was, nevertheless, observed in both MSX and LB. [C_8mpip][C_1COO] was completely inhibitory, like [C_8mpip]Br (Tables 5 and 6), perhaps unsurprising given the general toxicity of ionic liquids with octyl substituents (see also Table 4). This provides further confirmation that the toxicity of an ionic liquid is dominated by its most toxic component.[16b] Thus, like saccharinate salts,[16b] the toxicities of ethanoate salts are dominated by the toxicity of the cations.

We also investigated the effect of increasing the length of the alkanoate ion from 2 to 6 carbon atoms. Again, the toxicity of the sodium and potassium salts towards microorganisms has been investigated intensively, since the acids and their salts are used extensively as food preservatives.[18b] However, the higher homologues of the cholinium alkanoates were relatively non-toxic, although the growth rates in MSX and LB decreased when the alkyl chain length of the anion was increased.

Sulfates and dimethylphosphates

Alkyl sulfate salts are widely used as surfactants, and are relatively cheap. The derivatives with long alkyl chains disrupt bacterial cell membranes at high concentrations,[19] and the toxicity varies with the type of metal cation.[20] Therefore, we wished to analyse the toxicity of alkyl sulfate salts with organic cations. In a previous study of ionic liquid toxicity towards the anaerobe, *Clostridium butyricum*, alkyl sulfate salts were identified as non-toxic,[7] and alkyl sulfates and tosylates are also relatively non-toxic towards mammalian cells.[21] Therefore, we studied the toxicity of a much wider range of alkyl sulfates and tosylates towards *E. coli* (Table 7). In general, there were some discrepancies between the results of the agar diffusion tests and the growth rate measurements. The inhibition zones observed in the agar diffusion tests had large standard deviations. This is difficult to explain, although it is possible that the ionic liquids interacted with the cellulose filter paper, thus altering their diffusion through the agar medium, or alternatively did not intrinsically diffuse freely through the agar medium. Furthermore, there was a rather poor correlation between the mean size of the inhibition zones and the growth rates observed in MSX and LB media. For this reason, the agar diffusion test is rather unreliable for testing the toxicity of alkyl sulfate ionic liquids for *E. coli*, although better results were obtained with *C. butyricum*.[7] Thus, we base our conclusions primarily on the growth rate data, rather than the agar diffusion data.

Table 7 Effect of sulfate salts on growth of *E. coli*

Ionic liquid	Inhibition zone/cm	μ/% in MSX	μ/% in LB
[C_2mim][C_1OSO_3]	0	*n.d.*	*n.d.*
[C_2mim][C_2OSO_3]	0	93 ± 3.44	147 ± 7.66
[C_2mim][C_8OSO_3]	0.17 ± 0.15	68 ± 6.20	77 ± 15.99
[C_2mim][$C_1(OC_2)_3OSO_3$]	0	78 ± 0.62	88 ± 4.56
[C_2mim][tosylate]^S	0	101 ± 7.69	111 ± 6.94
[C_4mim][C_1OSO_3]	0	82 ± 1.57	79 ± 3.33
[C_4mim][C_2OSO_3]	0.06 ± 0.05	74 ± 2.07	69 ± 1.94
[C_4mim][$^iC_3OSO_3$]^I	0.1	73 ± 1.26	76 ± 0.62
[C_4mim][C_3OSO_3]	0.07 ± 0.06	78 ± 2.56	81 ± 0.39
[C_4mim][$C_1OC_2OSO_3$]	0	65 ± 0.69	63 ± 2.02
[C_4mim][$^iC_4OSO_3$]^{S,I}	0.1 ± 0.1	71 ± 6.04	47 ± 1.43
[C_4mim][$C_2OC_2OSO_3$]	0.06 ± 0.06	82 ± 2.21	71 ± 0.91
[C_4mpyrr][C_1OSO_3]^S	0.02 ± 0.02	85 ± 9.15	91 ± 6.50
[C_6mpip][C_1OSO_3]^S	0.5 ± 0.21	0	0
[N_{1124}][C_2OSO_3]^S	0	80 ± 8.55	44 ± 0.97
[N_{1124}][C_4OSO_3]^S	0.2 ± 0.12	89 ± 1.79	46 ± 3.49
[N_{1188}][C_1OSO_3]^S	0.8 ± 0.1	0	0
[N_{1128}][C_2OSO_3]^S	0.6	0	0
[N_{1288}][C_2OSO_3]^S	0.6 ± 0.1	0	0
[N_{1288}][C_6OSO_3]^S	0.1 ± 0.06	0	0
[N_{1288}][tosylate]^{S,I}	0.5 ± 0.06	0	0
[N_{1122OH}][C_2OSO_3]	0	94 ± 5.61	139 ± 11.45
[$N_{24(2OH)2}$][C_2OSO_3]	0	85 ± 4.33	46 ± 3.22
[$N_{1(2OH)3}$][C_1OSO_3]	0	106 ± 0.29	84 ± 0.65
[P_{1888}][C_1OSO_3]	0.1 ± 0.06	0	0
[C_4mpyrr][$(C_1O)_2PO_2$]	0.03 ± 0.03	97 ± 4.71	89 ± 0.73
[N_{1114}][$(C_1O)_2PO_2$]	0	102 ± 0.71	111 ± 1.28

Inhibition zones were measured using the agar diffusion test, and growth rates (μ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. *n.d.* – data not available. Data are the means of 3 replicates and the standard deviations are shown. All were water miscible liquids, except ^{I} – water immiscible or ^{S} – solid.

[C_2mim][C_1OSO_3], [C_2mim][C_2OSO_3] and [C_2mim][tosylate] were relatively non-toxic for *E. coli*. Furthermore, increasing the alkyl sulfate chain length had little effect. Although there was an inhibition zone with [C_2mim][C_8OSO_3], growth was only inhibited by 31% and 23% in MSX and LB, respectively. The errors were large for the latter cultures, possibly due to light scattering caused by the ionic liquid. The [C_2mim]$^+$ cation is much less toxic than the cations used previously with the octyl sulfate anion,[22] which further emphasises the importance of balancing the toxicity of both the anion and cation when designing non-inhibitory ionic liquids. Introducing ether linkages into the alkyl chain ([C_2mim][$C_1(OC_2)_3OSO_3$]) produced an ionic liquid which caused little growth inhibition and did not produce an inhibition zone in the agar diffusion test.

[C_4mim][C_1OSO_3] and [C_4mim][C_2OSO_3] were slightly more toxic than the equivalent [C_2mim]$^+$ salts. Including an extra carbon atom in the anion (rather than the cation; [C_4mim][C_3OSO_3]) had little effect on toxicity compared with ethyl sulfate. Branching had very little effect also ([C_4mim][$^iC_3OOSO_3$]) but increasing the chain length to isobutyl ([C_4mim][$^iC_4OOSO_3$]) increased

the toxicity slightly. Ether linkages ($[C_4mim][C_1OC_2OSO_3]$) increased the toxicity slightly compared with propyl sulfate or isopropyl sulfate, but the toxicity of $[C_4mim][C_2OC_2OSO_3]$ was lower than the equivalent isobutyl sulfate salt.

Like the respective $[C_2mim]^+$ and $[C_4mim]^+$ salts, $[C_4mpyrr][C_1OSO_3]$ was relatively non-toxic, but $[C_6mpip][C_1OSO_3]$ and $[N_{1\,1\,8\,8}][C_1OSO_3]$ were toxic. This may reflect the length of the alkyl chains on the cations. Indeed, $[N_{1\,1\,2\,4}][C_2OSO_3]$ was non-toxic and the alkyl sulfate could be extended to $[C_4OSO_3]^-$ without affecting the toxicity. On the other hand, all of the alkyl sulfate salts of $[N_{1\,1\,2\,8}]^+$ and $[N_{1\,2\,8\,8}]^+$ were toxic, as was the sulfonate salt, $[N_{1\,2\,8\,8}]$[tosylate]).

The effect of including alcohol groups in the cationic side chains was investigated. $[N_{1\,1\,2\,2OH}][C_2OSO_3]$ and $[N_{2\,4\,(2OH)2}][C_2OSO_3]$ caused slight growth inhibition, but $[N_{1\,(2OH)3}][C_1OSO_3]$ was non-toxic. $[N_{2\,(2O2O1)3}][C_2OSO_3]$ was also non-toxic. Therefore, increasing the number of hydroxyl groups or ether linkages appears to make the ionic liquid less toxic. This extends previous observations that the introduction of a single hydroxyl group, or *ether* or *ester* linkages into the cation can result in less toxic ionic liquids,[11h,23] suggesting that the presence of oxygen atoms in the side chains is generally beneficial.

Overall, cations with long alkyl chains were toxic when combined with alkyl sulfates, but the short chain homologues and variants which included ether or hydroxyl groups were less toxic or non-toxic. The structure of the alkyl sulfate could be varied widely, to include branching or ether groups, without affecting toxicity to a significant extent, but there was some evidence that toxicity increased as the alkyl chain length increased.

Thus, the alkyl sulfate anion appears to be very promising for the production of biocompatible ionic liquids. It is worth noting that the octyl sulfate anion is readily biodegradable,[24] but this is unlikely to affect the biodegradability of the cation: an ionic liquids is only as biodegradable as its least biodegradable component. The findings from this screening exercise suggest that further investigation of both short-chained sulfate and various sulfonate anions may be beneficial in the search for biocompatible and biodegradable ionic liquids.

Finally, the dimethyl phosphates, $[C_4mpyrr][(C_1O)_2PO_2]$ and $[N_{1\,1\,1\,4}][(C_1O)_2PO_2]$ were tested. On average, $[C_4mpyrr][(C_1O)_2PO_2]$ produced a small inhibition zone in the agar diffusion test, but the variation between replicates was very large. However, the growth rate measurements in LB and MSX media showed that the ionic liquid was relatively non-inhibitory for *E. coli*. $[N_{1\,1\,1\,4}][(C_1O)_2PO_2]$ was non-inhibitory. Therefore, dimethyl phosphates are potentially promising as non-toxic ionic liquids.

Water immiscible ionic liquids

The agar diffusion test was originally applied to test the toxicity of water-miscible ionic liquids.[7] However, we wished to examine the potential to use the test to study the toxicity of water-immiscible salts. In this study, we have avoided the use of hexafluorophosphate salts, because the anion has a low miscibility with water,[25] and hydrolyses in water to produce hydrogen fluoride. Instead, we focussed on ionic liquids containing the bis[(trifluoromethyl)sulfonyl]amide (or bistriflamide, $[NTf_2]^-$) anion, since they are extremely insoluble in water and have low viscosities. We also studied the toxicity of 1,4-bis(2-ethylhexoxy)-1,4-dioxo-butane-2-sulfonate (or docusate, $[AOT]^-$) salts and bis(2,4,4-trimethylpentyl)phosphinate $[(^iC_8O)_2PO_2]^-$ salts.

Table 8 Effect of bistriflamide salts on growth of *E. coli*

Ionic liquid	Inhibition zone/cm	μ/% in MSX	μ/% in LB
$[C_2mim][NTf_2]$	0.63 ± 0.12	0	0
$[C_4mim][NTf_2]$	0.77 ± 0.06	0	0
$[C_6mim][NTf_2]$	0.6 ± 0.1	0	0
$[C_8mim][NTf_2$	0.4 ± 0.15	0	0
$[C_4mpyrr][NTf_2]$	0.6 ± 0.12	0	0
$[C_{3O1}mpip][NTf_2]$	0.5 ± 0.1	0	0
$[N_{1\,1\,4\,8}][NTf_2]$	0.4 ± 0.1	0	28 ± 2.97
$[N_{1\,1\,4\,10}][NTf_2]$	0.07 ± 0.06	68 ± 6.53	89 ± 20.09
$[N_{1\,8\,8\,8}][NTf_2]$	0	98.0 ± 2.45	65.4 ± 0.34
$[N_{1\,12\,(2OH)2}][NTf_2]$	0.2 ± 0.06	0	0

Inhibition zones were measured using the agar diffusion test, and growth rates (μ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. Data are the means of 3 replicates and the standard deviations are shown. All of the salts were liquid at room temperature and were water immiscible.

For hydrophobic ionic liquids, the agar diffusion test is no longer a simple diffusion test *sensu stricto*, because the ionic liquids will not dissolve in the aqueous agar medium to a significant extent. Instead, we expected that hydrophobic ionic liquids would be transported over the surface of the agar as a film of oil. However, we also expected additional complexity. Firstly, the ionic liquids might differ in their ability to wet the filter paper disc (which is made of cellulose), and this would affect delivery of the ionic liquid to the cultures, both in terms of quantity and transport onto the surface of the agar. However, all of the ionic liquids used here were able to wet the filters. Secondly, the ionic liquids might interact with components of the culture medium, and this might hinder their smooth transport across the surface. Fortunately, the agar diffusion test can be easily bench-marked against growth rate measurements, since the presence of water-immiscible solvents does not usually affect the accuracy of growth rate measurements to a significant extent.[5,26] For the growth rate measurements, we used the ionic liquids above the solubility limit, to obtain a true biphasic system, as required for biotransformations using whole cells. However, we did encounter problems with docusate salts (*vide infra*).

Despite these concerns, the agar diffusion test provided a surprisingly good indication of the toxicity of hydrophobic ionic liquids when compared with growth rate measurements (Table 8). However, these ionic liquids produced very large inhibition zones compared with water-miscible ionic liquids. As noted above, this might be due to transport of a liquid film over the agar surface rather than diffusion through the gel itself.

The imidazolium-based bistriflamide salts caused complete inhibition of growth in liquid culture and produced some of the largest inhibition zones of any of the ionic liquids tested. This confirms earlier studies which showed that $[C_nmim][NTf_2]$ (n = 2–6) salts inhibit growth and affect membrane integrity.[5b,27] Our study also demonstrates that extending the alkyl chain to

Table 9 Effect of docusate, bis(2,4,4-trimethylpentyl)phosphinate and linoleate salts on growth of *E. coli*

Ionic liquid	Inhibition zone/cm	μ/% in MSX	μ/% in LB
$[C_2mim][AOT]$	0	57 ± 13.68	84 ± 34.18
$[C_4mim][AOT]$	0	88 ± 8.01	53 ± 37.95
$[C_6mim][AOT]$	0	57 ± 6.39*	198 ± 75.80
$[C_4mpyrr][AOT]^S$	0	62 ± 6.34	66 ± 7.94
$[C_4m_\beta py][AOT]$	0.3 ± 0.21	76 ± 24.51	37 ± 7.03
$[C_6m_\beta py][AOT]$	0.3 ± 0.15	0	0
$[P_{66614}][AOT]$	0	97 ± 1.75	94 ± 1.07
$[P_{88814}][AOT]$	0.2 ± 0.06	0	0
$[C_4m_\beta py][(^iC_8O)_2PO_2]$	0.4 ± 0.15	0	0
$[N_{1148}][(^iC_8O)_2PO_2]$	0.4 ± 0.06	0	0
$[N_{1888}][(^iC_8O)_2PO_2]$	0.1 ± 0.06	0	0
$[N_{1148}][linoleate]^S$	0.4 ± 0.15	0	0

Inhibition zones were measured using the agar diffusion test, and growth rates (μ) were measured in MSX or LB media in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. Data are the means of 3 replicates and the standard deviations are shown. All of the salts were liquid at room temperature (except ^{S} – solid), and were water immiscible. * – only two replicates available.

octyl also produces a toxic ionic liquid. This suggests that the toxicity of $[C_n mim]$ bistriflamides is attributed to the anion, since $[C_n mim]X$ ($n = 2$ or 4; X = halide or alkyl sulfates) were relatively non-toxic.

The water-miscible quaternary ammonium salts tended to become more toxic as the alkyl chain lengths in the cation increased (Tables 5 and 7). However, the opposite trend was observed with the equivalent, water-immiscible bistriflamide salts. Thus, short-chain tetraalkylammonium bistriflamide salts were toxic, whereas their long chain equivalents were not (Table 8). This trend seems to be analogous to the toxicity of alkanes, where short chain alkanes are toxic but long chain alkanes are not.[4,28] Furthermore, addition of a hydroxyl group produced an inhibitory ionic liquid (Table 8), whereas this decreased the toxicity of water-miscible ionic liquids (Table 5). It appears that when combined with a hydrophobic, toxic anion, the more hydrophilic the cation, then the more the anion will be "pulled" into aqueous solution. Thus, a small, hydrophilic cation probably increases the effective concentration of the bistriflamide anion in the agar, or in the liquid media, making the ionic liquid more toxic.

The diisooctylphosphinate salts, $[C_4m_\beta py][(^iC_8O)_2PO_2]$, $[N_{1148}][(^iC_8O)_2PO_2]$ and $[N_{1888}][(^iC_8O)_2PO_2]$ were also tested. They all produced large inhibition zones and all inhibited growth in MSX and LB (Table 9). Therefore, we conclude that they are toxic.

Although the agar diffusion test appeared to be a reliable qualitative method to screen hydrophobic ionic liquids for toxicity, it was difficult to obtain an accurate, quantitative comparison *via* growth rates in aqueous media, when the water-immiscible ionic liquids exhibited unusual phase behaviour. In particular, hydrophobic ionic liquids containing the docusate (AOT) or linoleate anions showed a strong tendency to form gels or pastes when added to water. This caused light scattering, and the resulting interference with the OD readings produced large errors. For example, the growth curve obtained in the presence of $[N_{4444}][AOT]$ had large error bars and the starting OD readings were also high due to the light scattering (Fig. 1; the growth curve in the presence of the water miscible ionic liquid, $[N_{1124}]Br$, is shown for comparison). Therefore, the growth rates calculated must be regarded as estimates, and should be used as an indicator of the presence or absence of growth, rather than as an absolute quantitative measure.

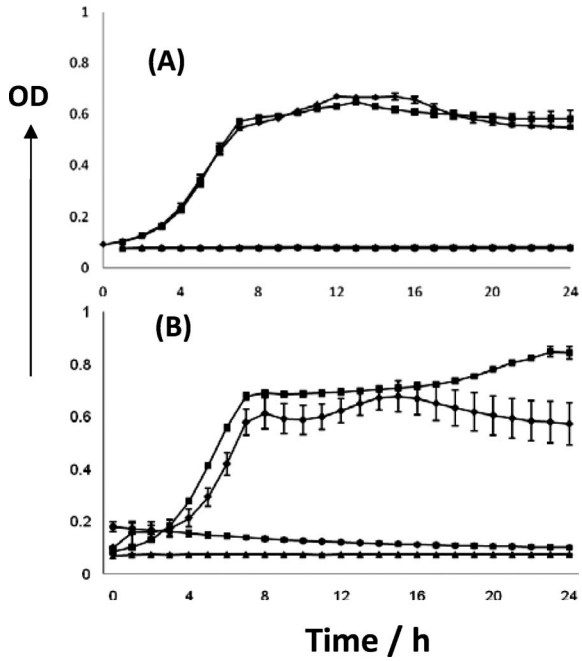


Fig. 1 Effect of gel-forming ionic liquids on growth of *E. coli*. *E. coli* was grown in MSX medium with (◆) and without (■) ionic liquid (2% v/v). A, $[N_{1124}]Br$; B, $[N_{4444}][AOT]$. Uninoculated controls with (●) and without (▲) ionic liquid control are also presented. Means of three replicates are shown and error bars are standard deviations.

In a number of cases, there were discrepencies between the agar diffusion test and the growth tests. Thus, $[C_4m_\beta py][AOT]$ produced a large inhibition zone even though there was significant growth in LB and MSX. By contrast, the large inhibition zone produced with $[C_6m_\beta py][AOT]$ correlated with growth inhibition in liquid media. $[N_{1124}][AOT]$ and $[N_{4444}]$ [AOT] also caused inhibition zones, even though there was growth in MSX and LB. Therefore, it is difficult to get reliable quantitative estimates of the toxicity of pyridinium and quaternary ammonium docusate salts using the agar diffusion test, and other, complementary methods will be needed to obtain robust measurements.

Observations on the agar diffusion test

In order to screen large numbers of ionic liquids for toxicity to a particular micro-organism, a simple and rapid method is required. The agar diffusion test is suitable, provided that the limitations of the methodology are appreciated. The agar screening method requires only small volumes of ionic liquids and generally available laboratory equipment. However, as discussed previously,[7] a major limitation of the method is in the classification of ionic liquids which produce small diffusion

zones. Although reliable for identification of biocompatible or highly toxic ionic liquids against microorganisms, those which fall between the two extremes can be difficult to quantify. In some cases, the ionic liquids appeared to interact with the filter papers or the culture medium, possibly leading to anomalous results. For example, some of the ionic liquids did not diffuse evenly across the agar plates, and thus produced non-circular inhibition zones, leading to possible measurement errors (Fig. 2).

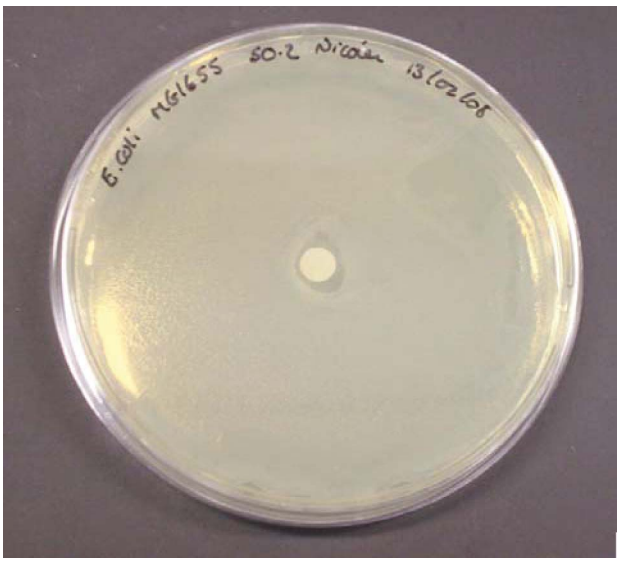


Fig. 2 Asymmetric inhibition zones in the agar diffusion test. An agar diffusion test was done using $[N_{1124}][C_4OSO_3]$. The photograph shows that the inhibition zone was asymmetric and showed a variable zone of clearing, rather than forming a uniform circle around the filter paper.

Additionally some of the tested ionic liquids caused the filter paper to turn brown (see, for example, Fig. 3), suggesting a chemical reaction, whilst others interacted with the culture medium, causing a "halo" or a precipitate to appear around the inhibition zone. It should also be noted that there may be problems when the ionic liquid is not absorbed by the filter paper, and care should be exercised when interpreting the results of studies with water-immiscible ionic liquids. Therefore, it is very important to record the appearance of unusual results, and where appropriate to verify the results by testing for growth in liquid media to ensure that potentially useful ionic liquids are not overlooked. In any case, it must be remembered that the methodology should be used as a first-stage, high throughput screen to identify the most promising ionic liquids. It should never be deployed as a substitute for detailed toxicology testing.[29]

Conclusion

By using a combination of agar diffusion testing and growth rate measurements, we have identified a large number of relatively non-toxic ionic liquids. In general, imidazolium salts with short alkyl chains were relatively non-toxic, especially when paired with alkyl sulfate anions. Methylpyrrolidinium salts were also very promising, whereas water-miscible quaternary ammonium salts were generally toxic. However, there was evidence that decreasing the alkyl chain length decreased their toxicity.

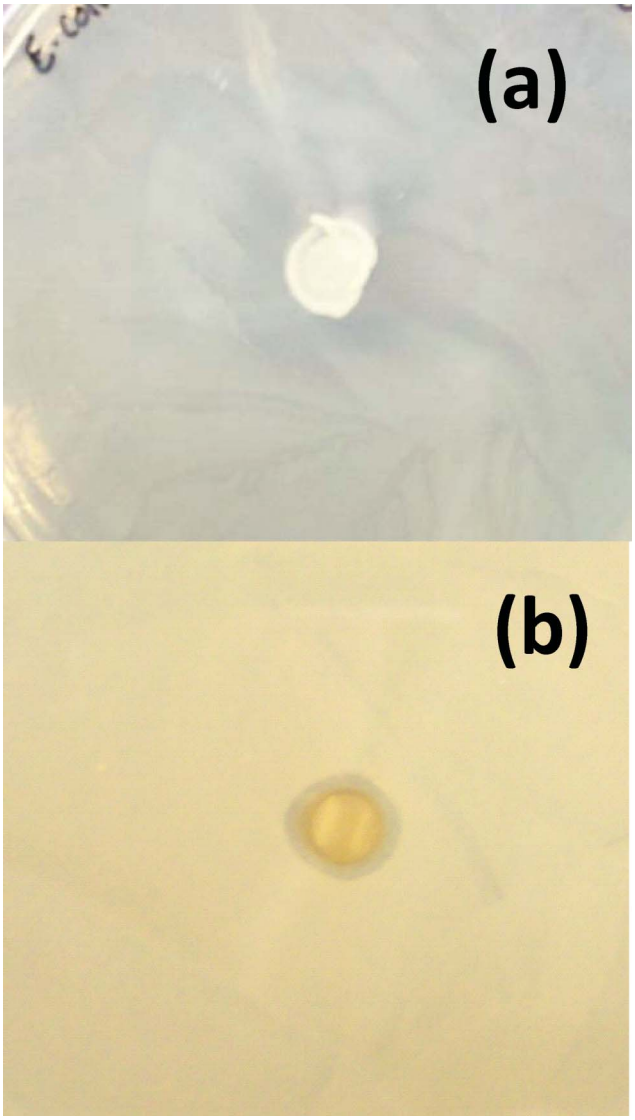


Fig. 3 Formation of precipitates and colour changes in the agar diffusion test. Photographs of the inhibition zones from agar diffusion tests; (a) white precipitate around the filter paper disc in the presence of $[C_6mim][AOT]$, (b) colour change in the presence of $[C_4mim][lactate]$ prepared using the silver synthesis.

Overall, the most promising cation class appeared to be the alkanolammonium salts, since these were generally non-toxic. In terms of the anions, alkyl sulfates and their derivatives tended to produce non-toxic water-miscible ionic liquids, whilst docusates produced non-toxic, water-immiscible ionic liquids, albeit with challenging phase behaviour. Interestingly, bistriflamides tended to make longer chain quaternary ammonium salts non-toxic, whereas other cation classes became more toxic compared with their water miscible counterparts. A small number of dimethyl phosphates was also tested, and tended to be non-toxic.

In conclusion, this study has revealed some very promising toxicity trends which will form a basis for rational design of new, non-toxic ionic liquids. Furthermore, we have already discovered a large number of ionic liquids with very low toxicity towards *E. coli*. We are now deploying these ionic liquids in biotransformations to produce fuels and chemicals.

Acknowledgements

This work was funded by the UK Biotechnology and Biological Sciences Research Council, *via* a studentship awarded to Nicola Wood and a Research Development Fellowship to Gill Stephens, and by QUILL and the Technology Strategy Board's Collaborative Research and Development programme.

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