Degradation of Xenobiotics in a Partitioning Bioreactor in Which the Partitioning Phase is a Polymer

Brian G. Amsden, Justyna Bochanysz, Andrew J. Daugulis

Department of Chemical Engineering, Queen's University, Kingston, Ontario, Canada K7L 3N6; telephone: (613) 533-2784; fax (613) 533-6637; e-mail: Daugulis@chee.queensu.ca

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Abstract: Two-phase partitioning bioreactors (TPPBs) are characterized by a cell-containing aqueous phase and a second immiscible phase that contains toxic and/or hydrophobic substrates that partition to the cells at subinhibitory levels in response to the metabolic demand of the organisms. To date, the delivery phase in TPPBs has been a hydrophobic solvent that traditionally needed to possess a variety of important properties including biocompatibility, nonbioavailability, low volatility, and low cost, among others. In the present work we have shown that the organic solvent phase can be replaced by inexpensive polymer beads that function in a similar fashion as organic solvents, delivering a toxic substrate to cells based on equilibrium considerations. Specifically, 3.4 mm diameter beads of poly(ethylene-co-vinyl acetate) (EVA) were used to reduce the aqueous concentration of phenol in a bioreactor from toxic levels (~2,000 mg/L) to subinhibitory levels (~750 mg/L), after which *Pseudomonas putida* ATCC 11172 was added to the system and allowed to consume the total phenol loading. Thus, the beads absorbed the toxic substrate and released it to the cells on demand. The EVA beads, which could be reused, were able to absorb 14 mg phenol/g EVA. This work has opened the possibility of using widely mixed cultures in TPPB systems without concern for degradation of the delivery material and without concern of contamination. © 2003 Wiley Periodicals. *Biotechnol Bioeng* **84**: 399–405, 2003.

Keywords: partitioning bioreactor; phenol; degradation; polymer; EVA

INTRODUCTION

The controlled biodegradation of toxic substrates is substantially hampered by the challenge of substrate delivery. If substrates are added at too high a concentration, the microbes could be inhibited or killed, and if added at too low a concentration the cells' metabolic activity could be reduced by substrate limitation. Substrate delivery is further complicated by the fact that as the cell population increases and/or the environmental conditions of pH, temperature, etc., change, the desired rate of substrate addition changes as well. These challenges have been largely met through the use of two-phase partitioning bioreactors (TPPBs) in which an immiscible organic solvent phase is used to provide delivery on demand to cells in the aqueous phase based on the system maintaining thermodynamic equilibrium. TPPBs have been shown to be very effective at degrading large amounts of toxic substrates added exogenously to the system and have also been used to deal with toxic organics occurring at high concentrations in water (Yeom et al., 2001), air (Davidson and Daugulis, 2003; Yeom and Daugulis, 2001a), and soil situations (Yeom and Daugulis, 2001b). The applications and performance of TPPBs have also been recently reviewed (Déziel et al., 1999; Malinowski, 2001).

TPPBs have additionally been shown to be very effective at delivering poorly water-soluble substrates to cells (Guieysse et al., 2001; Janikowski et al., 2002). For such substrates, degradation can often be limited by the lack of availability of the substrate to the organisms. The dissolution of the substrate in an immiscible organic solvent, and its subsequent dispersion within the aqueous phase, can provide a very large surface area, and hence availability, of the substrate to maintain biodegradation at its maximum, inherent rate.

Although the criteria that a delivery solvent must meet to be used in TPPB applications are quite extensive, numerous research groups have been able to successfully identify a variety of different solvents for their particular applications. One of the solvent criteria has been that the delivery solvent must be nonbioavailable, i.e., that it not be used as a substrate by the degrading organisms, so as to prevent preferential uptake of the solvent at the cost of the target substrate. Even though a recent article (MacLeod and

Correspondence to: A.J. Daugulis

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Daugulis, 2003) has shown that this requirement is not necessarily a ''hard'' criterion, nonbioavailability will likely remain as a condition that must be met by TPPB solvents. Related to this is the potential impact of contamination in TPPBs, which could result in degradation of the delivery solvent. Thus, the biodegradability of the solvent remains a concern.

These limitations could be overcome by using a polymer phase as the absorption/desorption component of the two-phase partitioning bioreactor. It is a well-known fact that polymers are capable of absorbing small molecular weight compounds (Crank and Park, 1968), this fact being the basis for many polymeric drug delivery systems (Baker, 1987). Such polymers can be very inexpensive, can be formed into many shapes and sizes, and, most importantly, are nonbiodegradable, thus minimizing concerns of solvent loss either by the organisms introduced intentionally into these systems or by possible contaminants. Thus, such delivery materials should be readily used in mixed culture situations, something that traditional TPPBs have been unable to accommodate.

Although liquid polymers have been used in TPPBs (e.g., silicone oils (Aalam et al., 1993; Guieysse et al., 2001)), these systems are still two liquid phase bioreactors. The polymers investigated in this work are solid at the reactor conditions, and thus possess several possible advantages. These advantages include ease of handling, complete recovery from the bioreactor, no potential for adsorption to or absorption into the reactor materials (for example, rubber gaskets and seals), and potential for modification to include properties such as temperature or solvent-induced swelling or contraction which may be beneficial to enhance xenobiotic uptake and delivery.

In the present work we show that small polymer beads comprised of poly(ethylene-co-vinyl acetate) (EVA) can act in a similar fashion as organic solvents in TPPBs, by having an affinity to absorb a toxic substrate, phenol, and release it on demand to the cells.

MATERIALS AND METHODS

Organism

The bacterium used in this study was *Pseudomonas putida* (ATCC 11172), which degrades phenol via the aerobic meta-cleavage pathway (Collins and Daugulis, 1996). *Pseudomonas putida* has been used extensively by us for phenol degradation in TPPBs (Collins and Daugulis, 1996; Vrionis et al., 2002a–c) utilizing organic solvents as delivery phases. Aqueous phenol concentrations above about 800 mg/L are toxic to these organisms.

Polymer

ELVAX40 (poly(ethylene-co-vinylacetate)) was supplied as a gift from Dupont Canada. This amorphous polymer has a density of 0.965 g/cm^3 and a vinylacetate content of 40%. The EVA was received as roughly spherical beads with an effective radius of 1.7 mm.

Media Preparations

Synthetic media consisting of mineral salts, trace elements, and iron chloride (as previously described) were used in all of the experiments (Vrionis et al., 2002b). Phenol and dextrose were used as carbon sources for maintenance of cultures on solid medium and for inoculum preparation purposes (Vrionis et al., 2002b). The medium for absorption and bioreactor studies consisted of the mineral salts formulation along with phenol, at a concentration of 2,000 mg/L, in 3 L of working volume.

Absorption of Phenol by EVA

Three hundred grams of EVA beads were added to 3 L of medium at a phenol concentration of 2,000 mg/L and stirred in a bioreactor at 30°C for 47 h. The concentration of the phenol at the end of this period was used, via mass balance, to determine the absorption capacity of the EVA beads (mg phenol/g EVA).

Bioreactor Studies

A Bioflo III Fermentor vessel (New Brunswick Scientific, Edison, NJ) was filled with 2,800 mL of bioreactor medium at a phenol concentration of 2,000 mg/L and autoclaved. The reactor was agitated at 400 rpm and kept at 30°C and a pH of 6.9 (using 2M NaOH). These conditions were maintained automatically by the Bioflo system throughout the duration of the experiments. A condenser was used to prevent volatilization of the reactor contents. After determining the capacity (i.e., mg phenol/g EVA) of the EVA beads to take up phenol, an amount of EVA required to reduce the phenol to a desired level was sterilized with UV radiation and added to the reactor. Samples were taken for 24 h to monitor the absorption of phenol by the EVA beads. Once the aqueous phenol concentration had reached its equilibrium value, 200 mL of 24 h *P. putida*-containing inoculum was added to the bioreactor. The system was aerated at 3 L/min, with the dissolved oxygen (DO) monitored by a DO electrode. Samples of the bioreactor broth were collected for phenol analysis and cell concentration at various times during the fermentation. To determine the reproducibility of this experiment, it was repeated with fresh EVA beads, and also with beads that had been used in a previous experiment.

Postbatch Fermentation Desorption Experiment

A portion (3.3 g) of the EVA used in the first and second fermentations was placed in a flask containing 100 mL of medium and the contents were agitated on a shaker

for 24 h at 30°C. The medium was then analyzed for phenol content.

Phenol Analysis

All phenol samples were analyzed using the 4-amino-antipyrine method (Vrionis et al., 2002b), which is sensitive to phenol concentrations at the 5 μg/L level. Absorbance readings were obtained using an Ultraspec 3000 spectrophotometer at 505 nm.

Cell Concentration

Cell concentration was determined by optical density measurements at 650 nm and the use of a previously prepared calibration curve relating OD to cell dry weight.

Physical Property Changes of EVA

EVA beads used once and twice were analyzed for changes in thermal properties. Ten mg EVA samples were subjected to a heating-cooling cycle from −70°C to 100°C to −70°C at a uniform rate of 10°C/min using a Seiko SSC/5200 Differential Scanning Calorimeter (DSC). The glass transition temperature of the polymer was measured as the inflection point in the endotherm using the Seiko software.

Analysis of the Surface of EVA Beads

Used EVA beads were analyzed using a scanning electron microscope in order to see if bacteria attached to the polymer during the fermentation. Standard SEM procedures were followed, i.e., the samples were mounted onto a metallic stand using double-sided adhesive tape and then gold-sputtered.

RESULTS AND DISCUSSION

Before discussing the results, the rationale for the choice of EVA as a sorbent for phenol should be provided. The solubility of a nonpolar organic solute in a nonpolar polymer can be estimated through the use of the following expression (Michaels et al., 1975):

$$\ln\phi = \frac{\Delta H_f}{R}\left(\frac{1}{T} - \frac{1}{T_f}\right) - (1 + \chi) \qquad (1)$$

where f is volume fraction of the solute in the polymer, Δ_f is the latent heat of fusion of the solute, T_f is the solute melting point, and χ is the polymer-solute interaction parameter. Use of Eq. [1] is valid provided that ϕ is low. For nonpolar systems, χ can be estimated using:

$$\chi = 0.34 + \frac{V_s}{RT}(\delta_g - \delta_p)^2 \qquad (2)$$

in which R is the gas constant, V_s is the molar volume of the solute, T is temperature, and δ_s and δ_p are the solubility parameters of solute and polymer, respectively (Painter and Coleman, 1994).

EVA has a solubility parameter of 9.4 $(cal/cm^3)^{1/2}$ (Mark, 1999), while the solubility parameter of phenol can be estimated to be 10.4 $(cal/cm^3)^{1/2}$ through the use of the group-contribution method (both at 25°C) (Painter and Coleman, 1994). The latent heat of fusion of phenol is 28.67 cal/g and its melting point is 40.9°C (Weast and Astle, 1982). Using these numbers, the weight fraction of phenol at saturation in EVA at 25°C is calculated to be 0.25. Thus, it would be expected that EVA would be a good sorbent for phenol.

In a preliminary study, EVA beads were added to 3 L of medium containing 2,000 mg/L phenol at 30°C and left for 47 h to achieve equilibrium. The equilibrium capacity of the EVA beads for phenol under these conditions was found to be 15.2 mg/g EVA. This information was used to determine the mass of beads that had to be added to the bioreactors to reduce the concentration from ~2,000 g/L to subinhibitory levels. Thus, for the first bioreactor experiment, 291.3 g of EVA beads were added to 2.8 L of phenol-containing medium and reduced the aqueous phenol concentration to 718 mg/L in 23 h (Fig. 1), which corresponds to a capacity of 12.3 mg phenol/g EVA. The bioreactor was inoculated with *P. putida* at 24 h. After a 13-h lag, the cell concentration began to increase and aqueous phenol concentration decreased until phenol was undetectable in the aqueous phase. Due to an equipment malfunction, the dissolved oxygen (DO) could not be measured during this experiment. The fairly long lag phase can be attributed to the relatively high level of phenol in the aqueous phase even after equilibration with the EVA beads; the length of the lag has been shown to correlate well with the level of phenol initially present. The cell concentration can be seen to increase in an exponential fashion and plateau at the point of substrate exhaustion. During this rapid growth period, the medium took on a characteristic yellow color, which is an indication of the formation of 2-hydroxy muconic semi-

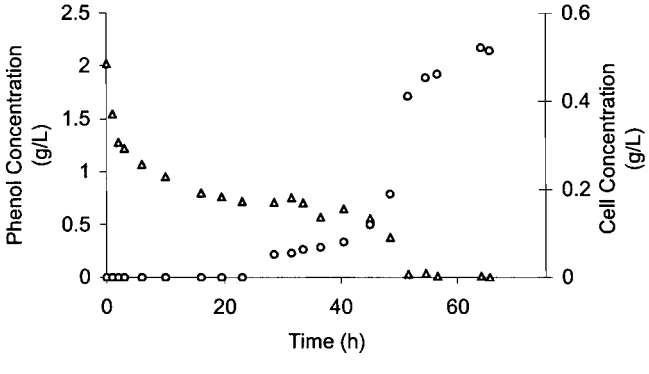

Figure 1. Bioreactor run 1. The open triangles represent phenol concentration in the bioreactor medium while the open circles represent cell concentration. The bioreactor was inoculated at 24 h.

aldehyde, a by-product of phenol degradation by the meta-cleavage pathway.

The cell yield (g dry weight cells per g phenol) for this experiment was calculated to be 0.26, which is similar to other work with this organism (0.20, Collins and Daugulis (1997), and 0.29, Vrionis et al. (2002a)). In the present work, and in the literature cited, wall growth was evident in all cases, possibly leading to an underestimate of the true cell yield. Electron microscopy studies of the used beads did not reveal any cells attached to the surface of the EVA (Fig. 2).

Figure 3 shows the results of a similar experiment in which 291.3 g of EVA beads were added to the bioreactor to reduce the phenol to below inhibitory levels. The beads were able to reduce the aqueous phenol concentration to 712 mg/L within 23 h (resulting in a phenol capacity of 12.4 mg/g EVA) before the system was inoculated with cells at 24 h. Again, after a lag of about 13 h, the cell concentration increased rapidly until the phenol in the system had been degraded. The DO profile was relatively flat, except near the point of maximum cell activity, when a drop in DO was clearly observable. It is likely that a reduction in aeration and/or agitation would have provided a more meaningful ''fingerprint'' of the cellular activity; however, the drop in DO at high cell activity, and the fact that at no time was DO limiting, are important observations. The yellow pigmentation was also present in this experiment during rapid cell growth and the yield was estimated to be 0.22. This value is again in agreement with other work undertaken with this organism, but again, wall growth was extensively observed in the bioreactor.

These beads were then used, after washing, in a subsequent experiment, run under identical conditions as the previous two, to examine the effect of reusing the beads. The results of this experiment are shown in Figure 4. In this case, the aqueous phenol level was reduced to 756 mg/L and the capacity of the beads was estimated to be 12.0 mg/g EVA, with very similar uptake of phenol, growth of cells, and DO profile as seen earlier. These results suggest that the beads can be readily reused, with

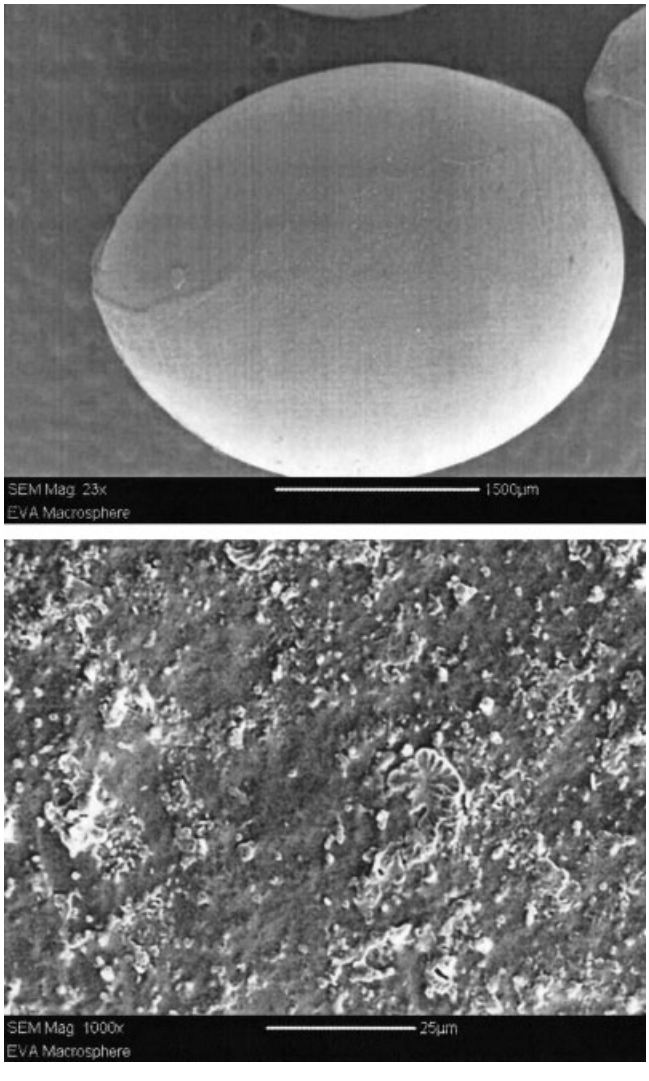

Figure 2. SEM pictures of EVA beads after being removed from the bioreactor at the completion of biodegradation of phenol. There is no evidence of cell adhesion to the beads.

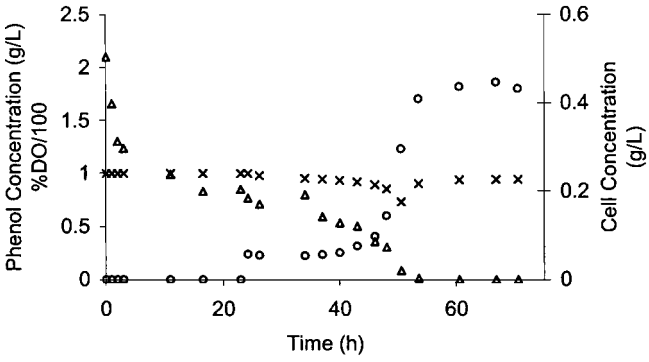

Figure 3. Bioreactor Run 2. The open triangles represent phenol concentration in the bioreactor medium, the x's represent % DO and the open circles represent cell concentration. The bioreactor was inoculated at 24 h.

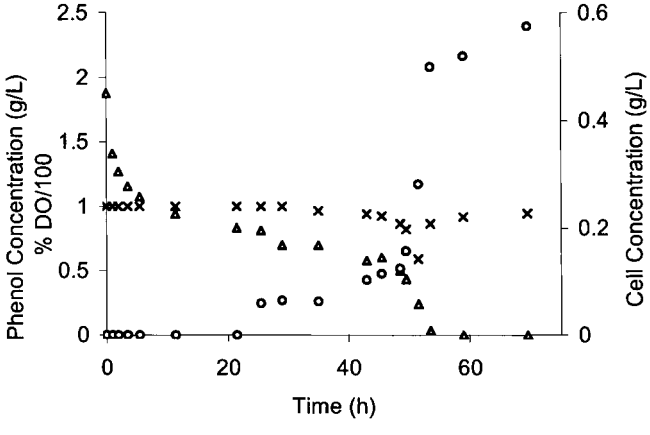

Figure 4. Bioreactor run 3, where it is demonstrated that the EVA beads can be reused to produce similar degradation results. The open triangles represent phenol concentration in the bioreactor medium, the x's represent % DO and the open circles represent cell concentration. The bioreactor was inoculated at 24 h.

virtually no compromise in their performance. The cell yield was estimated to be 0.29, again with the presence of extensive wall growth.

The performance of the once-and-twice-used beads was characterized in several ways. The absorption of phenol by the beads in the three experiments was normalized and plotted against time to generate an absorption curve. This is shown in Figure 5 for all of the data from the three bioreactor runs. As can be seen, there is good consistency of the data, suggesting that the beads performed in a similar way in terms of uptake of phenol, regardless of whether they were fresh or reused beads. The diffusivity of phenol within the EVA polymer can be calculated using the following equation (Crank, 1975):

$$\frac{M_t}{M_\infty} = 1 - \sum_{n=1}^{\infty} \frac{6\alpha(\alpha+1)\exp\left(\frac{-q_n^2 D_e^t}{r^2}\right)}{9 + 9\alpha + q_n^2\alpha^2} \tag{3}$$

in which M_t is the mass of phenol absorbed from the medium from a single bead at time t, M_∞ is the total mass of phenol absorbed by a bead, D_e is the average effective diffusivity of phenol within the polymer, r is the average radius of a bead, α is given by:

$$\frac{M_\infty}{VC_0} = \frac{1}{1+\alpha} \tag{4}$$

and q_n are the roots of:

$$\tan(q_n) = \frac{3q_n}{3+\alpha q_n^2} \tag{5}$$

In this manner, the diffusivity of phenol in EVA beads was found to be 3.73×10^{-9} cm^2/s from a least-squares fit of Eq. [3] to the accumulated absorption data of the bioreactor runs. This value was compared to literature values of solute diffusivities within EVA containing 40% vinyl acetate. The literature data included diffusion studies of water (Marais et al., 2000), benzocaine (Chen and Lostritto, 1996), hydrocortisone (Lee et al., 1985), and prednisolone (Michaels et al., 1975). Using the Stokes-Einstein equation as a basis, solute diffusivities within the same polymer are often plotted as log D_e versus log (molecular weight) to yield a straight line (Baker, 1987; Flynn et al., 1974). This was done using the literature data along with the calculated diffusivity for phenol and the results are shown in Figure 6. Based on this analysis, the calculated diffusivity of phenol is within the expected range. Furthermore, these data and analysis can be used in adapting the polymer TPPB to other xenobiotics.

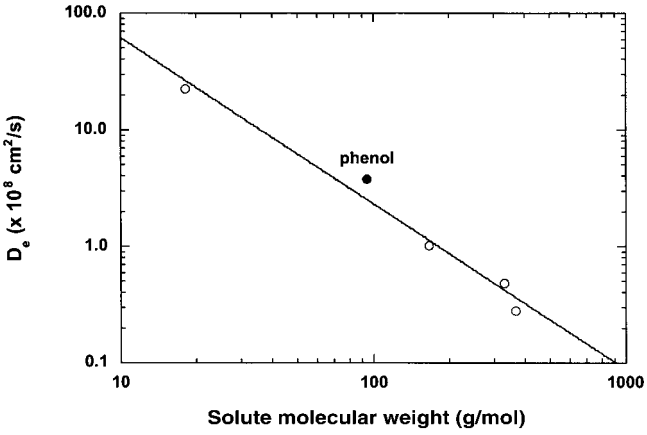

Figure 6. Comparison of calculated diffusivity of phenol to literature data.

Second, to confirm that all the phenol initially absorbed by the polymer was released from the polymer during the microbial consumption stage, two characterization techniques were used. In the first, beads used in a bioreactor run were resuspended and shaken in medium, the medium being sampled frequently and assayed for phenol. No phenol was detected in the medium even after 24 h. To confirm this result, the thermal properties of the EVA as received, after absorption of phenol, and after being used twice in the bioreactor were compared. The thermograms are shown in Figure 7.

The thermograms confirm that the polymer is amorphous, with a softening point near 70°C. More significant is a

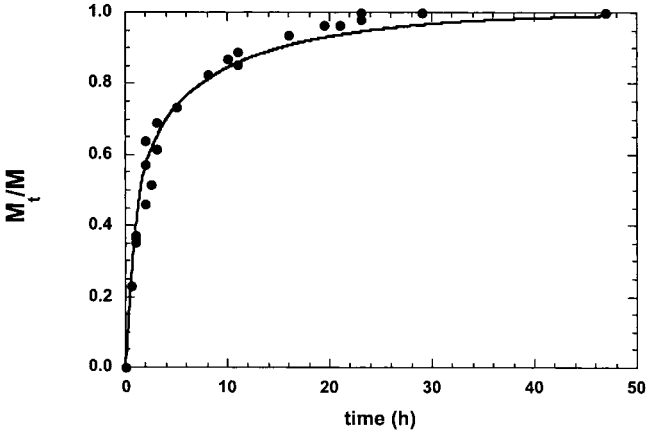

Figure 5. Absorption kinetics of phenol. The solid line represents the fit of Eq. [3] to the data.

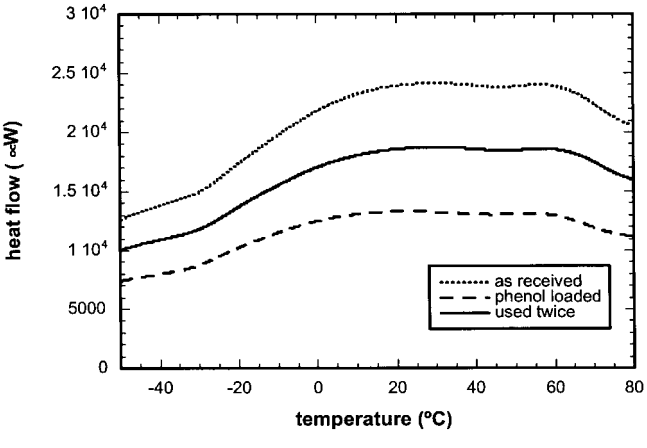

Figure 7. Thermograms of EVA samples as received, after absorbing phenol, and after being used twice in the bioreactor.

comparison of the glass transition temperatures. The glass transition temperature by definition is the inflection point of the endotherm. This point was calculated using the Seiko software supplied. The glass transition temperature of the polymer as received and after being used twice in the bioreactor were essentially the same (-16.5 ± 0.5°C as received vs. -16.2 ± 0.5°C after being used), while that of the phenol-loaded EVA was lower (–19.9°C). For reference, the glass transition temperature of EVA containing 40% vinyl acetate has been reported to be –17°C (Mukhopadhyay et al., 1993). In the presence of a dissolved solute, a polymer's glass transition temperature is lowered due to a plasticization effect, thus explaining the observed reduction of the glass transition temperature. The absence of this reduction in glass transition temperature for the used EVA indicates that essentially all the phenol has diffused from the EVA.

This work demonstrates the possibilities of using polymers as an effective partitioning phase in TPPBs. We believe that the use of polymers as a delivery phase in TPPBs is significant for several reasons. With proper thermodynamic matching of polymer to xenobiotic, for example, by matching solubility parameters as indicated by Eq. [2], the system can be adapted to many organic compounds and situations. Moreover, because commodity polymers such as EVA are completely nonbiodegradable, they can be used without concern for losses during microbial activity, and without concern for competition with the target substrate. The potential drawbacks associated with solvent bioavailability had previously prompted studies (Vrionis et al., 2002a–c) that showed that the organisms themselves could be genetically modified to make certain classes of delivery solvents nonbioavailable; the present work provides an alternative approach to addressing bioavailability concerns. In addition, the use of such inert polymers opens the door to the use of widely mixed populations of organisms in biotreatment reactors, as well as eliminating concerns of contamination. If contamination occurred, and the contaminant remained in the system, it is certainly possible that the organism(s) would aid in biodegradation of the target molecule since, from a selection pressure standpoint, their survival would require that they had some capability to metabolize the target substrate or its degradation product(s). In the widely mixed ecosystems occurring in the environment, it can be imagined that polymer shapes could be distributed in areas of high xenobiotic contamination to act as sponges to reduce contaminants to below toxic levels, and then to release them for biodegradation. These polymeric materials could then be collected and used again in other remediation situations.

Polymer delivery agents have additional potential advantages relative to organic solvents, such as being inexpensive (e.g., EVA costs ~US$3.40 per kg, compared to a price of US$80 per kg for 2-undecanone, a solvent previously used for phenol and *P. putida* (Collins and Daugulis, 1997)), nonvolatile, biocompatible, reusable, and being amenable to physical modification (size and shape) as well as chemical modification (e.g., cross-linking) which would allow better tailoring of the polymer(s) to each specific application. Being solids, the polymers could also be used in different reactor configurations, such as fluidized or packed bed arrangements, if this improved overall process performance. A clear limitation of solid polymer beads relative to organic solvents for use in TPPBs, however, is the lower diffusivity of target molecules in the delivery phase, which is seen in the relatively modest uptake rate of phenol from the aqueous phase shown in Figures 1, 3, and 4. This reduced diffusivity could lead to operational difficulties at times of high cell metabolic activity (mass transfer limitations) or in the case of imposed spikes or transients of substrate.

Notwithstanding the many advantages possessed by polymer delivery agents, it is not expected that they will eliminate the use of two-liquid phase TPPBs. We anticipate that, because of the vast array of possible organic delivery solvents available, they will continue to have the potential to provide much higher affinities and capacities for most toxic substrates. In addition, organic solvents have the capability to dissolve solid xenobiotics (such as polyaromatic hydrocarbons), a feature not possessed by polymer delivery systems. Finally, there are a number of possible remediation applications, such as removal of VOCs from gas streams, which would likely be more effectively accomplished via organic solvent TPPBs. Thus, we suggest that the use of polymer delivery systems is an important *addition* to the expansion of TPPB use, rather than a replacement of two-liquid TPPB systems.

CONCLUSION

This work has shown that EVA beads can be used to absorb phenol from aqueous solutions, reducing its concentration from toxic to subinhibitory levels. Moreover, it has also been shown that in the presence of *P. putida*, the beads will also release the phenol for degradation, allowing the cells to consume all of the original phenol loading. Physically, the beads were seemingly unaffected by this application and could be reused. The use of solid delivery agents in TPPBs provides numerous potential advantages relative to the use of two-liquid TPPB systems, perhaps most notably the potential to use widely mixed, and hence highly efficient, consortia in remediation applications. We are currently exploring the tailoring of polymers as delivery agents in TPPB systems and the use of microbial consortia in remediation applications facilitated by the use of such materials.

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