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Biodegradation of a phenolic mixture in a solid–liquid two-phase partitioning bioreactor

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Abstract A solid–liquid two-phase partitioning bioreactor (TPPB) in which the non-aqueous phase consisted of polymer (HYTREL) beads was used to degrade a model mixture of phenols [phenol, *o*-cresol, and 4-chlorophenol (4CP)] by a microbial consortium. In one set of experiments, high concentrations (850 mg l^{-1} of each of the three substrates) were reduced to sub-inhibitory levels within 45 min by the addition of the polymer beads, followed by inoculation and rapid (8 h) consumption of the total phenolics loading. In a second set of experiments, the beneficial effect of using polymer beads to launch a fermentation inhibited by high substrate concentrations was demonstrated by adding 1,300 and 2,000 mg l^{-1} total substrates (equal concentrations of each phenolic) to a pre-inoculated bioreactor. At these levels, no cell growth and no degradation were observed; however, after adding polymer beads to the systems, the ensuing reduced substrate concentrations permitted complete destruction of the target molecules, demonstrating the essential role played by the polymer sequestering phase when applied to systems facing inhibitory substrate concentrations. In addition to establishing alternative modes of TPPB operation, the present work has demonstrated the differential partitioning of phenols in a mixture between the aqueous and polymeric phases. The polymeric phase was also observed to absorb a degradation intermediate (arising from the incomplete biodegradation of 4CP), which opens the possibility of using solid–liquid TPPBs during biosynthetic transformation to sequester metabolic byproducts.

Introduction

Two-phase partitioning bioreactors (TPPBs) have been demonstrated to be effective tools for the bioremediation of toxic aromatic compounds such as benzene (Hamed et al. 2004), toluene (Davidson and Daugulis 2003), phenol (Collins and Daugulis 1997a,b), and PAHs (Marcoux et al. 2000). Traditional TPPBs employ a variety of liquids in the role of immiscible phase including organic solvents, ionic liquids, and silicone oil (Collins and Daugulis 1997a,b; Marcoux et al. 2000; Cull et al. 2000). Effective in their roles as a reservoir and delivery agent for substrates, liquid second phases may have limitations that hinder TPPB performance such as bioavailability, cytotoxicity, and fixed molecular structure. Cytotoxicity has been observed with systems utilizing ionic liquids, often times limiting the TPPB to the use of enzymes rather than more sensitive whole cells (Cull et al. 2000; Baumann et al. 2005). Cytotoxicity is also associated with immiscible organic solvents but may be avoided by careful selection of a solvent with an appropriately high log *P* value (Inoue and Horikoshi 1991; Brink and Tramper 1985; Malinowski 2001). Immiscible organic solvents, for use as a possible second phase, may also be bioavailable such that the solvent can serve as a carbon/energy source for the microorganisms present and, thus, may decrease the biodegradation of the intended, target contaminants. To minimize the possibility that the immiscible organic solvent may be bioavailable to the microorganisms, most TPPB systems using immiscible organic solvents are limited to the use of single pure microbial species that are chosen for their inability to utilize the solvent as a carbon/energy source.

Efforts to limit the cytotoxicity and bioavailability of immiscible organic solvents have resulted in the immobilization of organic solvents within calcium alginate hydrogels (Serp et al. 2003; Stark et al. 2003), thus, providing the benefit of increased target molecule affinity while limiting the toxic effects of the immiscible solvent. Silicone oil represents a liquid option in TPPBs that is neither cytotoxic nor bioavailable (Guieysse et al. 2001) but is limited in its use due to its relatively low solubility for many aromatic compounds (a result of its fixed molecular structure) and its difficulty in handling and re-use. Another option is the use of solid polymer beads for the absorption and delivery of toxic organic molecules to

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cells. Polymers have been shown to act as delivery agents for the in situ delivery of drugs (Hatefi and Amsden 2002) and have been observed to absorb inorganic (Bowen 1970) and organic molecules (David et al. 1989). Many polymers are resistant to microbial biodegradation (i.e., non-bioavailable), are chemically inert, may be molecularly altered (by the addition of functional groups), are easily re-used with no loss of performance, and are inexpensive, which are significantly beneficial features for the replacement of liquids as second phases in TPPBs.

Recently, polymer beads have been shown to be effective replacements for the immiscible organic liquid phase in TPPBs as was reported by Amsden et al. (2003) who demonstrated biodegradation of phenol in a solid–liquid TPPB via a single pure microbial species. By replacing the single pure microbial species with a microbial consortium, we were able to demonstrate enhanced rates of phenol biodegradation as well as highlight the non-bioavailable and non-cytotoxic nature of polymer beads as a sequestering phase (Prpich and Daugulis 2005). In addition, we have demonstrated the effects of polymer composition on affinity towards target molecules, suggesting the tailoring of polymeric matrices for the selective absorption of specific molecules (Prpich and Daugulis 2004).

The work presented here highlights the robust nature of a solid–liquid TPPB by demonstrating the biodegradation of multiple phenolic substrates via a microbial consortium. Work was undertaken to further characterize the absorption of a mixture of target phenols by a polymer matrix, as well as to show the migration of metabolic intermediates between the aqueous and solid phases.

Materials and methods

Chemicals and polymers

All chemicals used for the culturing of microorganisms were purchased from Fisher Scientific (Canada) except for tryptic soy broth (TSB) that was supplied by DIFCO (Canada). All other reagents were purchased from Fisher Scientific (Canada) with the exceptions of *o*-cresol (Sigma Aldrich, Canada) and 4-chlorophenol (4CP) (Avocado Research Chemicals, a subsidiary of Alfa Aesar, USA). HYTREL, a registered trademark of DuPont (Canada), was received as a donation. HYTREL is a thermoplastic polyester elastomer consisting of rice-sized beads 5 mm in length by 1.5 mm in diameter with a density of 1.17 g cm^{-3}.

Microorganism and culture conditions

A microbial consortium, obtained via selective enrichment, was used for the biodegradation of a mixture of phenols (phenol, *o*-cresol, and 4CP). The microbes were isolated from wastewater treatment facilities located at a pulp and paper mill, a chemical plant, as well as a commercial sample of hydrocarbon-degrading organisms. Initial growth of a mixture of these organisms occurred in TSB liquid medium to ensure healthy cultures. The TSB liquid cultures were grown for 24 h at 30°C and 180 rpm. Selective enrichment took place in 125-ml Erlenmeyer shake flasks containing 50 ml of mineral salts medium (MSM): (g l^{-1}) K_2HPO_4, 2.56; KH_2PO_4, 2.08; NH_4Cl, 1; $MgSO_4 \cdot 7H_2O$, 0.5; ferric ammonium citrate, 5×10^{-5}; $CaCl_2$, 5×10^{-6}; and 400 mg l^{-1} total phenolic substrate (200 mg l^{-1} phenol, 100 mg l^{-1} *o*-cresol, and 100 mg l^{-1} 4CP). The shake flasks were inoculated with 1 ml of TSB culture and were incubated at 30°C and 180 rpm for a 24-h period. Four serial transfers were performed, under identical conditions, to facilitate the isolation of a consortium capable of degrading the phenolic mixture. Consistent culture conditions were used for the preparation of fermentation inoculum as well as all other biological studies. Cultures were grown in 125-ml Erlenmeyer shake flasks containing 50 ml MSM and 400 mg l^{-1} of mixed phenolic substrates. The cultures were incubated for a 24-h period at 30°C and agitated at 180 rpm.

For the preparation of fermentation inocula, 400 ml of inoculum culture were centrifuged at 3,400 rpm (2,500×*g*) for 15 min. The supernatant was discarded and the resulting biological pellet was re-suspended in 50 ml of fresh carbon-free MSM medium. Preparations for all other biological studies were performed under similar conditions unless otherwise specified.

Characterization of the microbial consortium

Characterization of the phenolic-degrading microbial consortium was performed by Microbial Insights (Rockford, TN, USA). Denaturing gel gradient electrophoresis (DGGE) analysis was performed to determine the members of the microbial consortium. The analysis provided six distinct microbial affiliations which were then sequenced and identified using 16s rRNA techniques. The resulting sequences were compared to the Ribosomal Database Project (Michigan State University).

Bioproduction of degradation metabolite

Studies performed on a degradation metabolite (intermediate) were carried out on the supernatant of shake flask cultures grown on the phenolic mixture until a yellow/green color appeared. The contents of the shake flask were centrifuged at 3,400 rpm for 20 min to remove cellular material and filtered under vacuum through a 0.22-μm filter. Further processing of the supernatant involved removal of enzymes by centrifugation through a 5,000-Da (molecular weight cut off) filter (Millipore, USA).

Analytical methods

Aqueous samples containing phenols were prepared by centrifugation at 3,400 rpm (2,500×g) for 10 min followed

by filtration of 1 ml of supernatant through a 0.45-μm syringe filter (Waters, USA). Phenolics were extracted from the aqueous solution with an Oasis extraction cartridge (Waters) and 1 ml of methanol was used to elute phenolics from the column. A 0.2-μl methanol extract was then injected into the HPLC.

Phenol, *o*-cresol and 4CP concentrations were determined by high-pressure liquid chromatography (HPLC). A Spherisorb ODS-2 analytical column (4.6 um×250 mm) attached to a Waters 2487 UV–Visible Dual Wavelength Absorption Detector were used for analysis. An isocratic elution (35% (v/v) acetonitrile, 65% deionized water) was pumped through the column at a rate of 1 ml min^{-1} by a Waters 515 isocratic pump. Results were analyzed with Millenium[32] software (Workstation Version 3.05.01, Waters Corp., USA). Analysis of metabolites was performed by gas chromatography coupled with electronspray ionization mass spectrometry (GC–ESI-MS) detection as described elsewhere (Hollender et al. 1997).

Biomass and metabolite concentrations were analyzed with an Ultraspec 3000 UV/Visible Spectrophotometer, Biochrom, UK (UV/vis). Biomass concentrations were based on cell turbidity and were measured at 650 nm. The presence of metabolite was determined by removal of biomass with a 0.45-μm syringe filter (Waters, USA) followed by spectrophotometric measurement at 375 nm (Westmeier and Rehm 1987).

Polymer selection for absorption of phenols

It has been suggested that the absorption of phenols into a polymer matrix is most closely related to a solvent-extraction mechanism (Schumack and Chow 1987; El-Shahawi 1994). As phenol molecules consist of a hydrophobic aromatic ring and a polar hydroxyl functional group, both hydrophobic and hydrogen-bonding interactions may participate in their sorption into polymers and should be considered during the polymer selection process (Dmitrienko et al. 1999). Although a systematic strategy for the matching of appropriate polymer/target molecule systems has not yet been fully developed, a similar selection rationale, previously used for the matching of appropriate solvent/target molecule systems, was adopted for a preliminary assessment of the present system (Bruce and Daugulis 1991). The selection process involved using the extractive screening program (ESP) database (Bruce and Daugulis 1991), which is based on the UNIFAC (universal quasichemical functional group activity coefficient) group contribution method for predicting thermodynamic equilibrium to identify possible solvent classes possessing affinities for the target molecules. ESP screening of 1,300 different solvents yielded ester, ether and amine functional groups as being extensively dominant in the short list of possible extractive solvents. These results are in accordance with the expected sorption mechanism as the ester, ether, and amine functional groups are polar and capable of accepting hydrogen bonds. Furthermore, the findings are similar to those in literature where ether and ester-based polyurethanes (Anjaneyulu et al. 1990; Bowen 1970; Fong and Chow 1992; Rzeszutek and Chow 1998; Schumack and Chow 1987) as well as a HYTREL, a polyether-ester block copolymer (Prpich and Daugulis 2004) have demonstrated an ability to absorb phenols. Due to our previous experience with HYTREL, it was selected as the polymer for the extraction of phenol, *o*-cresol, and 4CP from aqueous solution.

Partitioning coefficient of phenols in HYTREL

To determine the partitioning coefficients for the three phenol species in HYTREL, six 125-ml Erlenmeyer flasks were prepared with each flask containing 70 ml of MSM and phenols with a total phenolic concentration of 500, 1,000, 2,000, 3,000, 6,000, and 9,000 mg l^{-1} (equal concentration of each constituent phenol). Six grams of fresh HYTREL polymer beads was added to each shake flask. The flasks were sealed with rubber stoppers and incubated at 30°C and agitated at 180 rpm for a 24-h period. After the incubation period, aqueous samples were analyzed for phenol concentrations, and a mass balance between initial and final aqueous phenolic concentration was used to determine the mass of phenols absorbed by the polymer beads. A least-squares regression of the uptake data was performed to determine the partitioning coefficients for each compound.

Effects of phenolic loading on culture growth

Tests to determine the effect of initial phenolic concentration on the lag time and subsequent growth of the microbial consortium were carried out in 125-ml Erlenmeyer shakes flasks under the previously described culture conditions. Initial phenolic concentrations were varied and the onset of growth was determined by medium turbidity with the appearance of the yellow/green metabolite indicating the biodegradation of 4CP.

Biodegradation of a phenolic mixture in a solid–liquid TPPB

Two types of TPPB fermentations were performed to demonstrate the ability of a solid–liquid TPPB to degrade a mixture of phenolic substrates. The initial fermentation was performed as a demonstration of concept for the delivery and biodegradation of high concentrations of phenol, *o*-cresol, and 4CP. In this experiment, high initial levels of phenols (850 mg l^{-1} each phenol) were reduced to sub-inhibitory concentrations before inoculation with the microbial consortium. The second type of fermentation was carried out to demonstrate the ability of polymer beads to aid in the recovery of an inhibited single-phase biological system through the reduction of inhibitory aqueous phase concentration of phenols. All fermentations were conducted in 5 l Bioflo III bioreactors (New

Brunswick Scientific, USA). The bioreactors had a working volume of 3 l consisting of MSM and were operated at an aeration rate of 0.6 l min^{-1} at 30°C and at pH 6.7 by addition of 3N KOH. Dissolved oxygen concentrations were monitored by a dissolved oxygen (DO) probe. The mass of polymer beads added to the bioreactor was calculated based on the partitioning coefficients of the phenols in the polymer beads (Fig. 1a) in an attempt to reduce initial phenolic loading to sub-inhibitory, aqueous phase concentrations. Upon completion of the fermentation, a mass of beads was removed from the bioreactor and desorption tests were performed to determine if any phenols remain within the polymer beads. Desorption tests were performed in water at pH 7 and pH 2 as well as in hexane at a volume approximately 100 times the volume of the beads.

Results

Absorption of phenols into HYTREL polymer beads

Experiments were performed with a mixture of phenols (phenol, *o*-cresol, and 4CP) being absorbed from aqueous solution with HYTREL polymer beads to determine the partition coefficients for each phenolic compound. Initial phenol concentrations varied over a practical concentration range for possible use in a TPPB from 150 to 3,000 mg l^{-1} for each individual compound, and the results are illustrated in Fig. 1a. Figure 1b shows a comparison between the partitioning of phenol in a polymer–aqueous environment where phenol is the sole compound and in a mixture.

To assess the re-usability of the polymer beads, a desorption test was performed on a mass of polymer beads (2 g) that contained a known quantity of phenol (1,000 mg l^{-1}). Multiple serial dilutions were carried out on the loaded beads and a mass balance was performed to determine the fate of the phenols. From the results (not shown), 95% of the phenol was desorbed and recovered from the polymer beads. To evaluate the performance of re-used beads, the unloaded polymers were placed into a fresh phenolic solution (1,000 mg l^{-1}) where they absorbed an identical quantity of phenol to that of the fresh beads. From these results, it is reasonable to suppose that TPPBs will not suffer any loss of performance due to the use of re-used polymer beads.

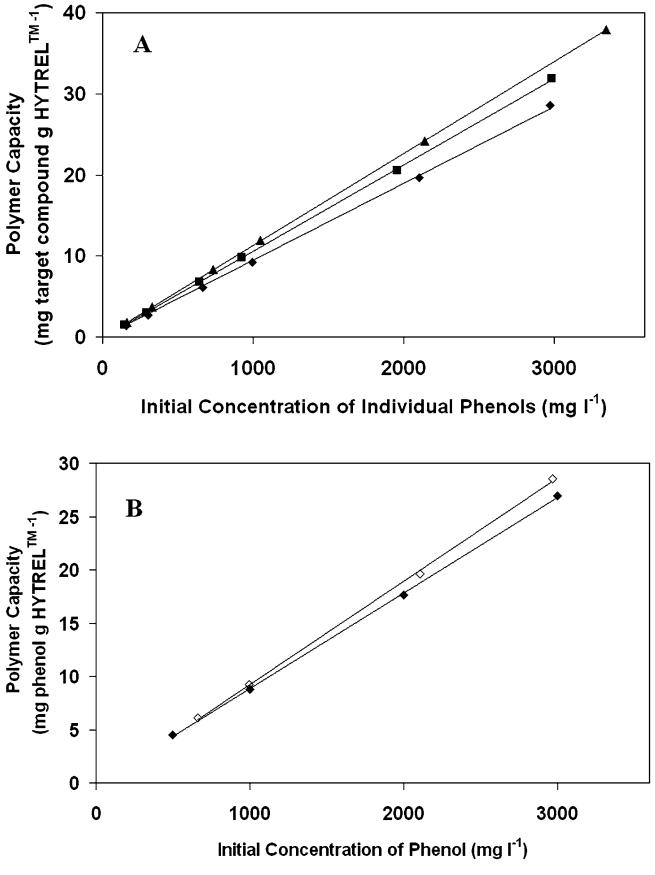

Fig. 1 **a** Partitioning of phenol (*diamonds*), *o*-cresol (*squares*), and 4CP (*triangles*) into HYTREL polymer beads. **b** Comparison of the absorption of phenol into HYTREL polymer beads as an individual species (*solid shape*) and in a mixture (*hollow shape*)

Isolation of a microbial consortium capable of degrading a phenolic mixture

To highlight the robust nature of a solid–liquid TPPB, a mixture of toxic phenols was degraded via a microbial consortium, which was constructed through selective enrichment. The selective enrichment was performed to create a healthy microbial consortium capable of degrading the mixture of phenol, *o*-cresol, and 4CP, with the biological sources for selective enrichment having been chosen for their exposure to phenolic contaminants. DGGE analysis, coupled with 16S rRNA identification, was employed to characterize the microbial consortium. DGGE analysis provided six prominent bands, which resulted in the identification of six isolates to the genus level. The prominent species present within the microbial consortium include two *Pseudomonas* sp., *Klebsiella* sp., *Citrobacter* sp., *Salmonella* sp., and two *Enterobacter* sp., of which the *Pseudomonas* sp. appear to be most abundant.

Tests were performed on the microbial consortium to characterize the population's ability to degrade the individual phenolic compounds, and the results indicated that the microbial consortium was capable of growth on phenol and *o*-cresol as the sole sources of carbon but not on 4CP. (Although the data from these experiments are not shown, the results illustrated in Fig. 2b support the preferential degradation of phenol.) The microbial consortium was capable of utilizing both phenol and *o*-cresol as the sole sources of carbon, but preferential degradation of phenol was observed and confirmed by HPLC analysis, when the two compounds were in a mixture. When the three compounds were in a mixture, phenol was initially consumed, followed by the cometabolic degradation of 4CP and the simultaneous competitively inhibited degradation of *o*-cresol.

Accumulation of 4CP metabolite

Biodegradation of the phenolic mixture resulted in yellow/green coloration of the culture medium and shake flask experiments were conducted to isolate the source compound responsible for the coloration. Cultures grown with phenol and *o*-cresol as the sole sources of carbon, as well as a culture grown on a mixture of phenol and *o*-cresol, produced a whitish coloration of the medium. A mixture of phenol and 4CP produced an initial whitish medium coloration (possibly due to phenol degradation) followed by yellow/green coloration (possibly due to the partial degradation of 4CP). Spectrophotometric analysis of this culture medium showed a peak absorbance at 375 nm and the maximum absorbance of the metabolite coincided with the disappearance of 4CP. GC–ESI-MS assessment of the culture medium detected the presence of a compound with molecular weight corresponding to that of 5-chloro-2-hydroxymuconic acid semialdehyde (HMSA). Further analysis of the ESI-MS spectrum indicated the presence of additional degradation metabolites that are a consequence of the *meta*-cleavage biodegradation pathway for 4CP for which HMSA is the initial degradation compound.

Under the assumption that the yellow/green coloration, with absorbance at 375 nm, may possibly be an inhibitory metabolite as a result of incomplete 4CP degradation, tests concerning the partitioning of the assumed metabolite between the aqueous and polymeric phases were performed. The results demonstrated (data not shown) that the absorbance (at 375 nm) between the control and experimental shake flask containing polymer beads differed by 30%, indicating that the polymer beads are responsible for the absorption of metabolite responsible for the medium coloration.

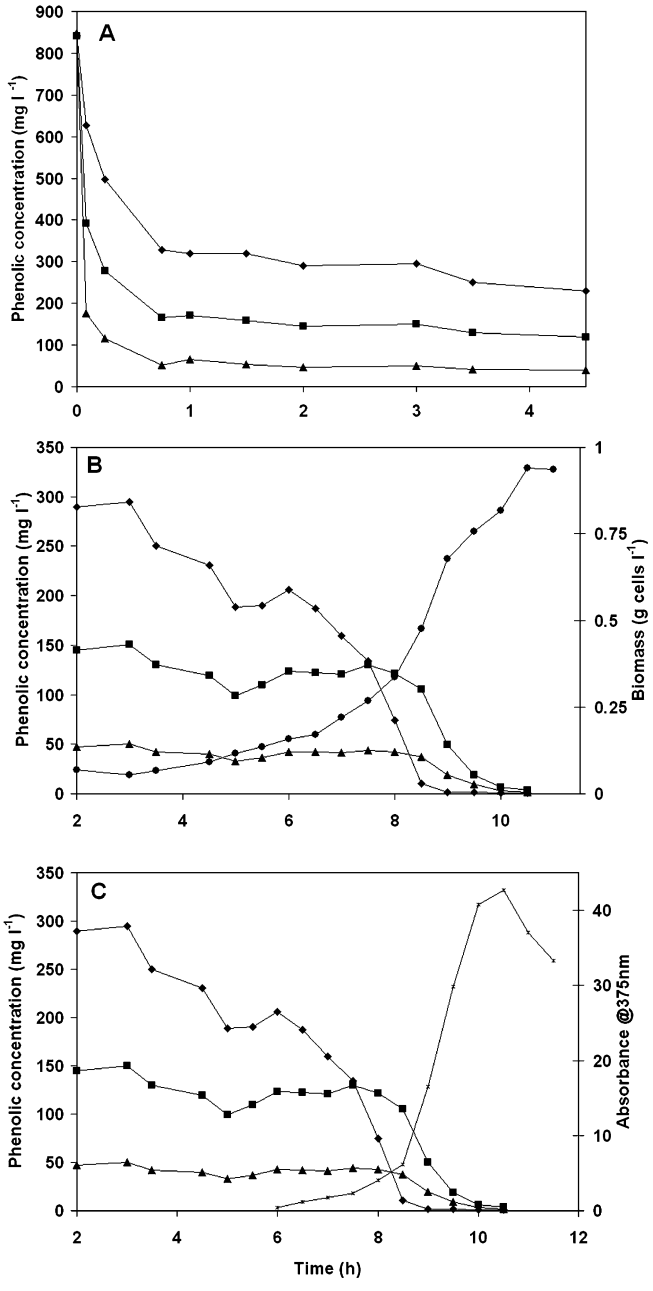

Fig. 2 **a**–**c** TPPB fermentation demonstrating the biodegradation of a phenolic mixture via a microbial consortium. **a** Initial uptake of phenols by HYTREL (t=0 to 5 h). **b** Biodegradation of phenols and subsequent accumulation of biomass after aqueous phase concentrations reduced to sub-inhibitory levels. **c** Biodegradation of phenols and build-up of HMSA due to the incomplete biodegradation of 4CP. Phenol (*diamonds*), *o*-cresol (*squares*), 4CP (*triangles*), biomass (*circles*), and HMSA (*x*)

Biodegradation of a phenolic mixture in a solid–liquid TPPB

A traditional TPPB fermentation was performed to assess the system's ability to degrade a mixture of phenols via a microbial consortium. The system was loaded with equal concentrations of phenol, *o*-cresol, and 4CP at approximately 850 mg l^{-1} each. HYTREL polymer beads at 225 g were added to the bioreactor for a final working volume of 3.2 l. The absorption of phenols into the polymer beads was rapid (approximately 45 min) and, as predicted previously (Fig. 1a), o-cresol and 4CP accumulated to a greater extent within the polymer beads. The phenolic uptake into the polymer beads (Fig. 2a) more clearly distinguishes the differential partitioning capabilities of HYTREL in the presence of multiple phenols, supporting the results obtained in Fig. 1a.

After a 2-h period to ensure that equilibrium had been achieved between the solid and aqueous phases, the TPPB was inoculated. The system experienced a 3-h lag phase after which time the microbes entered exponential growth preferentially degrading phenol, as indicated by the whitish medium coloration and the measured decrease in aqueous phase phenol concentration (Fig. 2b). As biomass increased and the aqueous phase phenol concentrations decreased, the expected degradation of *o*-cresol commenced, as observed by a decrease in aqueous-phase *o*-cresol concentrations and 4CP and by the increasing presence of HMSA. Absorbance of HMSA attained a maximum value that coincided with the disappearance of 4CP (Fig. 2c).

Dissolved oxygen data demonstrated that the system was not oxygen limited, as DO levels did not fall below 40% of saturation. Furthermore, the exponential growth of microbes suggests that the system was not mass transfer (substrate) limited. Upon completion of fermentation (t=12 h), a mass of beads was desorbed to determine whether any residual phenols remained within the beads. As no phenols were detected in the desorption solvents, therefore, it is reasonable to conclude that all the phenols that may have been exchanged within the aqueous medium of the bioreactor had been released. Additional analysis of the beads was performed by pyrolyzation of a used polymer bead at 600°C for 10 s followed by analysis of vapors by GC–ESI-MS and the results (not shown) indicate that no phenols were present.

To demonstrate reproducibility and re-usability of the polymer beads, a second fermentation was performed that used the polymers from the initial fermentation. The results indicate (data not shown) that the re-used polymers did not suffer any loss of capacity or performance and the overall system performed in an identical manner to the initial fermentation.

Alternative operation of a TPPB was investigated through the examination of a TPPB's ability to restore degradative capabilities of a single-phase system inhibited by high phenolic loading. To carry out the study, work was initially performed to determine the phenolic concentrations at which the microbial consortium would become inhibited and the effect that varying phenolic loadings have on the lag phase. Phenolic concentrations were selected based on experience, preliminary testing, and literature values (Wang and Loh 1999). Figure 3 illustrates the results of the experiments and it appears that total phenolic concentrations approaching 700 mg l^{-1} have a negative impact on the system while concentrations greater than 700 mg l^{-1} are significantly detrimental to degradation. At a total phenolic concentration of 900 mg l^{-1}, growth was observed after a 216-h lag phase. Therefore, fermentation experiments were designed to operate at initial phenolic concentrations great enough to demonstrate complete inhibition while showcasing the polymer's ability to return the system to more favorable operating capabilities.

Two fermentations were performed at differing initial phenolic concentrations to demonstrate reproducibility as well as to highlight the inhibitory effects of elevated phenolic levels. Two bioreactors were operated with initial total phenolic concentrations of 1,300 and 2,000 mg l^{-1} (equal concentrations of phenolic substituents). Each reactor was inoculated with 0.02 g CDW at t=0 and the systems were left for 14 h during which time no increase in biomass or disappearance of phenols was observed, thus, demonstrating inhibitory conditions (Fig. 4a,b). At 14 h, 175 g of re-used HYTREL polymer beads were added to the system and a rapid decrease in aqueous phase concentration of phenols was observed.

Fermentation #1 experienced a lag phase of approximately 14 h after the introduction of the polymer beads (Fig. 4a). At 28 h, the system entered exponential growth fueled initially by the rapid degradation of phenol. As the bulk of the phenol present in the system disappeared, degradation of o-cresol and 4CP began at an appreciable rate. As shown previously, o-cresol and 4CP were degraded simultaneously but only after aqueous phenol concentrations were depleted which is in agreement with previously conducted shake flask experiments.

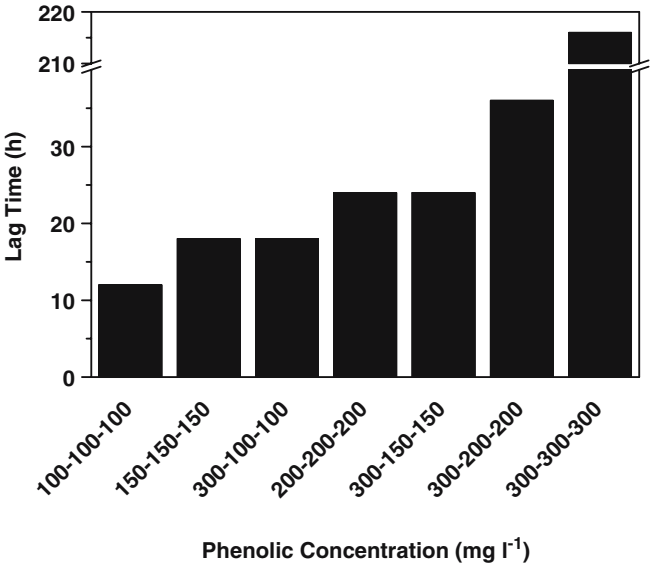

Fig. 3 Lag phase experienced by the microbial consortium due to increasing phenolic loading. *x-axis* notation represents phenol–*o*-cresol–4CP concentrations from left to right

Upon consumption of all available toxic substrate, the biomass concentrations leveled off for each system and remained constant until completion of the fermentations (t=50 h). The presence of possible 4CP metabolite, indicated by yellow/green coloration of the medium, was monitored beginning with its appearance at 28 and 35.5 h for fermentation #1 and #2, respectively. Each system experienced similar appearance and disappearance of the metabolite with an initial sharp spike, an absorbance plateau coinciding with depletion of 4CP, and, finally, a slow degradation over time until medium coloration returned to normal at t=50 h. The disappearance of the yellow/green color was more rapid in the bioreactors than was observed in shake flasks, suggesting that oxidation is a key component in the disappearance of the metabolite as the bioreactor contains a greater concentration of biomass and higher levels of dissolved oxygen compared to shake flasks.

In this experiment, polymer beads were added to the inoculated bioreactors after a 14-h period, but in the event that the system would have been able to recover on its own, possibly rendering the use of polymer beads unnecessary, shake flask experiments were set up in parallel to mimic the bioreactors without polymers. With identical phenolic loading, inoculum, medium, and operating conditions, the shake flasks were maintained for a 4-week period, over which time there was no observed cell growth or depletion of phenolic substrates. Fermentation #2 experienced a longer lag phase of approximately 20 h (Fig. 4b). The higher initial phenolic loading of fermentation #2 had a

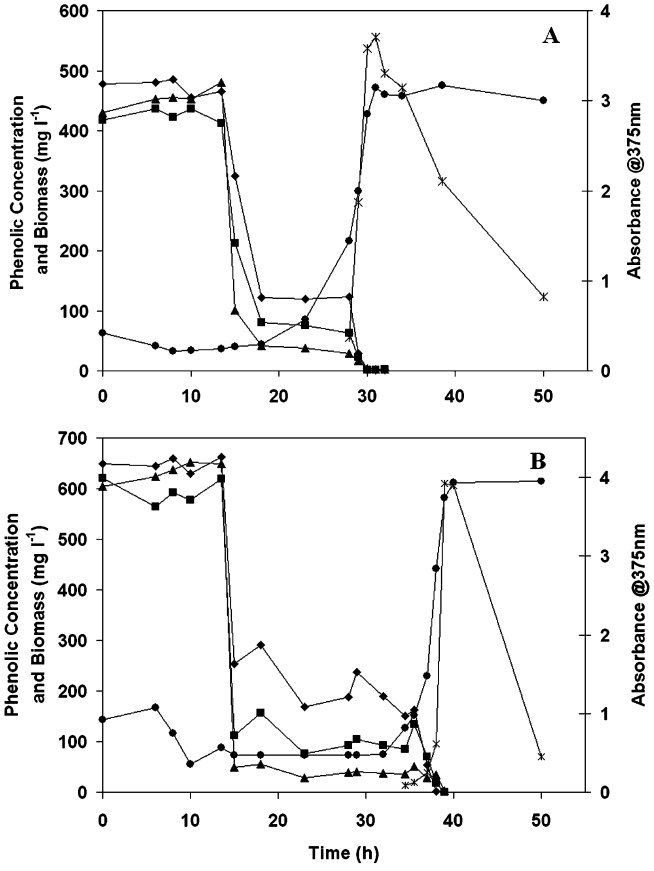

Fig. 4 TPPB operation with HYTREL polymer beads added to the reactor in an attempt to regain biological activity, which has been inhibited due to high concentrations of phenols. Initial phenolic loadings of 450 (**a**) and 600 mg/l (**b**) of each phenol were used and polymer beads were introduced at t=14 h. Phenol (*diamonds*), *o*-cresol (*squares*), 4CP (*triangles*), biomass (*circles*), and HMSA (*x*)

greater impact on the lag phase than the phenolic loading of fermentation #1.

Discussion

The literature has shown that organic molecules will partition between an aqueous and polymer phase, analogous to the partitioning of a solute between two immiscible liquid phases, and this relationship may be quantified by a partitioning coefficient (Anjaneyulu et al. 1990; Rzeszutek and Chow 1998). In a previous work, we were able to quantify the partitioning of phenol between aqueous and polymer phase (HYTREL), with phenol being the sole target compound in solution (Prpich and Daugulis 2004). A search of the literature resulted in many instances in which a number of target molecules were absorbed from solution via a polymeric matrix but, in each case, the target molecules were the sole molecules in solution (Anjaneyulu et al. 1990; Bowen 1970; Fong and Chow 1992; Rzeszutek and Chow 1998; Schumack and Chow 1987). Therefore, it was necessary to determine whether the presence of multiple target phenols in a mixture would have an effect on the partition coefficients of the individual phenolic species. The results showed that HYTREL demonstrated the greatest affinity for 4CP, followed by *o*-cresol and phenol. The increase in affinity among the phenols may possibly be explained by the increasing hydrophobicity of the compounds. Comparing the log P (partitioning coefficient of a compound in an octanol–water system) values for the phenols (phenol=1.46<*o*-cresol=1.95<4CP=2.39), there appears to be a correlation between hydrophobicity of the phenols and absorption affinity. Given that phenols are capable of both hydrophobic and hydrogen bonding interactions, it is reasonable to suppose that hydrophobicity plays a role in the sorption mechanism. The results of this study show the effect that hydrophobicity of the phenolic molecule has on the uptake into a polymer matrix and is in agreement with the literature (Rzeszutek and Chow 1998; Dmitrienko et al. 1999).

An assessment of possible interactions between the phenols and the effect this may have on the partitioning of the individual compounds showed that the presence of 4CP and *o*-cresol do not impact the partitioning of phenol between the aqueous and polymeric phases under the given conditions. Unlike surface adsorption, a phenomenon on which competition exists among target molecules for active sorption sites, polymers express individual partitioning coefficients for target species. In this manner, the absorption of individual target molecules in a mixture may be predicted based on the mass of polymer present as well as the mass of contaminant in solution.

As mentioned previously, a key benefit of solid–liquid TPPBs relative to TPPBs using immiscible organic solvents is due to the fact that the solid polymer phase is non-bioavailable and non-cytotoxic, which permits the use of microbial consortia. With a mixed microbial population present, there exists greater enzymatic diversity over previously operated liquid–liquid TPPBs using single, pure microbial cultures, which have been shown to result in enhanced rates of biodegradation of phenol (Prpich and Daugulis 2004). To highlight the robust nature of a solid–liquid TPPB, a mixture of toxic phenols was degraded via a microbial consortium.

Of the three compounds that comprise the phenolic mixture, 4CP has been shown to be the most recalcitrant to degradation and has been shown to be a non-growth substrate degraded cometabolically in the presence of glucose (Wang and Loh 1999), sodium glutamate (Wang and Loh 2000), and, most commonly, phenol (Saez and Rittmann 1991). Phenol has been hypothesized to be responsible for inducing the enzymes capable of carrying out 4CP degradation and, although the literature has indicated that 4CP alone may be able to induce the appropriate degradative enzymes, (Wang and Loh 1999) our results show that the microbial consortium was able to degrade 4CP only in the presence of phenol, supporting the premise that 4CP is a non-growth substrate requiring cometabolic metabolism.

Biodegradation of the phenolic mixture resulted in yellow/green coloration of the culture medium. The literature has shown that the presence of a yellow/green color is due to the incomplete degradation of 4CP and the

metabolite, having an absorbance at 375 nm, has been identified as 5-chloro-2-hydroxymuconic acid semialdehyde (Westmeier and Rehm 1987). The presence of HMSA suggests the degradation of 4CP via the *meta*-cleavage pathway (Kim and Hao 1999; Saez and Rittmann 1991). A mass spectrum of the suspected HMSA peak produced an output similar to that found in the literature (Hollender et al. 1997), providing additional evidence for the presence of HMSA. Furthermore, the GC–ESI-MS spectrum indicated the presence of additional degradation metabolites that are a consequence of the *meta*-cleavage biodegradation pathway for 4CP.

HMSA has been observed to inhibit metabolic activity (Westmeier and Rehm 1987) and, although the microbial consortium did not appear to suffer any adverse effects during our testing, it is possible that at elevated concentrations HMSA may become an issue. Partitioning of the metabolite from the aqueous phase may enhance the degradation of 4CP and the results showed that the polymer beads were capable of reducing the aqueous phase concentrations of HMSA. Therefore, the beads represent an effective option for the reduction of the potentially inhibitory metabolites. Although not a central feature of this work, the results suggest possible future projects involving the absorption of valuable metabolites in a biotransformation process.

Solid–liquid TPPBs have been demonstrated to effectively absorb and deliver phenol as a single substrate to microbial systems consisting of pure (Amsden et al. 2003) and mixed microbial populations (Prpich and Daugulis 2005) but, as yet, no studies have been performed concerning the partitioning and delivery of multiple substrates in a solid–liquid TPPB. Therefore, experiments were designed to demonstrate the ability of the TPPB to remediate a model mixture of phenols in both traditional and alternative modes of operation.

Traditional TPPB operation, as described in the previous section, is an effective means of demonstrating the ability of the TPPB system to degrade high concentrations of multiple toxic substrates via a microbial consortium under controlled conditions. Previous work has shown operation of a solid–liquid TPPB in fed-batch mode, where polymer beads were used to mitigate toxic shock loading with favorable performance (Prpich and Daugulis 2004). As high phenolic concentrations have been shown to affect biological systems by lengthening the lag phase or even terminating metabolic activity (Prpich and Daugulis 2004), sequestering of these toxic substrates is necessary and, therefore, one objective of this study was to use polymer beads to reduce inhibitory aqueous phase concentrations of phenols in a single-phase system in an effort to regain biodegradative capabilities. The results clearly demonstrate the advantage of using polymer beads for the reduction of elevated concentrations of toxic substrate to return a biological system to favorable operating conditions. Of importance is the significant lag phase exhibited by the microbes due to elevated initial phenol concentrations. The results suggest that as the initial phenolic concentration increases, the effect on lag phase is more profound and, although aqueous phase inhibitory concentrations may be alleviated (by the addition of polymers), the organisms still suffer some residual effects.

In conclusion, polymer beads have been shown to be an effective second phase option for the biodegradation of multiple toxic substrates in a TPPB. The polymer beads offer the ability to reduce inhibitory concentrations of contaminants, thus, moderating the negative impact that toxic loading may have on the lag time of the microorganisms. Therefore, in single-phase biological systems experiencing a loss of productivity due to elevated toxic loading, polymer beads may be employed as an economic and re-usable strategy for the reduction of high concentrations of contaminants and the return of the system to more favorable operating conditions. In addition, the observed absorption of HMSA by the polymer beads opens the possibility of using polymer beads as a means of partitioning toxic and valuable intermediate during the biotransformation of organic molecules.

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References

Amsden BG, Bochanysz J, Daugulis AJ (2003) Degradation of xenobiotics in a partitioning bioreactor in which the partitioning phase is a polymer. Biotechnol Bioeng 84:399–405

Anjaneyulu Y, Marayya R, Prabhakara R, Kumar PVS (1990) Removal and recovery of priority pollutant phenols from industrial effluents using polyurethane foam medium. Oil Chem Pollut 7:349–365

Baumann MD, Daugulis AJ, Jessop PG (2005) Phosphonium ionic liquids for degradation of phenol in a two-phase partitioning bioreactor. Appl Microbiol Biotechnol 67:131–137

Bowen HJM (1970) Absorption by polyurethane foams—new method of separation. J Chem Soc A 7:1082–1085

Brink LES, Tramper J (1985) Optimization of organic-solvent in multiphase biocatalysis. Biotechnol Bioeng 27:1258–1269

Bruce LJ, Daugulis AJ (1991) Solvent selection strategies for extractive biocatalysis. Biotechnol Prog 7:116–124

Collins LD, Daugulis AJ (1997a) Biodegradation of phenol at high initial concentrations in two-phase partitioning batch and fed-batch bioreactors. Biotechnol Bioeng 55:155–162

Collins LD, Daugulis AJ (1997b) Characterization and optimization of a two-phase partitioning bioreactor for the biodegradation of phenol. Appl Microbiol Biotechnol 48:18–22

Cull SG, Holbrey JD, Vargas-Mora V, Seddon KR, Lye GJ (2000) Room-temperature ionic liquids as replacements for organic solvents in multiphase bioprocess operations. Biotechnol Bioeng 69:227–233

David H, Bouzon J, Vergnaud JM (1989) Controlled absorption and release of an active agent by using Evac beads—effect of various parameters. Eur Polym J 25:1007–1011

Davidson CT, Daugulis AJ (2003) Addressing biofilter limitations: a two-phase partitioning bioreactor process for the treatment of benzene and toluene contaminated gas streams. Biodegradation 14:415–421

Dmitrienko S, Myshak EN, Pyatkova LN (1999) An empirical relationship between distribution coefficients of phenols by polyurethane foams and their octanol–water distribution constants and p*K*(a) values. Talanta 49:309–318

El-Shahawi MS (1994) Retention and seperation of some organic water pollutants with unloaded and tri-*n*-octylamine loaded polyester-based polyurethane foams. Talanta 41:1481–1488

Fong P, Chow A (1992) Extraction of aromatic-acids and phenols by polyurethane foam. Talanta 39:497–503

Guieysse B, Cirne MD, Mattiasson B (2001) Microbial degradation of phenanthrene and pyrene in a two-liquid phase-partitioning bioreactor. Appl Microbiol Biotechnol 56:796–802

Hamed TA, Bayraktar E, Mehmetoglu U, Mehmetoglu T (2004) The biodegradation of benzene, toluene and phenol in a two-phase system. Biochem Eng J 19:137–146

Hatefi A, Amsden B (2002) Biodegradable injectable in situ forming drug delivery systems. J Control Release 80:9–28

Hollender J, Hopp J, Dott W (1997) Degradation of 4-chlorophenol via the *meta* cleavage pathway by *Comamonas testosteroni* JH5. Appl Environ Microbiol 63:4567–4572

Inoue A, Horikoshi K (1991) Estimation of solvent-tolerance of bacteria by the solvent parameter log-*P*. J Ferment Bioeng 71:194–196

Kim MH, Hao OJ (1999) Cometabolic degradation of chlorophenols by *Acinetobacter* species. Water Res 33:562–574

Malinowski JJ (2001) Two-phase partitioning bioreactors in fermentation technology. Biotechnol Adv 19:525–538

Marcoux J, Deziel E, Villemur R, Lepine F, Bisaillon JG, Beaudet R (2000) Optimization of high-molecular-weight polycyclic aromatic hydrocarbons' degradation in a two-liquid-phase bioreactor. J Appl Microbiol 88:655–662

Prpich GP, Daugulis AJ (2004) Polymer development for enhanced delivery of phenol in a solid–liquid two-phase partitioning bioreactor. Biotechnol Prog 20:1725–1732

Prpich G, Daugulis AJ (2005) Enhanced biodegradation of phenol by a microbial consortium in a solid–liquid two phase partitioning bioreactor. Biodegradation 16:329–339

Rzeszutek K, Chow A (1998) Extraction of phenols using polyurethane membrane. Talanta 46:507–519

Saez PB, Rittmann BE (1991) Biodegradation kinetics of 4-chlorophenol, an inhibitory co-metabolite. Res J Water Pollut Control Fed 63:838–847

Schumack L, Chow A (1987) Extraction of aromatic organic-compounds by polyurethane foam. Talanta 34:957–962

Serp D, von Stockar U, Marison IW (2003) Enhancement of 2-phenylethanol productivity by *Saccharomyces cerevisiae* in two-phase fed-batch fermentations using solvent immobilization. Biotechnol Bioeng 82:103–110

Stark D, Kornmann H, Munch T, Sonnleitner B, Marison IW, von Stockar U (2003) Novel type of in situ extraction: use of solvent containing microcapsules for the bioconversion of 2-phenylethanol from L-phenylalanine by *Saccharomyces cerevisiae*. Biotechnol Bioeng 83:376–385

Wang SJ, Loh KC (1999) Facilitation of cometabolic degradation of 4-chlorophenol using glucose as an added growth substrate. Biodegradation 10:261–269

Wang SJ, Loh KC (2000) New cell growth pattern on mixed substrates and substrate utilization in cometabolic transformation of 4-chlorophenol. Water Res 34:3786–3794

Westmeier F, Rehm HJ (1987) Degradation of 4-chlorophenol in municipal waste-water by adsorptive immobilized *Alcaligenes Sp*-A-7-2. Appl Microbiol Biotechnol 26:78–83