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Enhanced biodegradation of phenol by a microbial consortium in a solid–liquid two phase partitioning bioreactor

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Abstract

Two phase partitioning bioreactors (TPPBs) operate by partitioning toxic substrates to or from an aqueous, cell-containing phase by means of second immiscible phase. Uptake of toxic substrates by the second phase effectively reduces their concentration within the aqueous phase to sub-inhibitory levels, and transfer of molecules between the phases to maintain equilibrium results in the continual feeding of substrate based on the metabolic demand of the microorganisms. Conventionally, a single pure species of microorganism, and a pure organic solvent, have been used in TPPBs. The present work has demonstrated the benefits of using a mixed microbial population for the degradation of phenol in a TPPB that uses solid polymer beads (comprised of ethylene vinyl acetate, or EVA) as the second phase. Polymer modification via an increase in vinyl acetate concentration was also shown to increase phenol uptake. Microbial consortia were isolated from three biological sources and, based on an evaluation of their kinetic performance, a superior consortium was chosen that offered improved degradation when compared to a pure strain of *Pseudomonas putida* ATCC 11172. The new microbial consortium used within a TPPB was capable of degrading high concentrations of phenol (≈2000 mg l^{-1}), with decreased lag time (10 h) and increased specific rate of phenol degradation (0.71 g phenol g^{-1} cell h). Investigation of the four-member consortium showed that it consisted of two *Pseudomonas* sp., and two *Acinetobacter* sp., and tests conducted upon the individual isolates, as well as paired organisms, confirmed the synergistic benefit of their existence within the consortium. The enhanced effects of the use of a microbial consortium now offer improved degradation of phenol, and open the possibility of the degradation of multiple toxic substrates via a polymer-mediated TPPB system.

Introduction

Phenol is an organic, aromatic compound that occurs naturally in the environment but arises more commonly, and detrimentally, from industrial activities such as petroleum processing, plastic manufacturing and the production of resins. A water-soluble compound, phenol is generally found to contaminate streams, rivers and lakes that are situated near, or are, the receiving waters of industrial factories.

Phenol is considered to be a toxic compound by the Agency for Toxic Substances and Disease Registry (Agency for Toxic Substance and Disease Registry 2003) and death among adults has been reported with ingestion of phenol ranging from 1 to 32 g. Although absorbed rapidly through the lungs, the low volatility of phenol and its affinity for water make oral consumption of contaminated water the greatest risk to humans.

A variety of techniques have been used for the cleanup of phenol contaminated waters and soils with bioremediation receiving the most attention due to its environmentally friendly approach and its ability to completely mineralize toxic organic compounds. Bioremediation strategies have generally

been limited by the toxicity of phenol to the microorganisms used within the system, and by substrate delivery. Measures have been taken to improve such limitations resulting in the advancement of novel bioremediation processes.

The two phase partitioning bioreactor (TPPB) concept (Daugulis 1997) has shown to be effective in resolving the limitations experienced with conventional bioremediation strategies (substrate toxicity and substrate delivery). The TPPB conventionally utilizes an immiscible liquid organic phase, which acts as a sponge that effectively absorbs and partitions large concentrations of toxic compounds from the aqueous phase. Absorption by the organic liquid decreases toxic concentrations to sub-inhibitory levels allowing for the rapid proliferation of microorganisms in the aqueous phase, and subsequent degradation of toxic substrates. As microbes degrade the substrates, equilibrium between the phases is disrupted resulting in a transfer of additional substrate molecules from the organic phase to the aqueous phase. This controlled release of toxic substrate molecules results in a self-regulated feeding of microorganisms that is determined by the their metabolic rate.

The majority of studies reported in the literature on the use of TPPBs have used pure organic solvents as the substrate delivery phase, and single species of organisms due to concerns of possible degradation of the organic phase if a mixed population of organisms were employed. This self-imposed metabolic limitation of using pure cultures is somewhat off-set by the fact that, through rational solvent selection (Bruce & Daugulis 1991), an organic delivery phase can be tailored to a substrate/organism pairing, usually with excellent performance. A few studies (Guieysse et al. 2001a; Marcoux et al. 2000; Villemur et al. 2000) have employed silicone oil as a delivery phase, due exclusively to its non-biodegradability, along with mixed cultures, however, the properties of silicone oil are, for better or for worse fixed; silicone oil thus provides no opportunity for enhancing important solvent properties such as substrate solubility, substrate distribution coefficient, viscosity, etc. as different substrates are selected. This fundamental limitation of the use of silicone oil has been amply demonstrated in TPPB systems degrading polyaromatic hydrocarbons (PAHs), in which rational solvent selection (MacLeod & Daugulis 2003) provided solubilities for PAHs substantially higher than are possible for silicone oil (Villemur et al. 2000).

Pure solvent/pure strain TPPBs have proven to be effective and efficient in the degradation of phenol (Collins & Daugulis 1997), benzene (Yeom & Daugulis 2001), toluene (Collins & Daugulis 1999), and PAHs (Janikowski et al. 2002; MacLeod & Daugulis 2003). As effective as these TPPBs have been, they do have several limitations, as noted above, which restrict operation to the use of pure strains of organisms. Pure cultures consisting of single strains of microorganisms have shown to be effective in the degradation of phenol (Bandyopadhyay et al. 1998; Beshay et al. 2002; Hamed et al. 2003; Seker et al. 1997; Vrionis et al. 2002), however, it has also been shown that microbial consortia can offer improved performance (Ambujom 2001; Guieysse et al. 2001b). The benefit of using a robust microbial consortium is often due to its capability of degrading a variety of toxic substrates without the accumulation of toxic intermediates (Acuna-Arguelles et al. 2003).

In order to accommodate the presence of a widely mixed microbial consortium in a TPPB, previous work (Amsden et al. 2003; Daugulis et al. 2003) has proposed the use of solid polymer beads in place of liquid organic solvents. The polymer beads, which are both biocompatible and non-biodegradable, are capable of absorption and desorption of target substrate molecules. Specifically, a co-polymer of ethylene and vinyl acetate (EVA) has been shown to be effective for the absorption of phenol in a TPPB (Amsden et al. 2003), and release to the cells based on their metabolic demand. The use of a solid polymer, which should enable the TPPB to accommodate a microbial consortium, does not require aseptic technique, as the introduction of any organism capable of degradation of the substrate is welcome and in fact could lead to enhanced degradation rates. Equally important, however, is the potential for such polymer delivery systems to also be tailored (as is the case for pure solvent delivery phases) to a particular target molecule through monomer selection, functionalization, co-polymerization, cross-linking, and polymer processing.

Activated carbon has also been used as a means to decrease excess concentrations of phenols from the bulk solution, thus promoting the growth and subsequent biodegradation of phenols. Activated

carbon relies on a surface adsorption phenomenon whereas polymer beads operate on the absorption, much like an organic solvent. In the case of activated carbon, microbes have been observed to readily attach themselves to the surface of the activated carbon in order to be in close proximity to the toxic substrate (Ha et al. 2000). In the case of polymer beads, absorption and release of phenols to the bulk solution is at a sufficient rate to sustain growth in solution thus eliminating the surface adhesion of microbes to the beads. Therefore, unlike activated carbon that predominately sequesters the phenol from solution without release, polymer beads, much like liquid organic solvents, remove and deliver toxic substrate upon demand.

The work performed in this study has examined the use, and characterization, of a microbial consortium for the degradation of phenol in a TPPB that uses a solid polymer as the delivery phase. As noted, the use of non-biodegradable polymers opens the possibility of using mixed populations of cells with potentially superior performance, without concerns for degradation of the delivery phase. Although it can be anticipated that a consortium will outperform a pure species in most instances, it was necessary in this study to demonstrate quantitatively that this in fact was the case. This study is part of a larger effort which includes rational polymer development, and the use of multiple toxic substrates in mixed culture/polymer TPPB systems.

Materials and methods

Organisms – culture enrichment

A mixed microbial population, selected from three candidate consortia, was used for the biodegradation of phenol in a TPPB. The three biological samples were chosen based on the assumption that they contain phenol-degrading organisms. Microbial consortia were isolated from: activated sludge from a wastewater treatment facility at a pulp and paper mill known to have prior phenol exposure, activated sludge from a wastewater treatment facility at a chemical plant known to have a wide variety of chemical exposures, and a commercial sample of hydrocarbon degrading organisms. Selective enrichment was performed with the samples to isolate for only those organisms capable of degrading phenol. As described later, one of the consortia was ultimately selected for use in the TPPB system after a comparative assessment of performance.

Samples were initially grown on carbon rich medium in order to restore activity in the microbial populations. Five grams of biological samples were grown in 125 ml Erlenmeyer flasks containing 50 ml tryptic soy broth (TSB). The flasks were agitated in an incubator at 30 °C and 180 rpm for a 24 h period and medium turbidity was used to indicate microbial growth. One milliter of aliquots were taken from the TSB medium and transferred to 125 ml Erlenmeyer flasks containing a mineral salts solution consisting of (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}. The sole carbon source was phenol at a concentration of 250 mg l^{-1}. The same mineral salts composition was used for all experiments.

Flasks were incubated at 30 °C and 180 rpm for a 24 h period and medium turbidity was used to indicate cell growth. Subsequent samples were transferred to fresh medium and grown under identical conditions to ensure that phenol-degrading consortia had been isolated. The isolated consortia were examined via a phase contrast microscope at 100× oil immersion to confirm the existence of diverse microbial cultures.

To ensure healthy cultures, a maintenance medium containing the mineral salts solution, 250 mg l^{-1} phenol and 11.8 mg l^{-1} of succinic acid was used to grow and maintain healthy cultures for cold storage. Cultures were stored in Erlenmeyer flasks at 4 °C. Importantly, succinic acid was used only in maintenance cultures and for the isolation of microbial strains. For TPPB seed cultures and studies, phenol acted as the sole source of carbon.

The benchmark for comparing the performance of the desired consortium was *Pseudomonas putida* ATCC 11172 as previously described (Vrionis et al. 2002).

Seed cultures

Seed cultures for use in the performance of consortia studies and the TPPB studies were grown in four 125 ml Erlenmeyer flasks containing 50 ml of maintenance medium and 500 mg l^{-1} of phenol.

The cultures were incubated at 30 °C, and 180 rpm for a 24 h period. The cultures were spun down at 3400 rpm (2600 RCF) for 10 min and re-suspended in 50 ml of fresh medium prior to inoculation.

Measurement of cell concentration

Cell turbidity was measured using an Ultraspec 3000 spectrophotometer at 600 nm, and concentrations were determined from previously constructed calibration curves.

Phenol analysis

Phenol concentration was determined using the 4-aminoantipyrine method (Yang & Humphrey 1975). The 4-aminoantipyrine method is effective at phenol detection to concentrations of 5 μg l^{-1}. Absorbences were measured at 510 nm using an Ultraspec 3000 spectrophotometer and subsequent concentrations were determined with a calibration curve. Sample preparation involved centrifugation at 3400 rpm (2600 RCF) for 10 min to eliminate interference from biomass before the supernatant was removed and analyzed. Samples containing low concentrations of phenol (below 1 mg/l) were filtered (0.22 μm) under vacuum to ensure all biomass was removed.

Kinetic studies

Kinetic studies (to determine specific growth rate, cell yield on phenol, specific substrate utilization rate, and lag period) of the three consortia were undertaken in New Brunswick Scientific Co., Bioflo III Fermentor vessels. The vessels had a working volume of 3000 ml of mineral salts solution and a phenol concentration of 600 mg l^{-1}. The systems were agitated at 400 rpm and the temperature and pH were maintained automatically at 30 °C and 6.8, respectively. Each fermentor was inoculated with an identical mass of 0.07 g cells dry weight (CDW) from each of the three consortia, and the system was aerated at 3 l min^{-1}. Dissolved oxygen was continuously monitored and samples were taken periodically and analyzed for cell and phenol concentrations.

TPPB studies

TPPB studies took place in a Bioflo III Fermentor as previously stated. The vessel had a working volume of 3000 ml and an initial phenol concentration of 2000 mg l^{-1}. The system was agitated at 600 rpm and temperature and pH were automatically maintained at 30 °C and 6.8, respectively. Five hundred and fifty grams of fresh, or reused, EVA beads were introduced to the system and allowed to equilibrate (in terms of phenol concentration) for a period of 15 h. Upon reaching equilibrium the system was inoculated with 50 ml concentrated seed culture and aerated at 2 l min^{-1}. Dissolved oxygen was monitored continuously, and samples were taken periodically and tested for cell and phenol concentrations. Due to equipment malfunction, dissolved oxygen measurements were only recorded for the first TPPB study that used fresh EVA beads.

De-sorption test

Upon completion of the TPPB studies, 20 g of EVA beads were removed and placed in a 1000 ml Erlenmeyer flask containing 500 ml of tap water. The flask was agitated at 180 rpm at 30 °C, and after 24 h aqueous samples were tested to determine the amount of phenol that had leached out of the beads and into solution.

Polymer capacity

ELVAX 25 (poly (ethylene-co-vinyl acetate)), supplied as a sample from Dupont Canada, was the inert polymer used for all TPPB fermentations. The polymer has a density of 0.965 g cm^{-3} and a vinyl acetate concentration of 25%. ELVAX 25 was chosen for all TPPB experiments due to its commercial availability. The polymer consists of small egg shaped beads with an approximate radius of 1.7 mm.

Tests were conducted to determine the capacity for phenol of EVA beads with varying concentrations of vinyl acetate. Testing occurred in 250 ml Erlenmeyer flasks containing 70 ml of mineral salts solution and a phenol concentration of 2000 mg l^{-1}. Polymer capacity tests were performed in a mineral salts solution to simulate actual conditions in a functioning TPPB system. Six grams of EVA beads were added to the solution and allowed to equilibrate for a 24 h period at 30 °C and 180 rpm. After the 24 h period an aqueous sample was taken, tested for phenol concentration, and a mass balance was performed

to determine the absorption capacity of the particular EVA bead.

Organism isolation

A serial dilution was performed on the selected microbial consortium grown in liquid culture. A 1 ml sample was taken from liquid culture and diluted in sterile water to a concentration of 10^{-6}. Two hundred and fifty microliter from each dilution was spread onto sterile agar plates consisting of mineral salts medium, 250 mg l^{-1} of phenol, 11.8 mg l^{-1} of succinic acid, and 15 g l^{-1} of Bacto agar using aseptic techniques. Plates were incubated at 30 °C for 24 h, after which time individual isolates were chosen from the plate for further isolation. Individual colonies were chosen based on their colony and cellular morphology when examined using a phase contrast microscope at 100× oil immersion. The individual isolates were re-streaked on fresh maintenance medium agar plates and incubated at 30 °C for 24 h. An additional re-streaking of individual isolates took place to ensure purity, and microscopy validated that single isolates had indeed been isolated on each plate. Grown plates were sealed with Parafilm and stored at 4 °C.

Preliminary identification of organisms

Individual colonies of each isolated isolate from the selected consortium were re-streaked on TSB agar plates and incubated at 30 °C for 24 h to ensure adequate and healthy growth of microorganisms. Identification of the individual microbial isolates was performed using the bioMereiux API 20 NE strips and the API identification database.

Performance of individual isolates and interactions

Individual isolates from the selected microbial consortium were further studied to determine the role that they play within the consortium. The individual isolates were also paired together in all possible combinations for a study on isolates interactions.

Cultures were grown in 125 ml Erlenmeyer flasks containing 50 ml of mineral salts medium and phenol at a concentration of 275 mg l^{-1}. The flasks were incubated at 30 °C and agitated at 180 rpm for a period of 24 h. Inoculum size for each flask was maintained at a constant mass by means of spectrophotometer measurement and cell dry weight calibration curves. Cell and phenol concentrations were monitored in each flask.

Results and discussion

Isolation of microbial consortia

Initial work focused on the isolation of a superior microbial consortium capable of degrading phenol. Again, as noted earlier, although microbial consortia are likely to outperform individual pure species of organisms, it was necessary in this study to verify, and demonstrate quantitatively, that this was the case; claiming superiority of a biological catalyst without proper verification (notwithstanding the likelihood of this being the case) cannot be scientifically justified. Three mixed microbial samples were chosen for isolation of phenol-degrading organisms by selective enrichment, which resulted in three unique consortia capable of degrading phenol. Contained within each consortium were, at a minimum, four distinct isolates, all of which were rod shaped organisms that appeared to exhibit motility. Further study of the individual isolates within one of the consortia was conducted after identifying the consortium having the most improved performance in terms of growth and phenol degradation.

Assessing consortium performance

In order to measure the performance of the three microbial consortia, the specific growth rate (μ), cell yield coefficient ($Y_{x/s}$), specific rate of substrate utilization (Q_s), and lag time were chosen as the criteria upon which performance was assessed. The performance of the three microbial consortia was compared to the growth characteristics of *Pseudomonas putida* ATCC 11172, a known and effective degrader of phenol (Amsden et al. 2003; Vrionis et al. 2002). Improved performance exhibited by a consortium over the single species of *Pseudomonas putida* ATCC 11172 would justify the use of a consortium for TPPB studies.

As seen in Table 1, consortium #1 (isolated from activated sludge taken from a pulp and paper mill) had the capability to degrade the greatest quantity

Table 1. Values for growth properties of three consortia relative to *Pseudomonas putida* ATCC 11172

	μ_{max} (h^{-1})	$Y_{x/s}$	Lag time (h)	Q_s (g phenol g^{-1} cells h^{-1})
Pseudomonas putida 11172	0.28	0.53	17	0.53
Consortium #1	0.47	0.67	11	0.71
Consortium #2	0.39	0.81	15	0.49
Consortium #3	0.30	0.7	15	0.43

of phenol per unit cell mass, per unit time (i.e. the highest Q_s), and this parameter was felt to be the most important measure of microbe performance. Consortium #1 also had the highest specific growth rate and the shortest lag period. The cell yield is higher than that of *Pseudomonas putida* ATCC 11172 but is slightly lower than those for consortium #2 (isolated from a commercial sample of hydrocarbon degrading organisms) and #3 (isolated from activated sludge taken from a chemical plant). The performance parameters determined for *Pseudomonas putida* ATCC 11172 are comparable to numbers achieved in previous studies (Amsden et al. 2003; Vrionis et al. 2002). In summary, these results show that a microbial consortium isolated from a sample of sludge taken from a wastewater treatment facility has improved performance for the degradation of phenol when compared to a single pure strain of a known and proven phenol degrader, and therefore should provide superior performance in a polymer TPPB system.

Enhancement of TPPB performance

The goal of this work was to improve on the biological performance of a TPPB that had previously made use of a solid polymer second phase (Amsden et al. 2003) and a pure strain of microorganism (*Pseudomonas putida* ATCC 11172). EVA, an inert co-polymer with a vinyl acetate concentration of 25%, was used as the second phase in the TPPB. To determine the quantity of polymer required to decrease aqueous phenol concentrations to sub-inhibitory levels, preliminary studies were conducted on the capacity of the polymer, and the absorption capacity was calculated to be 7.7 mg g^{-1} phenol EVA beads. From this value it was determined that a 3 l working volume reactor with a concentration of 2000 mg l^{-1} of phenol would require 550 g EVA beads to lower the phenol concentration to a sub-inhibiting level of about 700 mg l^{-1}.

Polymer modification, for example a change in the fraction of co-polymer, was found to affect the uptake of phenol, and this confirms the capability of polymer delivery TPPB phases to be rationally selected and modified to improve performance, similar to organic solvent TPPB systems. As shown in Figure 1, EVA blends with vinyl acetate concentrations of 9%, 23%, and 32% had absorption capacities of 3.6, 7.7, 11.9 mg g^{-1} phenol, respectively. Previous work (Amsden et al. 2003) showed that an EVA poly-

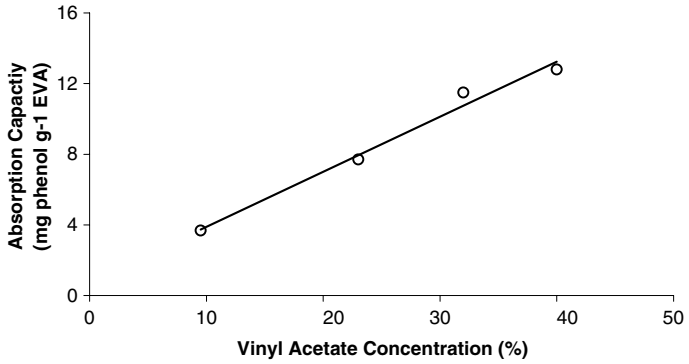

Figure 1. Absorption capacity of phenol as a function of vinyl acetate concentration in EVA.

mer with a vinyl acetate concentration of 40% had a phenol absorption capacity of 12.8 mg g^{-1} EVA. Figure 1 shows a linear relationship between increased absorption and increased vinyl acetate concentrations. It has been proposed (Anjaneyulu et al. 1990) that the absorption of phenol by a polymer is a result of hydrogen bonding between the functional group of the polymer and the polar hydroxyl group on the benzene ring. Therefore it is likely that the increase in functional groups arising from an increased percentage of vinyl acetate would result in an increase in the number of sites for which hydrogen bonding could occur. The data confirm those findings as an increase in the concentration of functional group directly correlates to a linear increase in the absorption capacity of the polymer for the target molecule phenol. The issue of polymer development and its effect on TPPB performance will be discussed later.

Figure 2 shows that when the EVA beads were added to the bioreactor an equilibrium concentration of 740 mg l^{-1} of phenol was attained; thus the EVA beads effectively reduced the phenol concentration to below inhibitory concentrations, allowing for the introduction of the microbial consortium. After seeding of the bioreactor, the consortium experienced a lag phase of approximately 10 h before exponential growth began. The lag phase may be attributed to the considerable level of phenol still present in the aqueous phase. From Figure 2 it can be seen that the bulk of phenol degradation occurred during a 5 h period (hours 25–30) after which substrate became depleted and cell concentration plateaued. It should also be noted that the exponential nature of the cell growth seen in Figure 2 indicates that substrate limitation did not occur, suggesting that the rate of diffusion of phenol from the EVA beads was sufficiently rapid so as not to limit cell growth. The consumption of oxygen, when plotted against time gave an excellent indication of the intense oxygen demand during the exponential growth phase although the dissolved oxygen concentrations did not drop below 60% and thus the system was not oxygen limited (Figure 2). Relative to the use of a pure strain of *Pseudomonas putida* and EVA beads (Amsden et al. 2003) the consortium had a reduced lag period and a more rapid uptake of phenol, as anticipated from the performance assessment summarized in Table 1.

The cell yield coefficient for this experiment was calculated to be 0.60 and the specific substrate utilization rate was found to be 0.79 g g^{-1} h^{-1}. These values are similar to earlier experimentally determined values for growth in a batch reactor (Table 1) and the values are an improvement compared to the $Y_{x/s}$ and Q_s rates for *Pseudomonas putida* ATCC 11172 operated under identical conditions in previous work (Amsden et al. 2003).

Biomass concentrations continued to increase for up to 10 h after the degradation of all available phenol (hours 30–40). Similar observations of continued growth of biomass have been reported with isolates of *Pseudomonas* and *Acinetobacter* grown on phenol (Hao et al. 2002; Saez & Rittmann 1991). The continued growth of biomass suggests the possible degradation of meta-

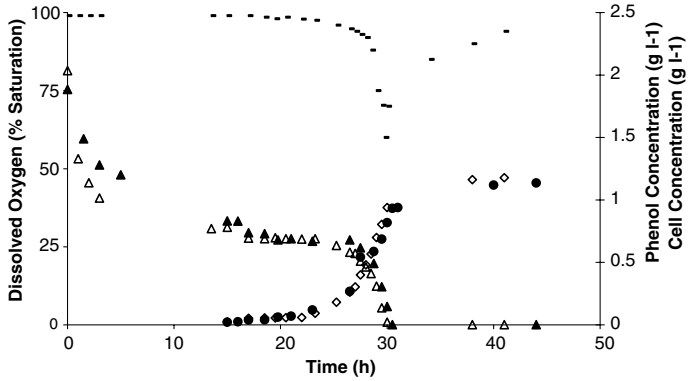

Figure 2. Replicated degradation experiments performed with a TPPB and EVA beads. Solid shapes represent first run with fresh EVA beads. Open shapes represent second run with reused EVA beads. (-) DO first run, (▲) Phenol first run, (△) Phenol second run, (◆) Cells first run, (◇) Cells second run.

bolic intermediates but analysis of intermediates was not undertaken to confirm this hypothesis.

Upon completion of the run, 20 g of used EVA beads were placed in 500 ml of tap water and allowed to equilibrate over a 24 h period, after which time a sample was drawn for detection of phenol. No phenol was detected indicating that all of the phenol was released from the beads and effectively degraded by the consortium. Biodegradation of phenol did not occur during these desorption tests, as microscopic and electron microscopic examination (Amsden et al. 2003) of the bead surfaces after their use in the bioreactors showed no adsorbed microbial cells.

A second fermentation was run in an identical fashion to the first with an initial phenol concentration of 2030 mg l^{-1}, but with 550 g of EVA beads used in the first fermentation. After a 15 h period (Figure 2) the beads reduced the phenol concentration to a sub-inhibitory level of 690 mg l^{-1} resulting in a capacity of 6.18 mg g^{-1} phenol. A period of 15 h was again chosen to ensure that equilibrium had been reached. From previous work with EVA (Amsden et al. 2003) and Figure 2 it can be seen that 80% of phenol absorbs in approximately 6 h. As the system nears equilibrium the concentration driving force decreases slowing the rate of phenol diffusion into the beads; thus even though a period of 15 h was provided, this was done merely to ensure that equilibrium was achieved, and to allow confirmation of EVA capacity. The system was inoculated and experienced a lag phase of approximately 10 h, after which time the system underwent an exponential growth phase of 5 h followed by a plateau in cell concentration. $Y_{x/s}$ and Q_s were calculated to be 0.58 and 0.82 g g^{-1} h^{-1}, respectively, which are similar to the values calculated from the first fermentation.

Upon completion of the fermentation, 20 g of EVA beads were removed from the reactor and placed in a flask containing 500 ml of tap water. No phenol was detected in solution after 24 h of contact. The fresh and reused EVA beads therefore showed similar absorption capacities and the ability to completely release all absorbed molecules of phenol, confirming that the EVA beads are capable of reuse with no loss of performance.

The two performance assessment fermentations were conducted under non-aseptic conditions. Previous work on TPPB systems utilizing organic solvents/pure cultures required the sterilization of medium and equipment to ensure contamination would not occur during the experiment (Collins and Daugulis 1997; Vrionis et al. 2002). If a system operating with a single pure microbial species were to be contaminated the result could yield inconsistent kinetic data, and in the case of TPPBs utilizing a liquid solvent, contamination may also result in the consumption of the organic phase. A microbial consortium eliminates the necessity for sterilization, as contamination with organisms capable of growing in an environment with such selection pressure will be beneficial at degrading the target molecules. The result of contamination would likely be an improved microbial consortium, which would be used for enhanced degradation. To determine if contamination may have occurred in these experiments, microscopy was used to view samples at various stages of the fermentations, but no new species appeared to be introduced during the experiment. As well, performance of the consortium remained constant after repeated fermentations indicating a similar microbial coznsortium.

Isolation and identification of consortium members

The selected microbial consortium was further studied to identify the individual isolates, and their contributions to phenol degradation. Four unique isolates were found to exist in the phenol-degrading consortium, all of which were gram-negative rods.

API 20 NE strips, specific for non-fastidious, gram-negative rods not belonging to the *Enterobacteriacaea*, were chosen as the method of identification. Were it deemed to be important to more precisely identify consortium members, 16S rRNA methods could have been used, however, more definitive identification would not change the outcome of this study. Table 2 contains the results of the biological tests conducted on the organisms. The results from the API strips were entered into the API 20 NE database and the organisms were identified. Isolate 1 was identified as *Pseudomonas putida* with excellent validity, isolate 2 was identified as *Acinetobacter baumannii* with very good validity, isolate 3 was identified as *Pseudomonas alcaligenes* with good validity and isolate 4 was identified as *Acinetobacter johnsonii* with excellent validity to the genus level.

Microscopy was used to further confirm the results of the API 20 NE identification. From microscopic observations at 100× oil immersion the *Acinetobacter* isolates appeared to be short rods that occurred in pairs or formed long chains. These observations are in accordance with previous work (Springer-Verlag Heidelberg 2003). Others (Schirmer et al. 1997) have confirmed the ability of *Acinetobacter* isolates to readily degrade phenol and hence it can be anticipated that *Acinetobacter* isolates may be in this consortium. Microscopy of the *Pseudomonas* isolates showed the organisms to be thin rods exhibiting motility. *Pseudomonas* isolates are readily connected to many bioremediation strategies involving aromatic compounds (Vrionis et al. 2002).

Table 2. Results from API 20 NE identification procedure

Tests	Substrates	Reaction	Results			
			Isolate 1	Isolate 2	Isolate 3	Isolate 4
NO_3	Potassium nitrate	Reduction of nitrates to nitrites	–	–	–	–
		Reduction of nitrates to nitrogen	–	–	–	–
TRP	Tryptophane	Indole production	–	–	–	–
Glucose	Glucose	Acidification	–	+	–	–
ADH	Arginine	Arginine dihydrolase	+	–	–	–
Urease	Urea	urease	–	–	–	–
ESC	Esculin	Hydrolysis (β–glucosidase)	–	–	–	–
Gelatin	Gelatine(with India ink)	Hydrolysis (protease)	–	–	–	–
PNG	*p*–Nitrophenyl–B–D–galactopyranoside	β–galactosidase	–	–	–	–
GLU	Glucose	Assimilation	+	–	–	–
ARA	Arabinose	Assimilation	–	+	–	+
MNE	Mannose	Assimilation	+	–	–	–
Manitol	Mannitol	Assimilation	–	–	–	–
NAG	*N*–acetyl–glucosamine	Assimilation	–	–	–	–
MAL	Maltose	Assimilation	–	–	–	–
GNT	Gluconate	Assimilation	+	–	+	–
CAP	Caprate	Assimilation	+	+	+	+
ADI	Adipate	Assimilation	–	–	+	–
MLT	Malate	Assimilation	+	+	+	+
CIT	Citrate	Assimilation	+	+	–	+
PAC	Phenyl–acetate	Assimilation	+	–	–	–
Oxidase		Cytochrome oxidase	+	–	+	–
Cell morphology			Rod	Rod	Rod	Rod
Gram reaction			–	–	–	–
Motility			+	–	+	+

Replicate experiments were conducted on the four individual organisms to further characterize the roles that they may play during the degradation of phenol (data not shown). Experiments were carried out with individual isolates as well as in all possible pairs of isolates. The results suggest some synergistic effects occurring between the organisms (e.g. 2 of the 4 isolates were not able to degrade phenol *per se*, but grew well when either of the other strains were present), which may aid in the biodegradation of phenol. Although details of these interactive effects are unknown at the present time the experiments did demonstrate that all members of the consortium do play a role in phenol biodegradation.

Conclusion

The results presented here are part of a larger study that has three objectives: to confirm that a microbial consortium can outperform a single species if an appropriate TPPB polymer delivery agent can be found; to rationally select and modify polymeric delivery agents for various substrates; and, to show the efficacy of such polymeric agents in mixed substrate/mixed culture TPPB systems. The use of a microbial consortium has been shown to improve the biological performance of a solid–liquid phase TPPB (objective one) when compared to a similar TPPB system operating with a single pure isolate. Although not unexpected, this validates the use of a non-biodegradable polymer delivery phase rather than an organic solvent, which could be susceptible to microbial attack in the presence of a mixed population of organisms. Improved specific rates, yield, and lag time were observed with the consortium in comparison to a pure species of *Pseudomonas putida* ATCC 11172. This translates into decreased cultivation times with increased masses of degraded target molecules.

A study of the microbial consortium revealed the existence of four individual microbial isolates. Tests conducted on the individual isolates showed that the *Pseudomonas* strains present were incapable of degrading phenol as individuals, but were able to degrade phenol when present with other strains. This suggests that all organisms within the consortium are important in the degradation of phenol.

The synergistic effects of the microbial consortium contributed to the complete degradation of phenol, the target molecule. The benefits of using a microbial consortium may be more apparent in a system in which multiple toxic substrates are present, and we are currently examining this scenario.

Solid polymer beads of EVA were used as the second phase in the TPPB and performed as anticipated with no loss in performance over repeated fermentations. It was observed that a direct correlation exists between the amount of the acetate functional group and the ability of the EVA beads to absorb phenol. Further polymer development is currently being undertaken (objective two, above) with efforts aimed at increasing polymer absorption capacity, quantifying the absorption rate, and rationally modifying polymer properties to be able to generate superior solid delivery agents for phenol and other xenobiotics substrates.

In addition to also investigating the use of multiple substrates in polymer TPPB systems, we are examining the prospect of a field test with mixtures of polymer beads. Specifically, we envisage possible sites containing mixtures of toxic substrates at levels that are sufficiently high so as to severely limit, or prevent, degradation by existing microbial populations. The addition of mixed polymer beads to reduce substrate concentrations to sub-inhibitory levels could result in the restoration of microbial activity, and substrate mineralization, with concomitant release of substrate by the polymer beads, leading to complete degradation.

Although polymer beads have performed well as the second phase within a TPPB, it should be noted that liquid organic solvents will still be advantageous for TPPB applications that require large absorption capacities, minimal diffusional resistances in the second phase (i.e. rapid uptake and release), and applications in situations in which the target molecule is relatively insoluble.

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