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Phosphonium ionic liquids for degradation of phenol in a two-phase partitioning bioreactor

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Abstract Six ionic liquids (ILs), which are organic salts that are liquid at room temperature, were tested for their biocompatibility with three xenobiotic-degrading bacteria, *Pseudomonas putida*, *Achromobacter xylosoxidans*, and *Sphingomonas aromaticivorans*. Of the 18 pairings, seven were found to demonstrate biocompatibility, with one IL (trihexyl(tetradecyl)phosphonium bis(trifluoromethylsulfonyl) amide) being biocompatible with all three organisms. This IL was then used in a two-phase partitioning bioreactor (TPPB), consisting of 1 l aqueous phase loaded with 1,580 mg phenol and 0.25 l IL, inoculated with the phenol degrader *P. putida*. This initially toxic aqueous level of phenol was substantially reduced by phenol partitioning into the IL phase, allowing the cells to utilize the reduced phenol concentration. The partitioning of phenol from the IL to the aqueous phase was driven by cellular demand and thermodynamic equilibrium. All of the phenol was consumed at a rate comparable to that of previously used organic-aqueous TPPB systems, demonstrating the first successful use of an IL with a cell-based system. A quantitative ^{31}P NMR spectroscopic assay for estimating the log *P* values of ILs is under development.

Introduction

Two-phase partitioning bioreactors (TPPBs) consist of a cell-containing aqueous phase and a second, immiscible phase (usually an organic solvent) that acts as a reservoir for the equilibrium-based delivery of substrate to the microorganisms (Daugulis 2001; Malinowski 2001). This reactor system is particularly suited to substrates that are cytotoxic, as the substrate is concentrated in the organic phase via equilibrium partitioning, maintaining a sub-inhibitory aqueous-phase concentration. This technology has found application in the destruction of biologically recalcitrant organic pollutants, including poorly water-soluble materials (Guieysse et al. 2001; Villemur et al. 2000), with substrate delivery being controlled by cellular demand. When the organic solvent removes an inhibitory product of cell metabolism, the process is known as extractive fermentation (Kollerup and Daugulis 1989).

The choice of the delivery phase which, as noted above, has almost exclusively been an organic solvent, is guided by consideration of cytotoxicity, bioavailability, substrate partitioning behavior, and cost. The ideal delivery phase therefore is non-toxic, non-bioavailable (i.e., not metabolized as a substrate), has a large partition coefficient (allowing high loading of substrate) and is inexpensive. In considering the use of TPPBs in bioremediation applications, environmental impact is a natural fifth consideration, and one criticism of TPPBs is their use of organic solvents to effect desirable substrate partitioning behavior. In these systems, organic solvents can reach the environment by adventitious emissions during the biodegradation process via stripping and exchange of bioreactor materials (i.e., feeding/withdrawal). In addition, the potentially volatile nature of these solvents is a threat to operator health and safety in the form of vapor inhalation and solvent flammability. Solid polymer beads have recently been proposed as alternative delivery phases to organic solvents (Amsden et al. 2003; Prpich and Daugulis 2004a, b).

Ionic liquids (ILs) have recently emerged as an alternative to organic solvents in traditional chemical processes (Wilkes 2002). Organic salts that are liquid at room temperature, most ILs display no measurable vapor pressure, eliminating the possibility of gaseous emissions (Wasserscheid and Welton 2003). This vapor pressure advantage renders the use of ILs greener, or more environmentally friendly, than organic solvents. The availability of halogen-free, metal-free and organic-solvent-free synthetic routes to ILs further enhances their

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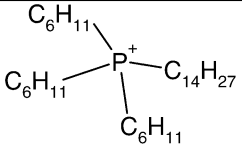
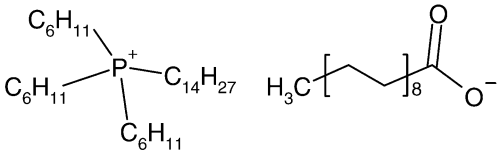
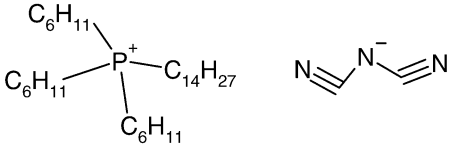
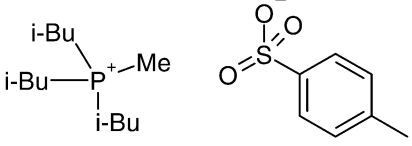
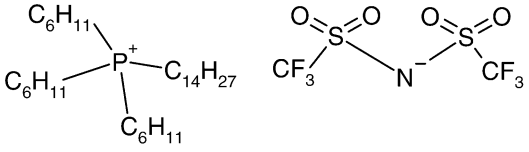
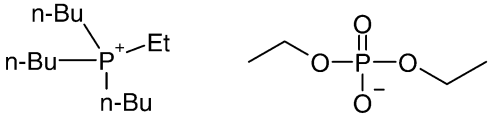
green nature (Varma and Namboodiri 2001; Golding et al. 2002; de Souza et al. 2002; Holbrey et al. 2002). In addition, their non-flammability makes them safer in practice than organic solvents.

Although imidazolium based ILs are the most widely available, pyridinium-, ammonium- and phosphonium-based ILs are also in use (Wasserscheid and Welton 2003). A wide variety of IL physical properties are available from these four classes of cations by altering the substituent alkyl chain lengths and by deliberate anion choice. Often inorganic, common anions found in ILs include chloride, dicyanamide, bis(trifluoromethylsulfonyl)amide, tetrafluoroborate, and hexafluorophosphate.

Beyond the green aspects of ILs, their application to TPPB systems is expected to expand the range of deliverable substrates, as ILs are known to dissolve an uncommon range of compounds. Individual ILs can solubilize a broad range of molecular species, from water to toluene (Wasserscheid and Welton 2003).

The use of ILs in biocatalysis applications (Krugl et al. 2003) has previously been investigated for enzymatic systems, with lipases applications having shown the most promise (Kaar 2003). In many cases, the research was directed toward determining the activity of enzymes with regard to single-phase, non-aqueous bio-transformations. To date, the possibility of whole-cell catalysis in the presence of ILs remains largely unexplored, with only a few published accounts. Cull et al. (2000) have shown that an IL (3-butyl-1-methylimidazolium hexafluorophosphate or [bmim]PF_6) can be used as the second phase in the biotransformation of 1,3-dicyanobenzene, in which the IL (or originally toluene) acts as a reservoir for the poorly water-soluble substrate. In their system, however, the cells were not actively growing, and the viability of the cells was assessed over only 90 min of exposure to the IL via an enzyme activity assay, which does not confirm that the IL used in this case was entirely biocompatible with the *Rhodococcus* strain used. Howarth et al. (2001) have attempted the asymmetric reduction of ketones by immobilized bakers yeast in the presence of wet [bmim]PF_6; the yields and enantioselectivities were fair for some substrates but not others. The use of dry [bmim]PF_6 gave very poor results, and the authors suggested that this was likely due to enzyme inactivation. Fadeev and Meagher (2001) reported that 3-butyl-1-methylimidazolium hexafluorophosphate ionic liquid inhibited an acetone–butyl alcohol–ethanol fermentation. Pernak et al. (2003) demonstrated that ILs can in fact show significant *antimicrobial* activity against cocci, rods and fungi, and have proposed a chemical basis for this phenomenon. This,

Table 1 Phosphonium ionic liquids used in this study

Trade Name/Proper Name	Structure
CYPHOS IL 101 Trihexyl(tetradecyl)phosphonium chloride	
CYPHOS IL 103 Trihexyl(tetradecyl)phosphonium decanoate	
CYPHOS IL 105 Trihexyl(tetradecyl)phosphonium dicyanamide	
CYPHOS IL 106 Tri-iso-butyl (methyl)phosphonium tosylate	
CYPHOS IL 109 Trihexyl(tetradecyl)phosphonium bis(trifluoromethylsulfonyl)amide	
CYPHOS IL 169 Tributyl(ethyl) phosphonium diethylphosphate	

along with the previous two reports, suggests that the demonstration of IL biocompatibility is not a trivial matter and must be established before such materials can be exploited as delivery phases in TPPBs.

This research sought to determine the biocompatibility of six phosphonium ILs with each of three xenobiotic-degrading bacteria. Phosphonium Ils, rather than the more frequently cited imidazolium ILs, were chosen for this study because it was expected that their greater hydrophobicity would lead to greater biocompatibility. Moreover, from a practical consideration of cost, phosphonium Ils are significantly cheaper and therefore more likely to be applied industrially. The three tested bacteria represent three different genera, have different sensitivities to organic solvents, have a wide substrate spectrum, including recalcitrant compounds such as aromatics, and have previously been used in organic aqueous TPPB systems. The work was also aimed at testing and comparing a model xenobiotic-degrading IL-water TPPB system (trihexyl(tetradecyl)phosphonium bis(trifluoromethylsulfonyl) amide) for the biodegradation of phenol by *Pseudomonas putida* ATCC 1172) with an aqueous-organic TPPB arrangement that has previously been shown to operate successfully.

Materials and methods

Chemicals and microorganisms

All ionic liquids were donated by CYTEC Canada and used as received. The formal name, trade name and chemical structure of each IL is given in Table 1. All reagents and medium components were purchased from Fisher Scientific (Ottawa, Canada).

Pseudomonas putida ATCC 11172 (Collins and Daugulis 1996; Vrionis et al. 2002a,b), *Achromobacter xylosoxidans* Y234 (Yeom and Daugulis 2001; Davidson and Daugulis 2003) and *Sphingomonas aromaticivorans* BO695 (Janikowski et al. 2002) were used in this study as pure cultures (stored at −80°C in DMSO). Stock cultures were plated on tryptic soy broth (TSB) agar and stored at 4°C. Liquid cultures were grown in a defined media with a single carbon source (2.0 g glucose l^{-1} or 0.25 g phenol l^{-1} as required) and a basal medium containing the following salts; 3.0 g K_2HPO_4 l^{-1} , 1.5 g KH_2PO_4 l^{-1} , 2.0 g $(NH_4)_2SO_4$ l^{-1} , 0.5 g $NaNO_3$ l^{-1} , 0.3 g $MgSO_4$ l^{-1} , 0.1 g $CaCl_2$ l^{-1} , 0.1 g KCl l^{-1} , and 1 ml of trace element solution (Davidson and Daugulis 2003). The salt solution was a composite of existing *Pseudomonas* (Vrionis et al. 2002a) and *Achromobacter* formulations (Yeom and Daugulis 2001). All medium components were dissolved in distilled water, had a final pH of 6.8, and were sterilized by autoclaving.

Ionic liquid biocompatibility

This experiment was conducted in order to determine which, if any, of the ILs were biocompatible with each of the three organisms tested. Prior to cultivation, liquid inocula of each species were prepared from single colonies grown on TSB agar. One colony was added to 50 ml of glucose medium in a 125-ml Erlenmeyer flask and incubated for 24 h at 30°C and 180 rpm.

The biocompatibility fermentations were completed at 50-ml scale with glucose medium and a 10% (v/v) 24-h inoculum. Immediately after inoculation, 3 ml of ionic liquid was added to each flask (except for a control flask that contained no IL). Cultivation was undertaken for 48 h at 30°C and 180 rpm, and each flask was sampled at t=0, at approximately the mid-point of the experiment, and at 48 h. Samples were centrifuged and the supernatant assayed for glucose. Glucose concentrations were measured at 650 nm using the dinitrosalicylic acid (DNS) assay (Miller 1959). After accounting for the partitioning of glucose in the water-IL system, a decrease (if any) in aqueous glucose concentration was taken as an indicator of biocompatibility. The uptake of glucose, rather than the formation of cells, was used as an estimate of biocompatibility, as several of the ILs formed a white dispersion/precipitate at the interface between the IL and the aqueous phase, making estimation of cell growth either by optical density or by cell dry weight impossible. Note that both substrate uptake and cell growth have been shown to be essentially interchangeable means of estimating biocompatibility (Vrionis et al. 2002a).

Phenol distribution testing

The phenol distribution coefficients ($K_{IL/aq}$) for two selected ILs were determined by dissolving a known amount of phenol in water and contacting with an equal volume of IL, with shaking, for 24 h at room temperature. The phenol concentration of the aqueous phase was then determined at 510 nm using the 4-aminoantipyrine method (Yang and Humphrey 1975) and the distribution coefficient was determined via mass balance. The limit of detection for this assay is 5 μg l^{-1}.

Phenol degradation in an IL-aqueous TPPB

The biphasic degradation of phenol was performed in a New Brunswick Scientific 2-l BioFlo fermentor with a start up volume of 1 l aqueous medium and 0.25 l of the chosen IL (trihexyl(tetradecyl)phosphonium bis(trifluoromethylsulfonyl)amide, or IL109). The temperature was maintained at 30°C, and agitation was provided at a rate of 800 rpm. Air flow was regulated at 1.0 vvm (based on aqueous volume) and was filter sterilized through a 0.2-μm filter. The pH was controlled at 7.0 with 6 M KOH. Based on the determined partition coefficient for phenol of IL109, the fermentation medium was originally

made up to a phenol concentration of 1,580 mg l^{-1}, which was reduced to a sub-inhibitory level of 400 mg l^{-1} after addition of 250 ml IL109. The fermentation inoculum comprised three sub-cultured flasks (total volume 0.135 l) grown on 250 mg phenol l^{-1} medium.

Results and discussion

Ionic liquid biocompatibility

The effect of six phosphonium ILs on glucose consumption by the bacteria *P. putida* ATCC 11172, *Alcaligenes xylosoxidans* Y234 and *S. aromaticivorans* BO695 was determined in 48-h batch cultures. Reduced glucose consumpion relative to the positive control (no IL) was taken as an indicator of toxicity; alternatively, if phosphonium ILs were shown to be biocompatible, their use in partitioning bioreactors may be justified. Each of the species tested was a known xenobiotic degrader, and all have already been used in organic-aqueous TPPB systems. The bacteria, the previously used xenobiotic substrate, and the log P_{crit} values for each cell are given in Table 2. The log P_{crit} of a cell, which must be determined experimentally, is a measure of the sensitivity of cells to the presence of organic solvents and is a common means of quantifying solvent-cell biocompatibility (Bruce and Daugulis 1991). Solvents with log P values above the log P_{crit} of a cell are invariably biocompatible with the organism, and thus the lower the log P_{crit} of a cell, the wider the range of solvents that can be used in two-phase systems.

Prior to beginning the biocompatibility experiment, it was necessary to quantify the partition coefficient, $K_{IL/aq}$, of glucose in each IL system (due to its limited solubility in organic solvents, glucose partitioning is not a factor in aqueous-organic TPPB systems) for an initial aqueous glucose concentration of 2 g l^{-1}. In this manner, any drop in the aqueous glucose concentration due to equilibrium partitioning can be separated from that due to cell metabolism. The aqueous concentration of glucose was measured via the DNS assay, while $K_{IL/aq}$ was calculated by material balance. The glucose partitioning data appear in Table 3. For those ILs that were miscible with the aqueous phase, no partition coefficient could be calculated. The large water/IL ratio of 17/1 used during biocompatibility testing ensured that actual aqueous glucose concentrations remained sufficiently high to support cell growth (also given in Table 3).

Of the 18 cell-IL combinations tested, seven showed biocompatibility via the glucose assay. Across species, similar trends were seen in IL biocompatibility; cell growth was unaffected by CYPHOS IL 109 in all cases while CYPHOS IL 103 and 105 were biocompatible with two of the three bacteria. Data from the six trials for each of the bacteria are presented in Figs. 1, 2, 3. It is apparent from the data that the bacteria with lower log Ps, that is, those bacteria which thrive in the presence of more hydrophilic organic solvents, are compatible with a wider range of ILs. Of the six phosphonium ILs tested, only CYPHOS IL 109 was biocompatible with the most sensitive (i.e., highest log P_{crit}) bacterium, *S. aromaticivorans*. By way of comparison to a widely studied organism, *Escherichia coli*, it may be expected that one or more of the ILs tested would be biocompatible with this organism, as its log P_{crit} is in the range of 3.4–3.8, depending on the strain (Inoue and Horikoshi 1991).

As noted earlier, cell-solvent biocompatibility has been well-correlated to log P; if a solvent's log P is greater than a cell's log P_{crit} then there is a significant likelihood of the solvent being biocompatible. In the present instance, estimation of the log P of an IL is problematic, since calculation methods (e.g., the hydrophobic fragmental constant method of Rekker and deKort 1979) would be difficult given the complex chemical structure of ILs and their unusual constituent groups, and experimental estimation are constrained by the lack of volatility of ILs precluding the use of gas chromatography. A new method for estimating the log P of ILs is proposed in "Conclusion."

Table 3 Glucose partitioning in phosphonium ionic liquids

Ionic liquid	Water miscible?	$[Glucose]_{aq}$ (g l^{-1})	$K_{IL/aq}$ (glucose)
101	No	1.9	0.3
103	No	1.5	5.3
105	No	1.6	4.1
106	Yes	2.0	–
109	No	1.7	2.9
169	Yes	2.0	–

Phenol distribution testing

From the biocompatibility tests, it was clear that IL 109 could be used with any of the bacteria for delivery of a toxic substrate in a TPPB configuration, while IL 103 and 105 could also be used with *A. xylosoxidans* Y234 and *P. putida* ATCC 11172. As we have generated extensive data with the use of *P. putida* 11172 in degrading phenol in a variety of aqueous-organic TPPB systems (Collins and Daugulis 1996; Vrionis et al. 2002a,b), it was decided to test this substrate-organism pairing in an aqueous-IL system. IL 105 was found to generate an extremely thick

Table 2 Xenobiotic degrading bacteria tested for ionic liquid biocompatibility

Species	Substrate(s)	Log P_{crit}	Reference
Achromobacter xylosoxidans Y234	Benzene, toluene, xylene	3.1	Yeom and Daugulis (2001)
Pseudomonas putida ATCC 11172	Phenol	3.4	Vrionis et al. (2002b)
Sphingomonas aromaticivorans B0695	PAHs	4.0	Janikowski et al. (2002)

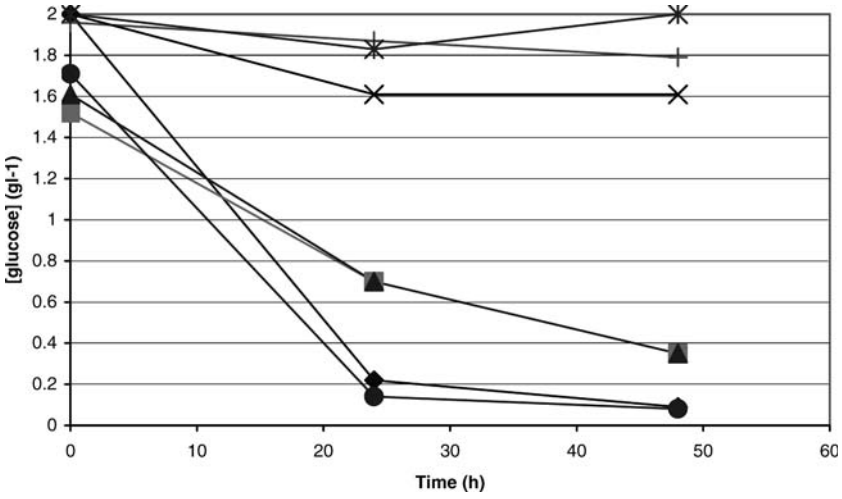


Fig. 1 The effect of 6% (v/v) ionic liquid (IL) on the glucose consumption of *Achromobacter xylosoxidans* Y234. *Filled diamonds* control, *plus signs* IL 101, *filled squares* IL 103, *filled triangles* IL 105, *cross marks* IL 106, *filled circles* IL 109, *asterisks* IL 169

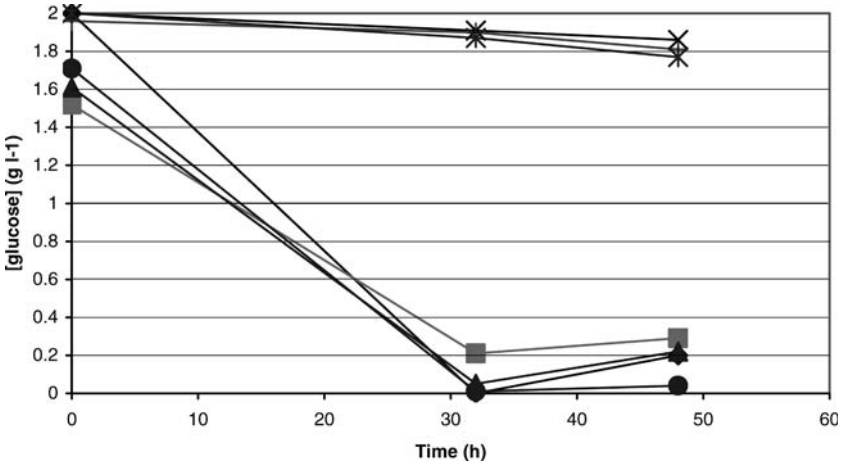


Fig. 2 The effect of 6% (v/v) IL on the glucose consumption of *Pseudomonas putida* ATCC 11172. *Filled diamonds* control, *plus signs* IL 101, *filled squares* IL 103, *filled triangles* IL 105, *cross marks* IL 106, *filled circles* IL 109,; *asterisks* IL 169

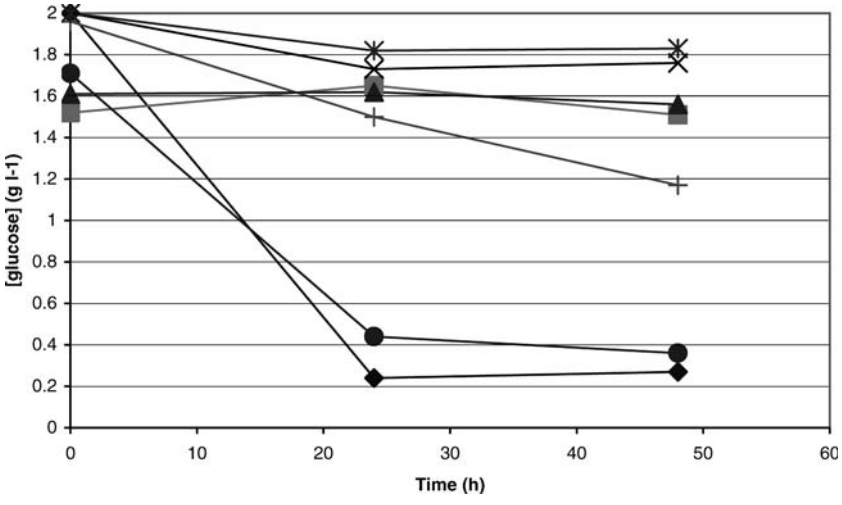


Fig. 3 The effect of 6% (v/v) IL on the glucose consumption of *Sphingomonas aromaticivorans* BO695. *Filled diamonds* control, *plus signs* IL 101, *filled squares* IL 103, *filled triangles* IL 105, *cross marks* IL 106, *filled circles* IL 109, *asterisks* IL 169

dispersion/emulsion of cells, aqueous phase and IL phase and was not considered further due to material handling concerns. The distribution ratios of phenol between IL 103 and 109 and water were therefore measured, and the $K_{IL/aq}$ values for phenol were found to be 18.2 and 11.8, respectively. These distribution ratios are lower than, but of the same order of magnitude to those of organic solvents (20–40) previously used in the TPPB degradation of phenol (Collins and Daugulis 1996; Vrionis et al. 2002a).

Although the partitioning data suggest that IL 103 is better suited to deliver phenol to the aqueous phase on the basis of loading capacity, IL 109 was selected as the delivery phase for the model fermentation. This was an arbitrary decision and was based on the fact that IL 109 is denser than water. Since all other TPPB systems using organic solvents have employed a delivery phase less dense than water, we wanted to determine what, if any, handling issues might emerge with a delivery agent with a higher density.

Phenol degradation in an IL-aqueous TPPB

With the microorganism, substrate and delivery phase set, only the aqueous concentration of phenol remained to be determined, and it was decided to aim for an initial aqueous phenol concentration of 400 mg l^{-1}, which is below the inhibitory concentration of *P. putida* (Vrionis et al. 2002a). For IL 109 with a phenol distribution coefficient of 11.8, and for 1 l aqueous volume and 0.25 l IL volume, it was calculated that 1,580 mg of phenol, if dissolved in the aqueous phase and allowed to equilibrate with the IL in the bioreactor, would give concentrations of phenol in the aqueous and IL phases of 400 mg l^{-1} and 4,700 mg l^{-1}, respectively. After 2 h of stirring to allow equilibration, the measured initial (i.e., prior to inoculation) concentration of phenol in the aqueous phase was 400 mg l^{-1} (reduced to 380 mg l^{-1} after inoculation). Note that the original loading of phenol (1,580 mg l^{-1}) in the 1 l aqueous volume would have been well above the toxic level for *P. putida* (Vrionis et al. 2002a), highlighting the importance of employing a second, delivery phase for degrading toxic substrates.

Figure 4 shows the decline in phenol concentration in the reactor for both the aqueous phase (measured) and IL phase (estimated), with complete phenol uptake occurring within 12 h. This rapid uptake is due to the relatively low aqueous phenol concentrations in the aqueous phase arising from partitioning of phenol into the IL. This performance (based on volumetric productivity) is very similar to prior TPPB experiments with organic liquids as the delivery phase (Collins and Daugulis 1996; Vrionis et al. 2002a,b), and suggests that the IL acted in an entirely similar fashion, initially taking up the majority of the target molecule due to its higher affinity, and delivering it to the cells on demand. As noted earlier, some emulsion/precipitation that arose with the use of this IL prevented estimation of the cell concentration; however, the turbidity

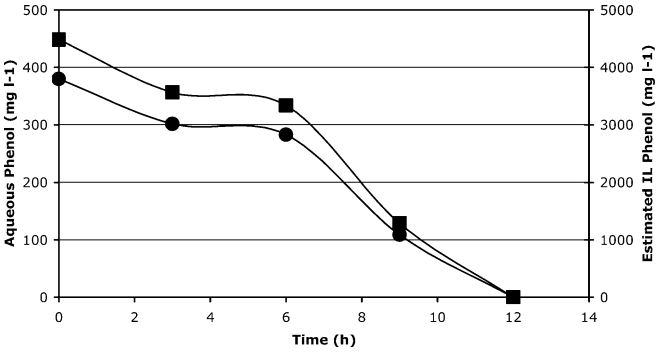


Fig. 4 Bioreactor two-phase partitioning bioreactor (TPPB) run with IL as delivery phase. *Circles* Measured aqueous phenol concentration, *squares* predicted IL phenol concentration

of the aqueous phase increased significantly during the phenol uptake, and the appearance (and disappearance) of a yellow pigment, previously identified as a temporary degradation intermediate, 2-hydroxymuconic semi-aldehyde (Vrionis et al. 2002b), suggest microbial degradation of the phenol. The use of a denser delivery phase did not result in any observed difficulties, with high-intensity mixing and dispersion of the two phase being readily achieved under the operating conditions employed.

Conclusion

Ionic liquids, although successfully used in a few enzymatic biotransformations, have not previously been shown to be similarly effective in cell-based systems. In this work six phosphonium ILs were tested for their biocompatibility with three xenobiotic-degrading organisms. A number of pairings showed good compatibility, and the results indicated that log *P* and a cell's log P_{crit} may play an important role in establishing appropriate pairings between ILs and microorganisms. Future studies include the measurement of log *P* (octanol/water partition coefficient) values for several phosphonium ILs and an evaluation of the reliability of predictions of IL biocompatibility based upon log *P* values. A quantitative ^{31}P NMR spectroscopic assay has been developed in preparation for this work.

One of the IL-organism pairings was successfully used to demonstrate phenol degradation in a TPPB in a manner and at rates very similar to previously shown aqueous organic TPPBs systems. We continue to explore further applications of ILs to microbial systems, particularly in the area of syntheses and biotransformations.

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