Biotechnology Letters **25:** 1203–1207, 2003.

Delivery of benzene to *Alcaligenes xylosoxidans* by solid polymers in a two-phase partitioning bioreactor

Andrew J. Daugulis*, Brian G. Amsden, Justina Bochanysz & Ahmed Kayssi
Department of Chemical Engineering, Queen's University, Kingston, Ontario, Canada, K7L 3N6
**Author for correspondence (Fax: (613) 533 6637; E-mail: Daugulis@chee.queensu.ca)*

Received 13 May 2003; Accepted 14 May 2003

Key words: benzene, degradation, partitioning bioreactor, polymers

Abstract

Toxic levels of benzene were decreased to sub-inhibitory levels in a bioreactor via absorption by polymer beads or cylinders (poly(ethylene-*co*-vinyl acetate) or poly(styrene-*co*-butadiene)). After inoculation with *Alcaligenes xylosoxidans*, the remaining benzene in the aqueous phase as well as the benzene taken up by the polymers was degraded to completion. The capacity of these polymers for benzene, and benzene diffusivity within the polymers were also determined.

Introduction

Two-phase partitioning bioreactors (TPPBs) have traditionally used immiscible and biocompatible organic solvents to deliver toxic or poorly soluble substrates to cells in the aqueous phase (Déziel *et al.* 1999, Malinowski 2001). In all cases, with one recent exception (MacLeod & Daugulis 2003), the delivery solvent was selected to be non-bioavailable (i.e. not used as a carbon source) so as not to interfere with degradation of the target substrate. For two-liquid phase TPPBs there has also been concern regarding the possibility of microbial contamination that could result in solvent degradation, and thus virtually all research on TPPBs has focussed on the use of axenic cultures.

The present work describes the characterization and use of two solid polymers, poly(ethylene-*co*-vinyl acetate), EVA, and poly(styrene-*co*-butadiene), SB, as delivery agents for benzene to cells of *Alcaligenes xylosoxidans* in a bioreactor. The structure of these two polymers is given in Figures 1A and B. EVA has previously been demonstrated by us to be effective as a substrate delivery agent in the TPPB biodegradation of phenol (Daugulis & Amsden 2003). SB was examined because its chemical structure more closely resembles benzene and so benzene solubility in SB should be greater than that in EVA. In general, the closer the solubility parameter of the polymer to the solute, the greater the affinity the polymer has for that compound. Benzene has a solubility parameter of 9.1 $(\text{cal cm}^{-3})^{1/2}$, while that of EVA and SB are 9.4 and 9 $(\text{cal cm}^{-3})^{1/2}$ respectively (Mark 1999). Furthermore, SB is the main component in automobile tires, that carries the possibility of using old tires as sorbents in TPPBs.

These solid polymers were able to absorb toxic concentrations of benzene from the aqueous phase, permitting inoculation by the degrading organism, and to release it on demand to the cells for complete biodegradation. Since these polymers are completely non-biodegradable (i.e. non-bioavailable) they have the potential to be used in TPPBs without concern for degradation, or interference with target substrate metabolism, and could also be readily used in mixed culture systems.

Materials and methods

Polymers

EVA (40% vinyl acetate) provided as a gift from DuPont Canada in the form of beads with average diameters of 3.4 mm, and SB (28% styrene, styrene-butadiene-styrene triblock) cylinders (Aldrich) with

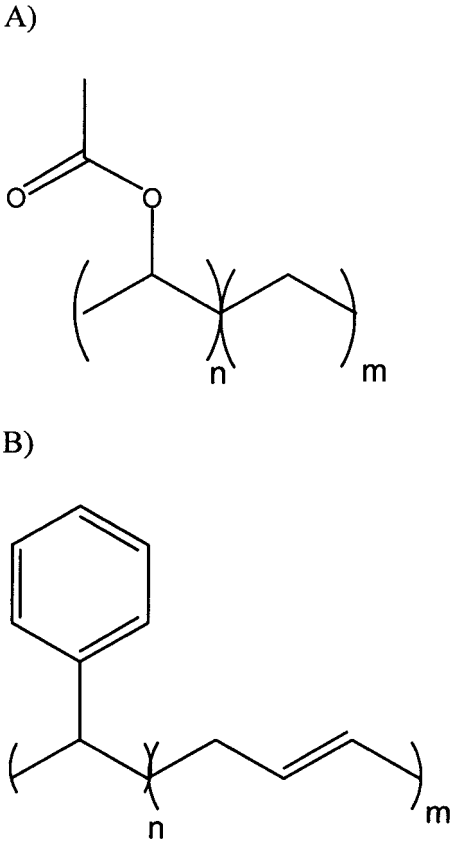

Fig. 1. Chemical structures of the polymers. (A) EVA, (B) SB.

an average diameter of 4 mm, and length of 1.2, were characterized in terms of their capacity to absorb benzene, and for benzene diffusivity. In addition, each type of polymer was used to absorb and release benzene to bacterial cells in a bioreactor.

Organism and growth medium

Growth of *Alcaligenes xylosoxidans* and its culture media have been described previously (Yeom & Daugulis 2001).

Diffusivity and capacity determinations

To determine diffusivity, benzene (to give 150 mg l^{-1}) was injected through Teflon-coated silicon septa into 160 ml serum bottles filled with medium and containing 3 g polymer. The bottles were shaken vigorously, and small samples (<1 ml, to minimize the creation of headspace) were periodically withdrawn for benzene analysis. The capacity (mg benzene per g polymer) of each polymer to absorb benzene was determined in a

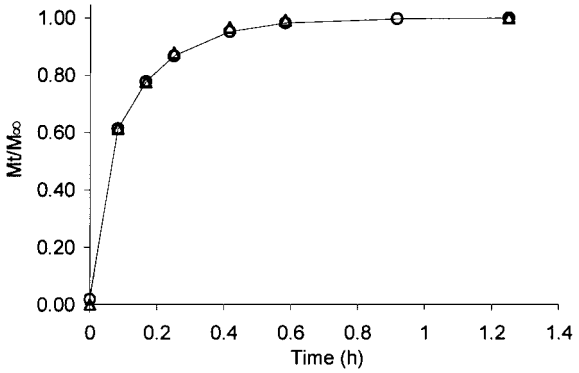

Fig. 2. Experimental (triangles) and predicted (circles) uptake of benzene by EVA beads.

similar fashion, except that only 2 samples were taken, one at 24 h and one at 72 h for each polymer.

TPPB experiments

An injection of 4 ml benzene was made to a sealed Bioflo III bioreactor (New Brunswick Scientific) containing 3 l growth medium. The system was operated at 30 °C, pH 6.6 and 450 rpm, and allowed to reach equilibrium (i.e. equilibration of benzene with the headspace and rubber components of the vessel), which took about 16–20 h. Either 310 g EVA beads, or 76.4 g SB cylinders sterilized by UV were then quickly added to the medium to absorb benzene. When the aqueous benzene concentration had stabilized in response to benzene uptake by the polymers, the bioreactor was inoculated with *A. xylosoxidans*, and recirculation of the air in the headspace was initiated to provide oxygen to the cells. The dissolved O_2 (DO) was continuously monitored, and 100 ml injections of pure O_2 were made into the headspace whenever the DO was decreased to 15% saturation due to cell metabolism. This recirculation of gas within the system was intended to eliminate any losses of benzene arising from volatilization and stripping that would be expected if the TPPB had been aerated in a conventional fashion.

Analytics

Benzene was analyzed by equilibrating aqueous samples with *n*-hexadecane, injecting 0.2 μl organic samples into a GC, and performing material balance calculations with an assumed partition coefficient of 140.1 (Yeom & Daugulis 2001). Cell concentrations were measured turbidometrically at 640 nm.

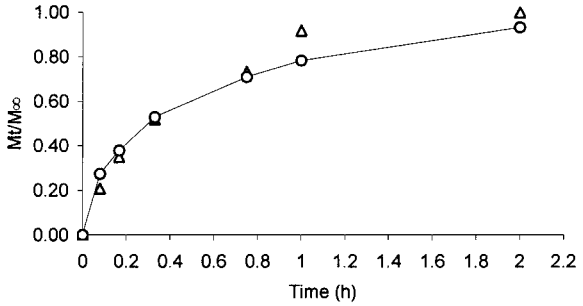

Fig. 3. Experimental (triangles) and predicted (circles) uptake of benzene by SB cylinders.

Results and discussion

Figures 2 and 3 show the absorption of benzene by EVA spheres and SB cylinders, respectively, along with the predicted uptakes according to Equations (1) and (4), respectively. The diffusivity of benzene within the EVA spheres can be calculated using the following equation (Crank 1975),

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

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 benzene within the polymer, r is the average radius of a bead, α is given by,

$$\frac{M_\infty}{VC_0} = \frac{1}{1+\alpha} \quad (2)$$

and q_n are the roots of,

$$\tan(q_n) = \frac{3q_n}{3 + \alpha q_n^2}. \quad (3)$$

The diffusivity of benzene within the SB cylinders can be calculated from a least-squares fit of the following equation (Crank 1975),

$$\frac{M_t}{M_\infty} = 1 - \sum_{n=1}^{\infty} \frac{4\alpha(\alpha+1)\exp\left(\frac{-q_n^2 D_e t}{r^2}\right)}{4 + 4\alpha + q_n^2\alpha^2}, \quad (4)$$

where, in this case, q_n's are the roots of the following,

$$\alpha q_n J_0(q_n) + 2J_1(q_n) = 0. \quad (5)$$

In Equation (5), J_0 and J_1 are the zero order and first order Bessel functions of q_n, respectively.

The diffusivities of benzene in EVA and SB thus obtained were determined to be 4.3×10^{-6} cm^2 s^{-1} and 2.23×10^{-6} cm^2 s^{-1}, respectively. For EVA, Kumar *et al.* (1997) have reported benzene diffusivity at 28 °C to be 2.8×10^{-7} cm^2 s^{-1}, which is roughly 10 times lower than found in this study. However, the EVA used by Kumar *et al.* contained 18% vinylacetate, and so was semi-crystalline (Salyer & Kenyon 1971). The presence of crystalline domains within the polymer reduces diffusivity as the solute must move around the crystal obstacles. For SB of similar styrene content (25%) as used in this study, George *et al.* (1996) have reported a benzene diffusivity of $0.5 - 0.9 \times 10^{-6}$ cm^2 s^{-1}. However, the SB used by George *et al.* was crosslinked. It is well established that crosslinking decreases solute diffusivity (Crank & Park, 1968). Therefore, the values obtained in this work are reasonable.

The capacities for benzene by EVA and SB were determined under these conditions to be 4.19 mg g^{-1} for EVA and 9.45 mg g^{-1} for SB. These capacities were the same (±5%) regardless of whether 24 h or 72 h samples were assayed. These capacity values were used to determine the approximate amounts of each polymer that had to be added to the bioreactors once they had equilibrated with benzene in the headspace, to reduce the aqueous benzene level to about 50 mg l^{-1}, which is substantially non-toxic to *A. xylosoxidans.* Thus, although EVA should absorb benzene faster than SB, SB has a greater sorbent capacity. Thus SB may be the better polymer in this application.

Figures 4 and 5 show the system equilibration, uptake, release and degradation of benzene for added EVA and SB, respectively. Figure 4 shows that equilibration of the headspace of the bioreactor resulted in a decrease in aqueous benzene concentration from approx. 1000 mg l^{-1} to about 525 mg l^{-1}. It is important to note that the benzene in the headspace was still retained in the closed fermentation system, and was available for transfer back into the aqueous phase (and degradation by the cells) based on equilibrium considerations. It is also significant that this equilibrated aqueous level is substantially higher than the toxic level for *A. xylosoxidans.* The addition of 310 g EVA spheres decreased the aqueous benzene concentration to approximately 52 mg l^{-1} in 2 h, a level that is non-toxic to the cells. After inoculation at 22 h, the cells grew rapidly, until plateauing at 36 h, at which time benzene was not detectable in the aqueous phase. Although there was some wall growth on the bioreactor, the cell yield for this experiment was estimated to

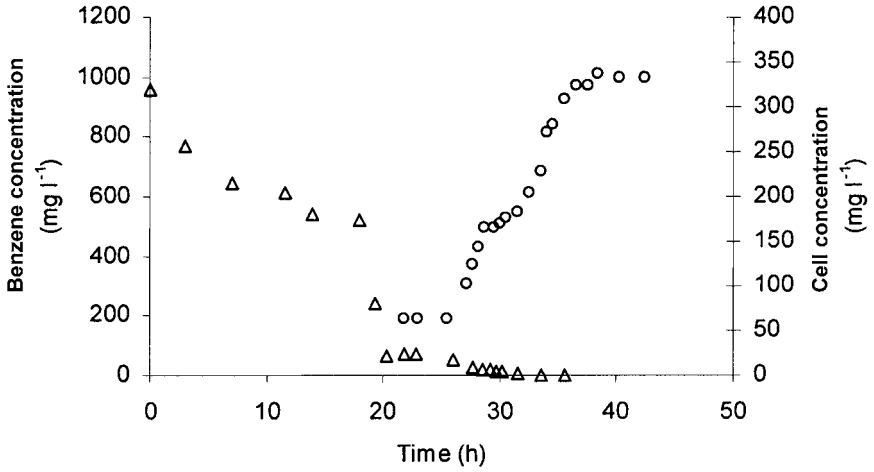

Fig. 4. Use of EVA spheres to absorb and release benzene (triangles) to cells (circles).

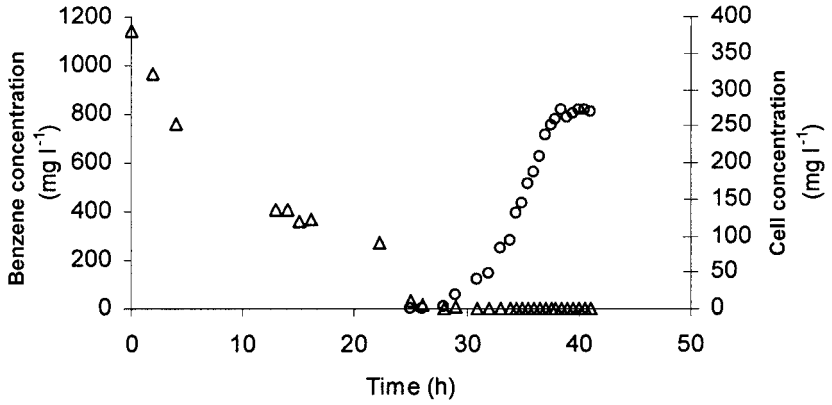

Fig. 5. Use of SB cylinders to absorb and release benzene (triangles) to cells (circles).

be 0.29 g g^{-1}, which is slightly lower than the value of 0.35 found earlier (Yeom & Daugulis 2001).

Figure 5 provides data for a similar experiment in which SB was used as the polymer to absorb, and release benzene. In this instance, equilibration with the headspace after about 15 h resulted in an aqueous benzene concentration of approx. 360 mg l^{-1} (again, a concentration far above the toxic threshold for *A. xylosoxidans*), but was reduced to about 35 mg l^{-1} by the addition of 76.4 g of SB cylinders. From the time of inoculation at 28 h, until the cell concentration plateaued at 38 h, benzene was consumed to undetectable levels, producing an estimated cell yield of 0.24 g g^{-1}, slightly below that found in the EVA experiment. Although not shown here, previous work in our laboratory with the use of EVA beads to deliver phenol to cells in TPPBs (Daugulis & Amsden 2003), has confirmed (by elution experiments and by examination of the polymer by differential scanning calorimtery) that all of the organic substrate diffuses from the polymers in response to a declining aqueous concentration of substrate arising from cell metabolism.

The above results have shown that polymers of significantly dissimilar composition have different affinities for target substrates, with styrene butadiene having slightly more than twice the capacity for benzene compared to ethylene vinyl acetate. This is a reflection of the greater similarity in molecular structure between benzene and SB, and suggests that rational polymer selection will be an important element in selecting superior solid delivery agents in TPPBs. In addition, polymers can be readily modified (e.g. via cross-linking) providing another opportunity for developing polymer delivery systems with desirable physical properties. In both cases, it has been shown that the 2 polymers tested behaved in a very similar fashion relative to a two-liquid TPPB system (Yeom *et al.* 2001) in terms of taking up and releasing a mi-

crobial substrate based on equilibrium considerations and cellular demand. Both EVA and SB are inexpensive materials, and are also non-biodegradable. This latter feature suggests that it is likely possible to use mixed cultures of organisms in TPPB systems, with the anticipated enhancement of performance expected of multiple organisms, without concern for degradation of the delivery agent or competition with substrate uptake. On a related note, concerns related to possible contamination of TPPBs may also now be unfounded.

We believe that polymer delivery agents for TPPBs, rather than eliminating two-liquid TPPB systems from consideration, will merely add to the possible applications of these reactor configurations. The continued use of two-liquid TPPBs is primarily due to the fact that solvents can be expected to have much higher capacities for most organic substrates than do polymers, and also because solvents can readily dissolve highly hydrophobic, solid substrates (e.g. PAHs) for delivery to microbes, a feature not readily available to polymeric materials. Finally, there are likely some applications of TPPBs, such as in the removal and degradation of VOCs from air streams (Yeom *et al.* 2000) that are more suited to two-liquid TPPB arrangements.

We are currently examining the use of mixed populations of organisms in polymer TPPB systems, and have initiated polymer development, formulation and modification studies to better understand the properties that will create superior solid delivery agents for TPPBs.

Acknowledgement

We are grateful to the Natural Sciences and Engineering Research Council of Canada for financial support of this work.

References

Crank J (1975) *The Mathematics of Diffusion*. New York: Oxford University Press.

Crank J, Park GS (1968) *Diffusion in Polymers*. New York: Academic Press.

Daugulis AJ, Amsden BG (2003) US Patent Application 010/367,822.

Déziel E, Comeau Y, Villemur R (1999) Two-phase bioreactors for enhanced degradation of hydrophobic/toxic compounds. *Biodegradation* **10**: 219–233.

George SC, Thomas S, Ninan KN (1996) Molecular transport of aromatic hydrocarbons through crosslinked styrene-butadiene rubber membranes. *Polymer* **37**: 5839–5848.

Kumar SA, Thomas S, Kumaran MG (1997) Transport of aromatic hydrocarbons through poly(ethylene-*co*-vinyl acetate) membranes. *Polymer* **38**: 4629–4640.

MacLeod CT, Daugulis AJ (2003) Biodegradation of polycyclic aromatic hydrocarbons in a two-phase partitioning bioreactor in the presence of a bioavailable solvent. *Appl. Microbiol. Biotechnol.* (in press).

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

Mark JE (1999) *Polymer Data Handbook*. Toronto: Oxford University Press.

Salyer I, Kenyon A (1971) Structure and property relationships in ethylene-vinyl acetate copolymers. *J. Polymer Sci.* **9**: 3083–3103.

Yeom SH, Dalm MCF, Daugulis AJ (2000) Treatment of high-concentration gaseous benzene streams using a novel bioreactor system *Biotechnol. Lett.* **22**: 1747–1751.

Yeom SH, Daugulis AJ (2001) Benzene degradation in a two-phase partitioning bioreactor by *Alcaligenes xylosoxidans* Y234. *Process Biochem*. **36**: 765–772.

Yeom SH, Louie J, Daugulis AJ (2001) The use of partitioning bioreactors for the treatment of high-concentration benzene solutions. *Can. J. Chem. Eng*. **79**: 785–790.