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ORIGINAL PAPER

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Use of a two-phase partitioning bioreactor for degrading polycyclic aromatic hydrocarbons by a *Sphingomonas* sp.

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Abstract A two-phase partitioning bioreactor (TPPB) utilizing the bacterium *Sphingomonas aromaticivorans* B0695 was used to degrade four low molecular weight (LMW) polycyclic aromatic hydrocarbons (PAHs). The TPPB concept is based on the use of a biocompatible, immiscible organic solvent in which high concentrations of recalcitrant substrates are dissolved. These substances partition into the cell-containing aqueous phase at rates determined by the metabolic activity of the cells. Experiments showed that the selected solvent, dodecane, could be successfully used in both solvent extraction experiments (to remove PAHs from soil) and in a TPPB application. Further testing demonstrated that solvent extraction from spiked soil was enhanced when a solvent combination (dodecane and ethanol) was used, and it was shown that the co-solvent did not significantly affect TPPB performance. The TPPB achieved complete biodegradation of naphthalene, phenanthrene, acenaphthene and anthracene at a volumetric consumption rate of 90 mg l^{-1} h^{-1} in approximately 30 h. Additionally, a total of 20.0 g of LMW PAHs (naphthalene and phenanthrene) were biodegraded at an overall volumetric rate of 98 mg l^{-1} h^{-1} in less than 75 h. Degradation rates achieved using the TPPB and *S. aromaticivorans* B0695 are much greater than any others previously reported for an ex situ PAH biodegradation system operating with a single species.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are released into the environment through the incomplete combustion of solid and liquid fuels (Ramdahl and Bjorseth 1985). Suspected to possess toxic, mutagenic and carcinogenic properties (Heitkamp and Cerniglia 1988; Keith and Telliard 1979; Yuan et al. 2000), PAHs require effective remediation strategies due to growing production from anthropogenic sources (Wilson and Jones 1993). The Sydney Tar Ponds, a large PAH-contaminated site in Nova Scotia, Canada, has generated great interest in the development of efficient and cost-effective methods for the removal and destruction of these compounds (Gordon 1997). While some in situ and ex situ PAH biodegradation methods are available (Dreyer et al. 1995; Fuchs and Braun 1995, Guiyesse et al. 2000; Jones 1992; Joyce et al. 1998; Oberbremer et al. 1990; Pinelli et al. 1997; Potter et al. 1999; Rockne and Strand 1998; Stefess 1998; Woo and Park 1999), many are costly and/or time-intensive. Biodegradation methods can utilize indigenous soil populations or mixed bacterial populations isolated from industrial waste. To date, only a few have been implemented for the treatment of PAH-contaminated soils with pure cultures and/or on a large-scale.

The ex situ biodegradation of PAHs has been studied in soil slurry bioreactors (Fuchs and Braun 1995, Oberbremer et al. 1990; Pinelli et al. 1997; Woo and Park 1999) with consortia and at large scale. Applications using a single PAH-degrading bacterial species have only been examined using small reactor volumes (<1.0 l). Two factors have limited the development of bioremediation of PAHs from soils, and both relate to the bioavailability of the PAHs to the bacterium. First, it is difficult to provide an adequate concentration of PAHs to the bacterium in an aqueous phase because of the low solubility of the PAHs. Second, contaminant PAHs in the environment are strongly sorbed to the soil (Ashok 1995; Smith et al. 1997). Without a desorption strategy, it is difficult to provide bacterial access to the PAHs in a sterile environment. Desorption of PAHs from soil by solvent extraction followed by biodegradation in a two-phase partitioning bioreactor (TPPB) has been identified as a possible solution to address both concerns.

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TPPB technology has been successfully used with other xenobiotic compounds such as pentachlorophenol (Munro and Daugulis 1997), phenol (Collins and Daugulis 1996) and benzene, toluene and *p*-xylene (BTX; Collins and Daugulis 1999). In this study, the use of *Sphingomonas aromaticivorans* B0695, a bacterium recently classified and identified as possessing the ability to degrade up to seven low molecular weight (LMW) PAHs (Fredrickson et al. 1995, 1999; Rockne and Strand 1998) was examined. The capability of a TPPB for PAH biodegradation using a solvent (with or without a co-solvent) coupled with a prior solvent extraction step was investigated.

Materials and methods

Chemicals

The PAHs (naphthalene, phenanthrene, anthracene and acenaphthene) and specified solvents used in this study were obtained from Sigma-Aldrich, Canada. All salts and *n*-dodecane were purchased from Fisher Scientific.

Bacterial culture

S. aromaticivorans B0695, previously isolated from deep subsurface sediment of the Atlantic Coastal Plains and recently identified (Fredrickson et al. 1999), was generously provided by Dr. David Balkwill, Florida State University. It is available from the Subsurface Microbial Culture Collection (SMCC) as SMCC B0695 at Florida State University.

Medium and culture conditions

Maintenance medium was based on a modified Luria Bertani broth developed by Lantz et al. (1995) and contained 10.0 g l^{-1} tryptone; 5.0 g l^{-1} yeast extract; 1.0 g l^{-1} glucose; 0.2 g l^{-1} $MgSO_4 \cdot 7H_2O$; 0.02 g l^{-1} $CaCl_2$; 1.0 g l^{-1} KH_2PO_4; 1.0 g l^{-1} K_2HPO_4 and 1 ml of a trace elements solution. The trace elements solution (containing 16.20 mg l^{-1} $FeCl_3 \cdot 6H_2O$, 9.44 mg l^{-1} $CaHPO_4$, 0.15 mg l^{-1} $CuSO_4 \cdot 5H_2O$, and 40.0 mg l^{-1} citric acid) was prepared as a stock solution at a 100-fold concentration and diluted to give the amounts indicated. Stock cultures were maintained on the maintenance medium with the addition of 15.0 g l^{-1} agar and sub-cultured monthly. Inoculum was prepared using the maintenance medium for all experiments. During PAH degradation experiments, the medium formulation was altered to ensure that PAHs were the main carbon source. The concentrations of tryptone and yeast extract were reduced (as described for each experiment) and glucose was omitted entirely and instead LMW PAHs (naphthalene, phenanthrene, anthracene and acenaphthene) were added via the solvent phase in varying quantities to replace the glucose. For culture maintenance, all medium components were dissolved in distilled water and the pH was adjusted to 6.2 using 2 M H_2SO_4. All medium was autoclaved prior to use.

Analytical procedures

In all experiments, the optical density of the aqueous phase, used to track the cell density, was measured using a Brinkmann PC 800 colorimeter at 650 nm. PAH concentration changes in the organic phase were monitored by diluting the sample 10 times in dichloromethane (DCM) in order to improve peak elucidation and injecting 1.0 µl of the diluted sample directly into a Perkin Elmer gas chromatograph (GC) with a flame ionization detector. Due to the low water solubility of naphthalene, phenanthrene, acenaphthene and anthracene, the disappearance of these compounds in the aqueous phase was not measured. The GC column used was a 30 m Agilent J&W DB-5.625. The four PAHs were detected using the following temperature program: the column temperature was held at 40°C for 2 min, then increased by 5°C/min to 160°C, then increased by 20°C/min to 270°C and held at constant temperature for 15 min. The injector and detector were maintained at 250°C and 300°C, respectively.

Solvent selection

The log P of a solvent is defined as the logarithm of its partition coefficient when placed into a two-phase system consisting of octanol and water. The lower the solvent log P, the more hydrophilic and therefore the more likely that the toxic effects of a solvent will affect a bacterium growing in the aqueous phase. The critical log P for an organism is defined as the log P of a solvent at which organism growth in the aqueous phase is not adversely affected by the presence of the solvent. The critical log P of the bacterium was determined using a method previously described by Collins and Daugulis (1999). Following the determination of the critical log P value, a group of solvents was selected using a UNIFAC-based extractant screening program (ESP) (Bruce and Daugulis 1991) that contains thermodynamic data on over 1,500 solvents. Solvents were selected using the following criteria: a log P value greater than the previously determined critical log P value; a high boiling point; a high partition coefficient; and a low water solubility. The bioavailability of these solvents to the bacterium was tested as described by Collins and Daugulis (1999) and those solvents that could be used as a carbon source by the bacterium were excluded from consideration for use in the TPPB system. The short-list of solvents available for use was re-examined based on cost, biocompatibility, lack of toxicity and commercial availability and a final solvent selection was made.

Removal of PAHs from soil using solvent extraction

Using the selected solvent, soil extraction tests were carried out. A wet clay sample (10% moisture content) was spiked with eight PAHs to give an overall concentration of 8 mg PAH g $soil^{-1}$. To effectively create a simulated contaminated soil, PAHs (acenaphthene, anthracene, fluorene, naphthalene, phenanthrene, pyrene, benzo[a]pyrene and fluoranthene), were dissolved in a 1:1 DCM:acetone solution, and then added to air-dried soil and allowed to tumble for 2 h. The soil was placed in a flat pan and the solvent was allowed to evaporate. The soil was then rehydrated to give a final water content of 10%. The soil sample was split into four portions and four solvent combinations were tested: dodecane; dodecane/5% ethanol; dodecane/10% ethanol; and DCM/acetone. Solvent extraction was carried out by placing 10 ml of solvent in contact with 5 g of the spiked soil in a 40 ml vial. After shaking for 30 min, 1 ml of solvent was collected for PAH analysis using an HP 5890 gas chromatograph with a flame ionization detector.

Abiotic operation

The 5 l New Brunswick Scientific BioFlo III fermentor, used for all lab-scale fermentations, was set up to simulate a fermentation experiment. Distilled water (3 l) was placed in the fermentor. Dodecane (250 ml), the solvent ultimately selected for use in the TPPB, was loaded with 1.25 g naphthalene, 1.25 g phenanthrene, 0.25 g acenaphthene and 0.25 g anthracene and added to the fermentor. An initial solvent phase sample was taken to determine the concentration of PAHs. The fermentor was then operated at a high rate of aeration (1.00 vvm; volume of air/volume aqueous phase/min) and agitation (400 rpm) for 4 days. Rates of aeration in this work are based on an aqueous working volume of 3 l. Organic

phase samples were taken every 24 h and analyzed to determine whether PAH losses due to volatilization were occurring.

Batch fermentation 1: Proof of concept

An initial experiment to determine the ability of *S. aromaticivorans* B0695 to degrade PAHs was carried out using two LMW PAHs: naphthalene and phenanthrene. Medium (3 l) was prepared in the fermentation vessel and autoclaved. All nutrients (except tryptone, yeast extract and glucose) were doubled from concentrations outlined in the Medium and Culture Conditions section to prevent nutrient limitation. Glucose was omitted entirely and 0.5 g l^{-1} tryptone and 0.25 g l^{-1} yeast extract were added to provide required micronutrients. Inoculum preparation was the same for all fermentations. Cells were taken from a plate culture and placed in 100 ml of maintenance medium. Following a 24 h (late exponential phase) growth period, 10 ml of cells were taken from the flask culture and placed in a second flask containing 100 ml of medium. After an additional 24 h growth period, the inoculum was added to the fermentor. Dodecane (500 ml) loaded with 4.5 g of naphthalene and 4.5 g of phenanthrene was then added. Samples of the aqueous and organic phases were taken twice per day for 4 days and analyzed for PAH concentration in the solvent phase and the optical density of the aqueous phase. The fermentor temperature was maintained at 30°C and the concentration of dissolved oxygen in the fermentor was monitored. The rate of aeration, initially at 0.33 vvm, was raised in response to developing oxygen limitation. At 12 h, aeration was increased to 0.67 vvm and at 22 h it was increased again to 0.83 vvm. The pH of the fermentation broth was recorded, but pH control was not undertaken.

Batch fermentation 2: Use of ethanol as a co-solvent

To determine the effectiveness of using ethanol as a co-solvent in a TPPB, two fermentors were run simultaneously for 72 h. Both were prepared as described in Batch Fermentation 1 with the following changes. In the first fermentor, the solvent (500 ml of dodecane) was loaded with 1.97 g of naphthalene, 2.09 g of phenanthrene, 0.54 g of anthracene and 0.50 g of acenaphthene. In the second fermentor, the solvent [475 ml of dodecane and 25 ml of ethanol (5% v/v ethanol)], was loaded with 2.00 g of naphthalene, 2.00 g of phenanthrene, 0.56 g of anthracene and 0.50 g of acenaphthene. The initial aeration rate in both fermentors was 0.67 vvm but was increased to 1.33 vvm at 31 h to alleviate oxygen limitation. Agitation, initially at 200 rpm, was increased to 250 rpm at 10.5 h and further increased to 325 rpm at 31 h. The pH was controlled at 6.2 using 0.5 M KOH and 0.5 M H_2SO_4, and the temperature was maintained at 30°C.

Batch fermentation 3: Degradation of four LMW PAHs

The ability of the bacterium to degrade a combination of PAHs was examined. Following initial attempts, increased concentrations of medium components necessary for successful biodegradation were identified and added to the system. Sterile medium (3 l) containing triple the concentration of nutrients, 1.5 g l^{-1} of tryptone, 0.75 g l^{-1} yeast extract and no glucose was prepared. Inoculum (110 ml) grown on maintenance medium and 500 ml of dodecane, loaded with an initial concentration of 3.64 g naphthalene, 3.48 g phenanthrene, 0.54 g anthracene and 0.47 g acenaphthene, was added to the fermentor. The temperature was maintained at 30°C and the pH was maintained at 6.2. In response to developing oxygen limitation, the rates of aeration and agitation were increased throughout the experiment. The rate of aeration was between 0.67 and 1.00 vvm and the rate of agitation was between 275 and 400 rpm. At 12 h, 0.25 ml of Antifoam 204 was added to control the high level of foam production observed in the fermentor. The fermentation was allowed to continue until the PAH concentration in the organic phase was depleted.

Fed-batch fermentation: degradation of a high concentration of PAHs

This fermentation was performed using medium conditions identical to those outlined in Batch Fermentation 3. PAH substrates (5.6 g naphthalene and 4.9 g phenanthrene) were dissolved in 500 ml of dodecane and added to the fermentor. Following the complete disappearance of the original PAHs, an additional 5.0 g naphthalene and 5.0 g of phenanthrene were added to the fermentor at 35 h. At this time, an autoclaved 500 ml solution with additional tryptone, yeast extract and salts was added, containing the same amounts as initially added at time 0. The fermentor was run for a total of 85 h.

Results

Determination of critical log P and bioavailability of solvents

The critical log P for *S. aromaticivorans* B0695 was found to be 4.0 (data not shown). After determining this value, ten solvents, each with a high boiling point (>130°C), a log P value >4.0, a high partition coefficient and a low solubility in the aqueous phase were selected using ESP. Each solvent selected represented a class of compounds (e.g., ketones and alkanes) since solvents may interact differently with the bacterium depending on their chemical structures. The solvents examined were Adol 85 NF (an industrial grade of oleyl alcohol), diethyl sebecate, dipentyl ether, jasmone, 2-undecanone, *n*-hexadecane, 1-decene, limonene, 2-methyl undecanal and *n*-dodecane.

The solvents were subjected to bioavailability experiments, in which cells were grown in a carbon-free medium, and in the presence of the solvent. Solvents on which cells displayed growth were eliminated from further consideration (data not shown). Six solvents were found to be non-bioavailable and some of their key properties are shown in Table 1. Of the six solvents, limonene, jasmone and dodecane were identified as attractive choices for use in the TPPB because of their relative non-toxicity and their additional potential for use in a soil solvent extraction application. Since a solvent minimum of 500 ml is required to operate the 5 l reactor, jasmone was ruled out as a potential solvent due to its high cost. Biocompatibility of limonene and dodecane to *S. aromaticivorans* B0695 was then investigated to ensure that the lack of growth observed in the bioavailability experiment was not due to solvent toxicity. It was found that cell growth was inhibited in the presence of limonene but not dodecane. The final solvent selected for use in the TPPB with *S. aromaticivorans* B0695 for the degradation of PAHs was dodecane.

PAH removal from soil using solvent extraction

The results of the single-pass solvent extraction using dodecane are presented in Fig. 1. The DCM/acetone mixture (DCM + 10% acetone) is commonly used to ex-

Table 1 Characteristics for solvent selection for use in a two-phase partitioning bioreactor (TPPB)

Characteristic	*cis*-Jasmone (90%)	*r*-Limonene (97%)	2-Undecanone (99%)	*n*-Hexadecane (99%)	1-Decene (94%)	*n*-Dodecane (99+%)
Other names		Carvene; cyclohexene	Methyl nonyl ketone			Dihexyl; bihexyl
CAS no.	488–10–8	5989–27–5	112–12–9	544–76–3	872–05–9	112–40–3
Log P	4.05	4.15	4.09	8.67	5.19	6.60
Density (g/ml)	0.940	0.841	0.825	0.770	0.741	0.749
Melting point (°C)	Not found	–96.9	12.0	18.0	–66.0	–9.6
Boiling point (°C)	134.0	176.0	231.0	287.0	166.5	216.0
Solubility (mg l^{-1})	1.68×10^{2}	1.38×10^{1}	Not found	9.00×10^{-4}	1.48×10^{-1}	3.70×10^{-3}
Price (CDN $)	473.76/500ml[a]	50.50/500ml[a]	88.80/500ml[a]	186.14/500ml[b]	128.50/500ml[a]	58.00/500ml[b]
Safety		Skin irritant, sensitizer	Irritant	Severe irritant	Irritant	Irritant
Uses	In perfumery	Wetting and dispersing agent, manufacturing resins	Pesticide	Component of plastic seal (e.g., Handi-wrap)	Breakdown product in synthetic engine oils	Found in fuel oil

[a] Sigma-Aldrich Fine Chemicals, 2000/01 catalogue – price was converted to CDN $/500 ml for comparison
[b] Fisher Scientific, 2000/01 catalogue – price was converted to CDN $/500 ml for comparison

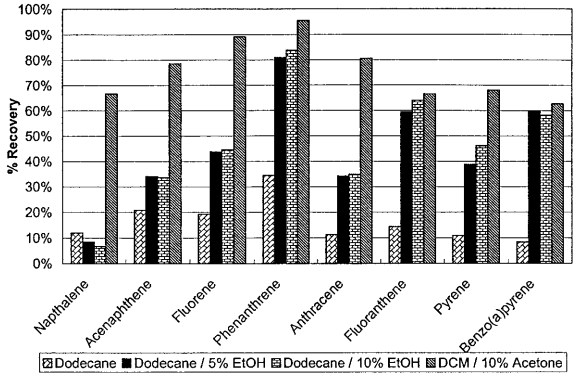

Fig. 1 Solvent extraction of various polycyclic aromatic hydrocarbons (PAHs) using dodecane, dodecane/ethanol and dichloromethane (DCM)/acetone

tract PAHs and other organic compounds for laboratory analysis and was used in this work for comparative purposes (Dean 1996; Keith and Telliard 1979; Saim et al. 1998). Although a DCM/acetone mixture provides good recovery, the toxicity of DCM would preclude the use of this solvent combination in large-scale soil treatments (SAIC Canada, personal communication). With the exception of naphthalene, the addition of ethanol to the dodecane as a co-solvent improves PAH extraction, as shown in Fig. 1. Other experiments have demonstrated that, as expected, a second pass of the solvent increases the PAH recovery further (data not shown). The PAH recovery may also depend on the type of soil present. In this case, wet clay, one of the more difficult soil types to treat, was used. It is suspected that solvent extraction on contaminated, aged soil would produce lower recoveries of PAHs; however, our comparison between solvents for extraction, using a DCM/acetone mixture as the benchmark comparison would remain valid provided that the same soil sample is used for all testwork.

Abiotic operation of the fermentor

After 96 h of operation at a high rate of aeration and agitation, with sampling every 24 h, no losses of the four PAHs present were detected from the organic phase. It can be concluded that, largely due to the condenser, depletion of PAHs from the bioreactor during fermentations does not occur due to volatilization.

Batch fermentation 1: Degradation of 9 g of LMW PAHs (naphthalene and phenanthrene)

The PAH concentration used in this fermentation far exceeds most previously reported initial concentrations in PAH biodegradation studies. As shown in Fig. 2, the cells exhibited little or no lag phase and showed good growth during the initial 12 h of fermentor operation. A nearly linear increase in growth was observed. Oxygen limitation occurred within the first 12 h and, although aeration and agitation were increased in an effort to alleviate this situation, it continued until the majority of the carbon source had been used.

A small amount of foam production began to occur after 56 h and biomass was observed entrained in the foam. As shown in Fig. 2, the appearance of foam corresponds to the disappearance of the naphthalene, the point at which the more rapidly consumed carbon source disappears. The cell growth rate also slowed at 56 h and eventually leveled off. Following 56 h, the creamy yellow color initially observed to indicate a healthy cell

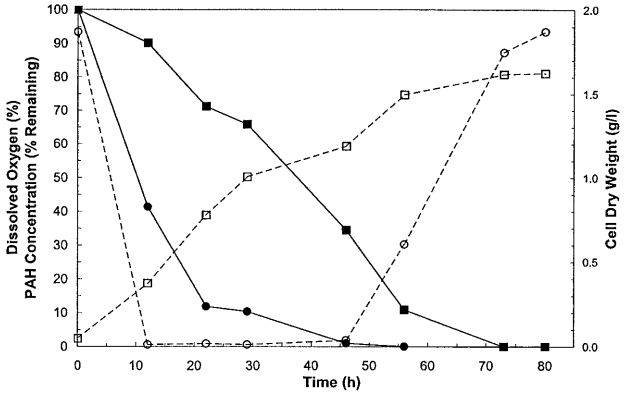

Fig. 2 Proof of concept – degradation of two low molecular weight (LMW) PAHs in a two-phase partitioning bioreactor (TPPB). Changes in the organic phase naphthalene (*closed circles*) and phenanthrene (*closed squares*) concentrations, and in aqueous cell dry weight (*open squares*) and % dissolved oxygen (*open circles*) in the fermentor are shown

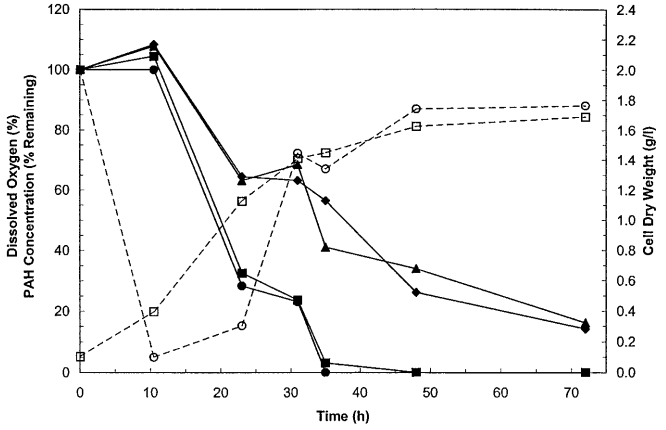

Fig. 3 Degradation of four PAHs using a TPPB with a dodecane/ethanol solvent phase. Changes in the organic phase naphthalene (*closed circles*), phenanthrene (*closed squares*), anthracene (*closed diamonds*) and acenaphthene (*closed triangles*) concentrations, and in aqueous cell dry weight (*open squares*) and % dissolved oxygen (*open circles*) in the fermentor are shown

population deepened to a light brown color and finally to a dark earthy brown color observed at the 72 h sample. The final color change occurs very rapidly (~15 min) and is linked to PAH (carbon-source) depletion. The 72 h sample was taken immediately after the color change was observed. Throughout the fermentation and all subsequent fermentations, microscopic examination and plating of the cells were undertaken to ensure that the culture remained pure. Results showed that the culture was uncontaminated throughout the fermentation, even following the observed pigment change.

The concentrations of naphthalene and phenanthrene were reduced to 0 g l^{-1} in the organic phase within 56 and 72 h, respectively. No degradation intermediates were detected by GC in this or any other experiments. The linear regions in the respective data series in Fig. 2 show that the volumetric consumption rates of naphthalene and phenanthrene are almost constant.

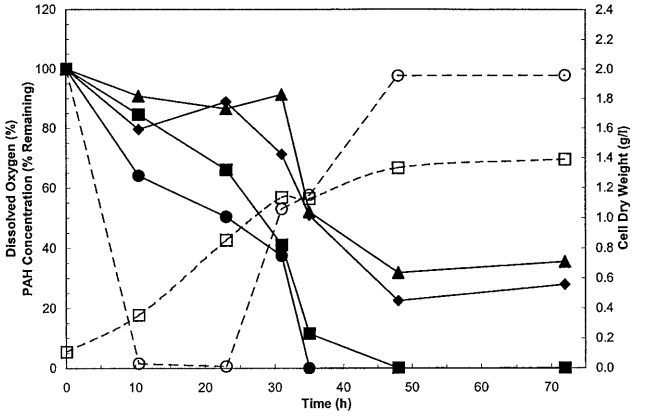

Fig. 4 Degradation of four PAHs using a TPPB with a dodecane solvent phase. Changes in the organic phase naphthalene (*closed circles*), phenanthrene (*closed squares*), anthracene (*closed diamonds*) and acenaphthene (*closed triangles*) concentrations, and in aqueous cell dry weight (*open squares*) and % dissolved oxygen (*open circles*) in the fermentor are shown

Batch fermentation 2: Degradation of 5.1 g of four LMW PAHs using a dodecane/ethanol solvent combination

The addition of ethanol to dodecane noticeably increased the recovery level of PAHs from soil in the solvent extraction experiments (Fig. 1), and the use of dodecane/ethanol (and just dodecane) as the TPPB solvent was examined.

As shown in Figs 3 and 4, both fermentors exhibited a large increase in cell density and, as in Batch Fermentation 1, good growth occurred in the first 12 h, with the fermentor containing the solvent mixture showing a greater increase. In both cases, cell growth increased from 0 to 35 h at which point it started to level off, corresponding to the depletion of the easily biodegraded PAHs, naphthalene and phenanthrene.

Foam production was observed at 35 h in both fermentors, although foaming was much greater in the co-solvent configuration, along with a small amount of brown sediment in the foam. The fermentation was allowed to continue until 72 h when the distinctive color change from creamy yellow to dark brown was observed. Oxygen limitation occurred in both reactors between 10 and 25 h. Concerted efforts to reduce oxygen limitation were successful following a subsequent increase in both aeration and agitation.

Degradation of the PAHs in both reactors was similar but not identical. In both cases, naphthalene and phenanthrene were biodegraded at similar rates and acenaphthene and anthracene, while biodegraded more slowly, also

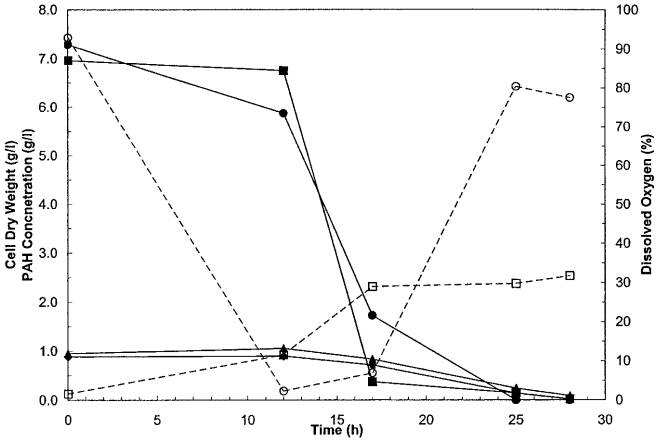

Fig. 5 Complete degradation of four PAHs in a TPPB using new nutrient conditions. Changes in the organic phase naphthalene (*closed circles*), phenanthrene (*closed squares*), anthracene (*closed diamonds*) and acenaphthene (*closed triangles*) concentrations and in cell dry weight (*open squares*) and % dissolved oxygen (*open circles*) in the fermentor are shown

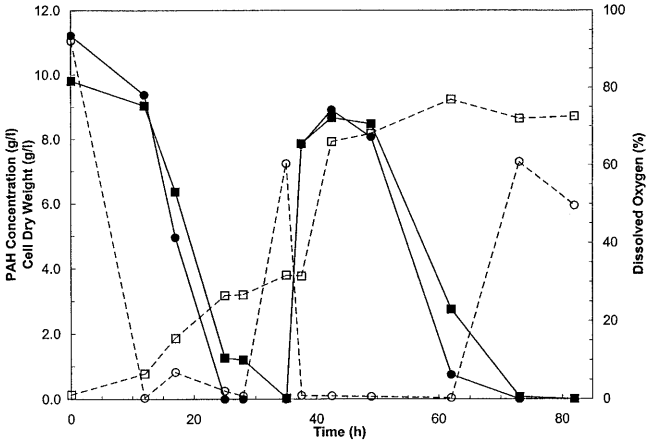

Fig. 6 Degradation of a high concentration of PAHs in a TPPB using a sequential feeding strategy. Changes in the organic phase naphthalene (*closed circles*) and phenanthrene (*closed squares*) concentrations, and in aqueous cell dry weight (*open squares*) and % dissolved oxygen (*open circles*) in the fermentor are shown

disappeared at similar rates. Anthracene and acenaphthene were not completely biodegraded but both showed at least a 65% reduction from the original concentration after 48 h.

Batch fermentation 3: Degradation of 8.0 g of four LMW PAHs

After the development of an altered medium formulation (increased concentrations of tryptone and yeast extract), the ability of *S. aromaticivorans* B0695 to more completely degrade anthracene and acenaphthene was re-examined. Despite the use of high rates of aeration and agitation conditions, oxygen limitation still occurred as shown in Fig. 5. Biodegradation of PAHs showed a lag phase of approximately 12 h. Cells experienced slow initial growth in the first 12 h after which rapid growth occurred, accompanied by a rapid biodegradation of the PAHs. All four PAHs were completely biodegraded in approximately 28 h. At 28.75 h, a large increase in foam production, a color change in the bioreactor from creamy yellow to an olive green color, and the appearance of dark brown biomass appearing on the baffles and in the foam was observed.

Fed-batch fermentation: Degradation of 20.0 g LMW PAHs (naphthalene and phenanthrene)

The highest mass successfully biodegraded in our previous experiments was a total of 9.0 g of LMW PAHs in Fermentation 1. It was therefore decided that a larger amount would be added to the fermentor in two batches, for a total of 20.0 g of PAHs. A nutrient spike was also added to the fermentors when the second batch of PAHs was added.

As shown in Fig. 6, there was a small lag phase that occurred in the first 12 h, followed by rapid cell and PAH degradation. As before, this system rapidly became oxygen limited, but aeration and agitation were not increased further. Cell growth slowed as the overall concentration of PAHs in the dodecane neared 1 g l^{-1}. This was accompanied by a rise in the dissolved oxygen as shown in Fig. 6. At 35 h, when the PAH concentration had become depleted, additional PAHs were added to the reactor. The bioreactor responded to the new batch of PAHs with a rapid increase in cell density and a rapid decrease in dissolved oxygen. The second spike of PAHs was again completely biodegraded in 35 h. Foam production in the fermentors was a challenge during this fermentation and was controlled by the addition of a single aliquot of 0.25 ml of Antifoam 204 at 12 h.

Discussion

The literature has revealed that ex situ attempts using microbial consortia to biodegrade PAHs have produced extremely slow consumption rates, and the time required for remediation varies from a few days to several months (Dreyer et al. 1995; Fuchs and Braun 1995; Guiyesse et al. 2000; Joyce et al. 1998; Oberbremer et al. 1990; Pinelli et al. 1997; Potter et al. 1999; Rockne and Strand 1998; Stefess 1998; Woo and Park 1999). In this work the potential use of a TPPB for biodegradation of PAHs was investigated using a single strain of a bacterium mentioned repeatedly in the literature as possessing PAH-degradation capabilities (Barkay et al. 1999; Lantz et al. 1995; Shi et al. 2001; Willumsen and Arvin 1999; Willumsen et al. 1998; Ye et al. 1996; Zylstra and Kim 1997).

Initially, screening experiments were carried out for the selection of an organic phase that could be success-

fully used with the bacterium *S. aromaticivorans* B0695 in a TPPB. The critical log P, 4.0, of the bacterium was within the expected range of 3.0–4.0 based on previous work in this laboratory (Collins and Daugulis 1999; Munro and Daugulis 1997). Dodecane was chosen as the most suitable solvent based on the bacterium's ability to grow in its presence and its inability to use it as a carbon source. Dodecane, a straight chain alkane, is low in cost, relative to the other six solvents considered (Table 1), has a log P value significantly above the critical log P value and is insoluble in water.

Following solvent selection, the utility of this solvent for PAH extraction from soil was examined. The use of solvents for the extraction of xenobiotics is not a novel process and it has been demonstrated to be successful in field-scale applications (Hall and Sandrin 1990; Jones 1992; Rulkens et al. 1998). However, solvent selection for this process has hitherto been considered without regard to other processes that may be required for further treatment. It was determined that dodecane could be successfully used in solvent extraction applications particularly when enhanced by a second solvent, ethanol (Fig. 1). It is suspected that ethanol, which is water miscible, may recover PAHs that are not easily accessible by the hydrophobic solvent, dodecane (Nardella et al. 1999; Rulkens et al. 1998). The eventual goal is to couple the solvent extraction and TPPB technologies to create a full-scale, cost-efficient system to remove and destroy PAHs from soil.

The capability of this bacterium, *S. aromaticivorans* B0695, to effectively degrade two LMW PAHs (naphthalene and phenanthrene) was shown by an initial experiment (Fig. 2) in the TPPB. The bacterium completely biodegraded 4.5 g of both compounds in less than 75 h at a volumetric consumption rate of 40 mg l^{-1} h^{-1}. This rate of degradation corresponds to an overall volumetric rate for naphthalene of 33 mg l^{-1} h^{-1} and an overall phenanthrene consumption rate of 21 mg l^{-1} h^{-1}. Since the fermentation was oxygen limited when most of the biodegradation occurred, oxygen transfer became the rate-limiting step. This is confirmed by the almost linear increase in cell growth and decrease in PAH concentrations in the aqueous phase. The rates achieved in this fermentation are an order of magnitude higher than those previously reported in the literature for PAHs in ex situ mixed bacterial population bioreactor schemes and, to the best of our knowledge, are the first volumetric consumption rates reported for a single bacterial species in a bioreactor. With the exception of Oberbremer et al. (1990), whose work focused primarily on hydrocarbons and a single PAH (naphthalene), the highest previously reported volumetric rate is 2.4 mg l^{-1} h^{-1} obtained from the biodegradation of a mixture of 16 PAHs (Dreyer et al. 1995).

Another measure of system performance is the yield coefficient $Y_{x/s}$ (g cells produced/g substrate consumed). Since the majority of previous work on PAH biodegradation has been completed using mixed populations, very few yield coefficients are reported in the literature. Saner et al. (1996) reported a yield coefficient, $Y_{x/s}$, of 0.6 g biomass/g phenanthrene for a mixed culture in a closed agitated soil fermentor. However, unsterile soil and indigenous soil organisms were used and it cannot be concluded that all biomass growth occurred due to the model contaminant (phenanthrene) that was added to the soil and not a carbon source already present. The yield coefficient for this fermentation was found to be 0.55 g cells/g substrate and is comparable to the value reported by Saner et al. (1996) for a mixed culture. However, aqueous phase optical density measurements taken to determine cell concentration can be underestimated due to cell growth that occurs on the baffles and fermentor walls, and bacteria that become entrained in the solvent layer and/or foam. For these reasons, it is believed that the true yield coefficient for this fermentation was higher than the measured value.

The effect of using a dodecane/ethanol co-solvent in a TPPB was examined since it was shown (Fig. 1) that this combination greatly increases the efficacy of solvent extraction. The observed increase in cell density in the fermentor containing the co-solvent was expected since it has been previously shown in our laboratory that *S. aromaticivorans* B0695 is able to achieve some cell growth using ethanol as a carbon source. This was confirmed by the yield coefficients generated for the fermentation. In the reactor containing the co-solvent, $Y_{x/s}$ for the overall system was 0.88 (g cells produced/g PAH consumed). This can be directly compared to the single solvent fermentation that had an overall $Y_{x/s}$ of 0.67. Both values achieved in this experiment are higher than the yield coefficient found previously in Batch Fermentation 1. Aeration and agitation were much higher in these fermentations and as a result, growth occurring on surfaces in the fermentor may have been reduced. Less foam production was also observed in this fermentation compared to the first fermentation.

In the fermentor with a co-solvent, there was an initial lag of approximately 12 h before PAH degradation occurred. We believe that this lag occurs because the bacterium was using ethanol for growth before commencing to utilize the PAHs. The overall calculated volumetric PAH consumption rate over 72 h was 23 mg l^{-1} h^{-1}. Individual PAH degradation rates were determined from 0 h; naphthalene and phenanthrene were completely biodegraded in approximately 35 h at a rate of 39 mg l^{-1} h^{-1}, and anthracene and acenaphthene were biodegraded to approximately 15% of their initial concentration, but as in the presence of dodecane alone, degradation occurred much more slowly. The volumetric rate for anthracene and acenaphthene biodegradation was 2.0 mg l^{-1} h^{-1}. The rates achieved in the presence of a co-solvent show little difference to the rates achieved without ethanol. As shown in Fig. 3, naphthalene and phenanthrene were almost completely biodegraded in 35 h with a volumetric rate of 38 mg l^{-1} h^{-1} and acenaphthene and anthracene, degraded at a much slower rate than naphthalene and phenanthrene, were biodegraded at a rate of 1.6 mg l^{-1} h^{-1}. In the presence of a co-solvent, the overall total

volumetric rate for the PAHs achieved over 72 h was 22.0 mg l^{-1} h^{-1}. It is suspected that the distinct cessation of acenaphthene and anthracene degradation at 48 h (as shown in Figs. 3 and 4) could, in both cases, be attributed to a nutrient deficiency in the medium. Oxygen limitation was again a feature of this fermentation, resulting in almost linear increases in cell growth and decreases in PAH concentration. From these results, it appears as though the presence of ethanol as a co-solvent, does not inhibit cell growth or PAH degradation. This confirms that a successful linkage of solvent extraction and TPPB technology is possible. Further work in our laboratory has showed that methanol (5%) could also be successfully employed as a co-solvent with dodecane (95%) in a TPPB.

A subsequent fermentation was conducted in an effort to achieve complete biodegradation of acenaphthene and anthracene. The possible salt and nutrient limitation was addressed by increasing the concentration of yeast extract, tryptone and salts in the medium and the complete biodegradation of the four PAHs (naphthalene, phenanthrene, acenaphthene and anthracene) was directly attributed to these changes. An overall volumetric consumption rate of 95 mg l^{-1} h^{-1} was achieved. Naphthalene and phenanthrene were degraded at a rate of 85 mg l^{-1} h^{-1} and acenaphthene and anthracene were degraded at a rate of 11 mg l^{-1} h^{-1}. These rates show an almost 2-fold increase in previously obtained volumetric consumption rates. Despite a high rate of aeration and agitation, oxygen limitation occurred causing it to become, as before, the rate limiting step in cell growth and PAH biodegradation.

The calculated overall yield coefficient was 0.90 g cells/g PAH. This fermentation was carried out at much higher agitation and aeration and, for this reason, any possible wall growth and baffle growth was again reduced. Antifoam 204 was added, which kept foaming to a minimum and reduced cell loss from the aqueous phase. Additionally, higher concentrations of complex medium components were added to the fermentor. It is expected that growth on complex nutrient and/or nitrogen sources will lead to a higher concentration of cells than growth achieved on a minimal salts medium.

Finally, using the new medium formulation, a fed-batch fermentation was undertaken in an attempt to degrade a high concentration of LMW PAHs. The initial rate for volumetric consumption was 100 mg l^{-1} h^{-1} over the first 35 h. The second spike of PAHs reached a maximum concentration at 42.5 h and the PAHs were completely degraded 35 h following the addition of the second spike of PAHs at an almost identical rate of 95 mg l^{-1} h^{-1}. Since the second spike of PAHs was degraded at the same rate (and not more rapidly, as one would expect due to a higher cell concentration), oxygen limitation was clearly occurring in this system. This means that the observed rates are not the maximum achievable volumetric rates. This fact is further confirmed by the linear regions of Fig. 6 showing PAH biodegradation and cell density. The overall volumetric consumption rate for the fermentation was 98 mg l^{-1} h^{-1} and is comparable to the rates achieved in the previous fermentation.

The overall yield coefficient for this fermentation was also determined to be 1.00 g cells/g substrate, which is higher than any of the yield coefficients previously reported. Comparable to the results achieved in the previous experiment, addition of Antifoam 204 at 12 h likely reduced cell mass previously entrained in the foam and additional nutrients and salts eliminated bacterial synthesis requirements and increased the amount of growth achieved. This experiment is significant because it showed that *S. aromaticivorans* B0695 was capable of degrading 20.0 g of LMW PAHs in a very short period of time. In future experiments, it is our hope that a higher level of PAH loading in the solvent phase could be achieved, which would reduce the need for a frequent feeding strategy.

In summary, the results achieved in this work show great promise for the ability to achieve rapid biodegradation of PAHs in a TPPB. The volumetric consumption rate of 95 mg l^{-1} h^{-1} obtained here is, to our knowledge, the highest rate obtained for LMW PAH degradation using a single species. One significant challenge encountered throughout this work was oxygen limitation. As a result, it is believed that the calculated volumetric consumption rates achieved underestimate the true capability of the bacterium *S. aromaticivorans* B0695 to biodegrade PAHs using a TPPB, and if oxygen limitation can be avoided, the volumetric consumption rates should be substantially improved. TPPB technology has proved to be an efficient and effective method of degrading PAHs. The initial steps taken to link a solvent extraction process directly to a TPPB system have been successful and further work to scale-up and connect these two processes is ongoing.

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