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Kinetics of BTEX biodegradation by a coculture of *Pseudomonas putida* and *Pseudomonas fluorescens* under hypoxic conditions

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Abstract

Pseudomonas putida and *Pseudomonas fluorescens* present as a coculture were studied for their abilities to degrade benzene, toluene, ethylbenzene, and xylenes (collectively known as BTEX) under various growth conditions. The coculture effectively degraded various concentrations of BTEX as sole carbon sources. However, all BTEX compounds showed substrate inhibition to the bacteria, in terms of specific growth, degradation rate, and cell net yield. Cell growth was completely inhibited at 500 mg l^{-1} of benzene, 600 mg l^{-1} of *o*-xylene, and 1000 mg l^{-1} of toluene. Without aeration, aerobic biodegradation of BTEX required additional oxygen provided as hydrogen peroxide in the medium. Under hypoxic conditions, however, nitrate could be used as an alternative electron acceptor for BTEX biodegradation when oxygen was limited and denitrification took place in the culture. The carbon mass balance study confirmed that benzene and toluene were completely mineralized to CO_2 and H_2O without producing any identifiable intermediate metabolites.

Introduction

Benzene, toluene, ethylbenzene, and three isomers (*ortho*-, *meta*-, and *para*-) of xylene, collectively known as BTEX, are widely used as industrial solvents for organic synthesis and equipment cleansing. BTEX are the major aromatic components in many petroleum products and are frequently found in groundwater and industrial waste streams (Lee et al. 1988). They are among the priority pollutants to be removed from many superfund sites. Understanding the biodegradation of BTEX in the natural environment is thus of interest and is important to the development of *in situ* bioremediation and bioreactor technologies for removing BTEX. It is also essential for the prediction of the extent to which contamination will spread and the effectiveness of intrinsic bioremediation cleanup operations.

Previous BTEX biodegradation studies usually used a consortium of undefined mixed cultures (Dyreborg et al. 1996; Fan & Scow 1993; Kelly et al. 1996; Kolb & Wilderer 1997). Many pure culture isolates, including various strains of *Pseudomonas putida*, have also been studied, but they often could not degrade all BTEX compounds simultaneously and efficiently under hypoxic conditions (Burback & Perry 1993; Cruden et al. 1992; Dararat & Riffat 1999; Goudar et al. 2000; Keener & Arp 1994; Komukai-Nakamura et al. 1996; Lee et al. 1994; Löser & Ray 1994; Lu et al. 2000; Mason et al. 2000; Moriya & Horikoshi 1993; Phelps & Young 1999; Yerushalmi et al. 1999). Furthermore, aerobic biodegradation is often limited by oxygen available in

the environment. For instance, BTEX usually cannot be completely mineralized by *P. putida* alone when oxygen is limited, which is often the situation existing in natural ecosystems (Lee et al. 1994). Some bacteria, such as *P. fluorescens*, can denitrify and use nitrate as an electron acceptor for BTEX biodegradation under hypoxic or anaerobic conditions (Mikesell et al. 1994; Wilson & Bouwer 1997). To date, no kinetic study of BTEX biodegradation has been done with a simple, defined coculture of the above two *Pseudomonas* species that can completely degrade (mineralize) all BTEX compounds under hypoxic conditions.

In this work, the kinetics of BTEX biodegradation by the coculture of *P. putida* and *P. fluorescens* was studied using a synthetic medium containing BTEX as sole carbon sources in serum bottles, without aeration to prevent stripping (or evaporative) losses of BTEX compounds. Several important factors, including: (1) hydrogen peroxide (H_2O_2) as an additional oxygen source, (2) substrate (BTEX) concentration, and (3) nitrate as an alternative electron acceptor under hypoxic conditions, were investigated and their effects on BTEX biodegradation are reported here. Carbon mass balances using ^{14}C-labeled benzene and toluene as representative substrates were also performed to demonstrate that complete mineralization of BTEX was indeed achieved with these microorganisms in the presence of nitrate and under hypoxic conditions.

Materials and methods

Cultures and media

The coculture (Munox bacteria™ 112, Osprey Biotechnics, Sarasota, FL) consisting of *P. putida* and *P. fluorescens* used in this study was grown in a defined mineral salts medium (MSM), which contained (per liter) 0.5 g $(NH_4)_2SO_4$, 0.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 9.8 mg $CaCl_2{\cdot}2H_2O$, 10 mg $MnSO_4H_2O$, 8 mg $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$, 2 mg $ZnSO_4{\cdot}7H_2O$, and 20 mg nitrilotriacetic acid. After the pH of MSM was adjusted to around 7, the MSM (100 ml) was placed into 125-ml serum bottles, sealed with aluminum crimped Viton stoppers (Fisher Scientific Co., PA), and autoclaved at 121 °C for 15 min. The Viton stopper was used because it is resistant (impermeable) to BTEX. Each BTEX compound was then aseptically added to the serum bottle using a microsyringe directly from the stock solution to give a desired final concentration. Hydrogen peroxide and nitrate were also included in the MSM in the experiments to study their effects on BTEX biodegradation. Details about the medium preparation can be found elsewhere (Shim 1997; Shim & Yang 1999).

Biodegradation kinetic studies

The original coculture was first adapted to grow in the MSM with toluene as the sole carbon source in a 125-ml serum bottle. After several transfers with gradually increasing toluene concentrations, the culture showed good growth on toluene. The adapted culture was then studied for its ability to degrade benzene (B), toluene (T), ethylbenzene (E), or *o*-xylene (*o*-X) as a sole carbon source. For kinetic studies, each serum bottle containing 100 ml of the MSM (pH preadjusted to 7) was aseptically injected with one substrate (B, T, E, or *o*-X) directly from the stock solution using a microsyringe to give a desired final concentration. Unless otherwise noted, each serum bottle (95 ml MSM) was inoculated with 5 ml of a fresh culture pre-grown on toluene (or the same individual BTEX compound as the substrate studied) and incubated in an incubator shaker (25° C, 110 rpm). Liquid sample aliquots were periodically withdrawn to measure optical density (OD), pH, and BTEX concentrations. The pH of the medium generally dropped slightly from the initial value of 7 to 6.4 at the end of each batch experiment.

Effects of substrate concentrations

The effects of substrate concentrations on cell net yield, specific growth rate, and substrate degradation rate were studied for individual BTEX compounds with concentrations up to 1000 mg l^{-1}. The cell net yield was determined from the slope of cell concentration versus substrate concentration plot. The specific growth rate was determined from the slope of the semi-logarithmic plot of OD versus time. The substrate degradation rate was determined from the initial slope (or maximum slope) in the plot of substrate concentration versus time.

Effect of H_2O_2

The effect of H_2O_2, as an additional oxygen source, on BTEX biodegradation was studied by

aseptically injecting H_2O_2 stock solution (30%) to give a final concentration of 0.01%, which would provide an equivalent of ~47 mg l^{-1} additional oxygen in the liquid medium. Parallel experiments without H_2O_2 were also carried out at the same time for direct comparison.

Effect of nitrate

The effect of nitrate on benzene biodegradation under hypoxic conditions was studied using benzene (150 mg l^{-1}) as the sole carbon source. Ammonium sulfate, $(NH_4)_2SO_4$, (500 mg l^{-1}) in the MSM was replaced with NH_4NO_3 (300 mg l^{-1}) or KNO_3 (800 mg l^{-1}) as the major nitrogen source. The amount of each nitrogen source was chosen to give the same total nitrogen concentration at approximately 110 mg N l^{-1}. The effect of nitrate as an alternative electron acceptor on benzene biodegradation was also studied, using various concentrations (0–5000 mg l^{-1}) of NH_4NO_3.

Carbon mass balance study

Radiolabeled, [Ring-UL-^{14}C], benzene and toluene, as representative BTEX compounds, were used to study the carbon mass balance in BTEX biodegradation. [Ring-UL-^{14}C] benzene and toluene (specific activity, 19.3 and 5 mCi $mmol^{-1}$ each; purity, greater than 99%) were purchased from Sigma Chemical Co. (St. Louis, MO). The radiochemicals were dissolved and stored in HPLC-grade methanol (4.4×10^5 and 2.2×10^5 dpm μl^{-1} each). The mineral salts media (50 ml in serum bottles) containing NH_4NO_3 (500 mg l^{-1}) as the major nitrogen source with nitrate also as the alternative electron acceptor were supplemented with both labeled and unlabeled benzene or toluene (total final concentrations: 100–500 mg l^{-1}). The serum bottles were inoculated with aliquots of the culture obtained from the fibrous-bed bioreactor (for the details about the fibrous-bed bioreactor, refer to Shim & Yang 1999) and incubated on a shaker (25 °C, 110 rpm). Each serum bottle also contained a KOH trap (1.5 ml vial containing 1 ml 0.5 N KOH), to absorb CO_2 produced from benzene or toluene biodegradation. The radioactivity in the culture medium was quantified for the initial sample (right after inoculation). At the end of 4-week incubation period, the radioactivity in the KOH trap, sorbed to the rubber stopper, and remaining in the culture medium were quantified. To recover and quantify sorbed [^{14}C]-benzene and [^{14}C]-toluene, the stoppers were extracted with ethyl acetate overnight. The liquid culture samples were centrifuged for 10 min at 10,000 rpm, to separate the cell biomass from the liquid medium. The radioactivities associated with the cell biomass and water-soluble metabolites or non-utilized substrate in the liquid medium were counted separately.

Parallel experiments were carried out with non-inoculated media that served as negative controls, and with inoculated media containing only unlabeled benzene or toluene (in the same concentration range studied), which served as positive controls. Liquid sample aliquots were periodically withdrawn from serum bottles containing unlabeled benzene or toluene, to measure OD, pH, and benzene or toluene concentrations. The results from control experiments using unlabeled benzene or toluene were used to compare with the ones using labeled benzene or toluene, in terms of cell biomass (cell net yield), abiotic losses of substrate, and concentrations of benzene or toluene.

Analytical methods

Cell concentration

Free cell concentration was measured as OD at 600 nm using a spectrophotometer (Model 340, Sequoia-Turner). Cell concentration was estimated from a correlation between OD and cell dry weight. One unit of OD was found to be equivalent to 0.64 g cell dry weight per liter (Shim 1997).

BTEX concentrations

Unless otherwise noted, BTEX compounds were analyzed using a gas chromatograph (Varian 3400) equipped with a flame ionization detector. The column used was a glass column (6 ft × 2 mm i.d.; Alltech C6191) packed with Carbograph 2 (80/100 mesh, Alltech 1728). Nitrogen was used as the carrier gas at a flow rate of 40 ml min^{-1}. The temperatures for column, injector, and detector were 220 (isothermal), 220, and 250° C, respectively. One μl of liquid (aqueous phase) sample was injected with a liquid-tight microsyringe (Hamilton 14-815-1). The detection limit for each BTEX compound was 5 μg l^{-1}. The concentrations of BTEX in high-concentration samples were also analyzed by micellar electrokinetic capillary chromatography (MEKC) with an auto-sampler,

without further dilutions of samples with high BTEX concentrations. A high performance capillary electrophoresis (HPCE) system (Thermo Separation Products, CA) equipped with an UV detector (Cathodeon Ltd., Cambridge, UK) and a fused-silica capillary tube (44 cm length, 50 μm i.d.; Polymicro Technologies Inc., AZ) was used. The buffer system contained Na_2HPO_4/borate/sodium dodecyl sulfate (SDS) (10/20/60 mM, respectively), pH 7.5. The sample was placed into capillary with a 3-s hydrodynamic injection at 25 kV (typically producing 45 μA). The detailed MEKC analytical procedure for BTEX can be found elsewhere (Shim 1997; Shim et al. 2004).

^{14}C liquid scintillation counting

Quantification of ^{14}C in liquid medium was performed by adding 1 ml of the sample (KOH solution, ethyl acetate extract, or culture fluid) to 15 ml of scintillation cocktail, ScintiVerse-BD (Fisher Scientific, PA), and by subsequent counting in a Beckman LS 6800 liquid scintillation counter (Fullerton, CA).

Results and discussion

BTEX biodegradation kinetics

Figure 1 shows typical biodegradation kinetics for cells grown on toluene as the sole carbon source. In general, at low initial substrate concentrations (< 50 mg l^{-1}), cells continued to grow until all substrates were consumed (data not shown). However, at higher substrate concentrations, BTEX degradation was not complete and cell growth stopped long before all substrates could be consumed, although the coculture grew on toluene well initially (Figure 1). Benzene, ethylbenzene, and *p*-xylene showed the similar trend (data not shown). The incomplete BTEX biodegradation could be attributed to oxygen depletion in the culture medium. Theoretically, for every 1 g of BTEX substrate, about 3 g of oxygen would be required for its complete mineralization aerobically. In this study, however, the total amount of oxygen in the serum bottle, including the headspace (25 ml), was not sufficient for complete biodegradation when BTEX concentration was higher than 100 mg l-1 (Shim & Yang 1999).

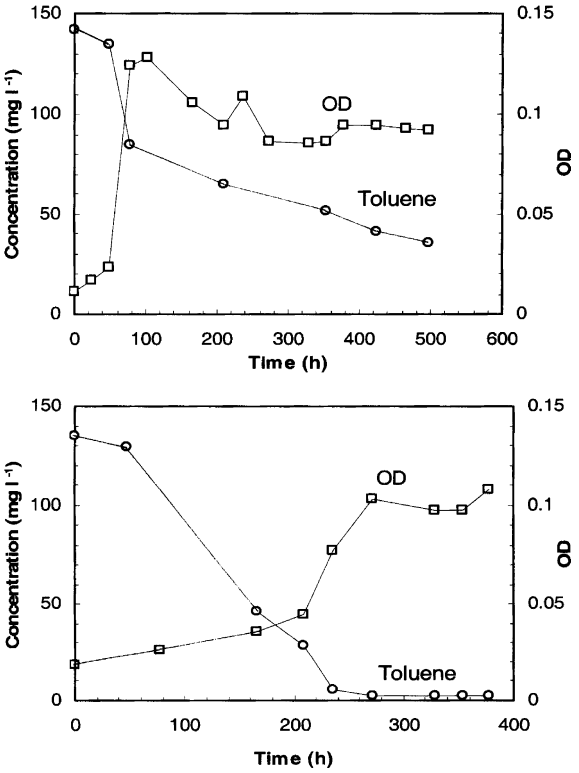

Figure 1. Kinetics of toluene biodegradation by the coculture of *P. putida* and *P. fluorescens* in serum bottles, without (top) and with (bottom) H2O2.

Effect of H_2O_2

Hydrogen peroxide (H_2O_2) is often used as an additional oxygen source to facilitate aerobic biodegradation processes for the removal of toxic organic compounds from the environment (Lee et al. 1988). It can be dissociated into O_2 and H_2O by catalase present in aerobic microorganisms. As shown in Figure 1, complete toluene biodegradation was achieved when H_2O_2 (0.01%) was added to the medium. Similar trends were observed for benzene, ethylbenzene, and *p*-xylene (data not shown). At 0.01%, H_2O_2 did not have significant chemical interactions with BTEX or any other components of the medium (data not shown).

As can be seen in Figure 1, growth kinetics with or without H_2O_2 were somewhat different. In general, without H_2O_2, cell growth was faster initially, but BTEX could not be completely degraded due to oxygen limitation. On the other hand, with H_2O_2, initial cell growth was slower, often with a long lag phase, but growth continued

and reached a higher cell concentration (OD) and complete BTEX degradation was achieved. The overall biodegradation rate was also faster with the addition of H_2O_2 in the medium (Table 1). Therefore, H_2O_2 stimulated BTEX biodegradation because of the additional oxygen provided to the medium. With 0.01% H_2O_2 in the medium, 47 mg l-1 of oxygen would be available for BTEX biodegradation. The stimulatory effect of H_2O_2 on BTEX biodegradation was also found in previous studies (Thomas & Ward 1989). However, H_2O_2 also showed an inhibitory effect on cell growth (Fiorenza & Ward 1997), with a longer lag phase (Figure 1) and slightly lower specific growth rate (Table 1). It has been reported that H_2O_2 has a toxic effect on subsurface microorganisms, even at a concentration of as low as 0.003% (Anid et al. 1993). Thus, it is important to optimize the concentration of H_2O_2 to promote BTEX biodegradation while avoiding its toxic effect on cell growth.

Effects of substrate concentration

The effects of substrate concentration on the specific growth rate (μ, h^{-1}), substrate degradation rate (D, mg l^{-1} h^{-1}), and cell net yield (Yx/s, mg mg^{-1}) were studied. Batch time course data (as shown in Figure 1) from various initial substrate concentrations up to 1000 mg l^{-1} were used to estimate μ, D, and $Y_{x/s}$. Similar to the previously reported results for μ and D (Shim & Yang 1999), $Y_{x/s}$ also increased initially with increasing substrate concentration but then decreased as the substrate concentration continued to increase, as shown in Figure 2, indicating substrate inhibition. Compared to benzene, toluene, and ethylbenzene,

Table 1. Effects of H_2O_2 on specific growth rate and BTEX degradation rate

Substrate	Specific growth rate (h^{-1})		Degradation rate (mg l^{-1} h^{-1})	
	With H_2O_2	Without H_2O_2	With H_2O_2	Without H_2O_2
Benzene	0.0330	0.0406	2.165	0.588
Toluene	0.0349	0.0368	1.319	0.991
Ethylbenzene	0.0327	0.0350	0.608	0.297
p-Xylene	0.0124	0.0164	0.626	0.580

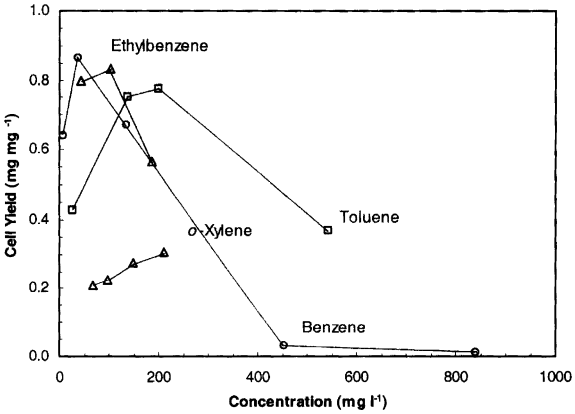

Figure 2. Effect of substrate concentration on cell net yield.

the cell net yield for *o*-xylene was significantly lower (Figure 2), implying higher toxicity at similar concentrations.

Most of previous kinetic studies of BTEX biodegradation by *Pseudomonas* species were done at low substrate concentrations and showed Monod kinetics (Chang et al. 1993; Langenhoff et al. 1996; Oh et al. 1994). However, it is clear from this study that BTEX at high concentrations are inhibitory and can be toxic to cells, which could be a problem for bioremediating high-concentration waste streams often generated at industrial sites.

Effect of nitrate

The possible denitrification effect on BTEX biodegradation was studied using benzene as the sole carbon source and different nitrogen sources, NO_3^- and NH_4^+. As shown in Figure 3, the medium containing NH_4NO_3 as the nitrogen source gave faster and complete benzene biodegradation, whereas $(NH_4)_2SO_4$ only accomplished partial (68%) benzene biodegradation. Potassium nitrate (KNO_3) also provided higher benzene biodegradation rate and efficiency (86%) than those from $(NH_4)_2SO_4$. This higher efficiency for benzene biodegradation in the presence of NO_3^- was attributed to the fact that *P. fluorescens* could degrade benzene with nitrate as an alternative electron acceptor when oxygen in the medium was depleted. Thus, the initial oxygenation reaction utilizing molecular oxygen initially available only resulted in incomplete degradation and the further biodegradation occurred utilizing nitrate as the alternative electron acceptor when the oxygen in

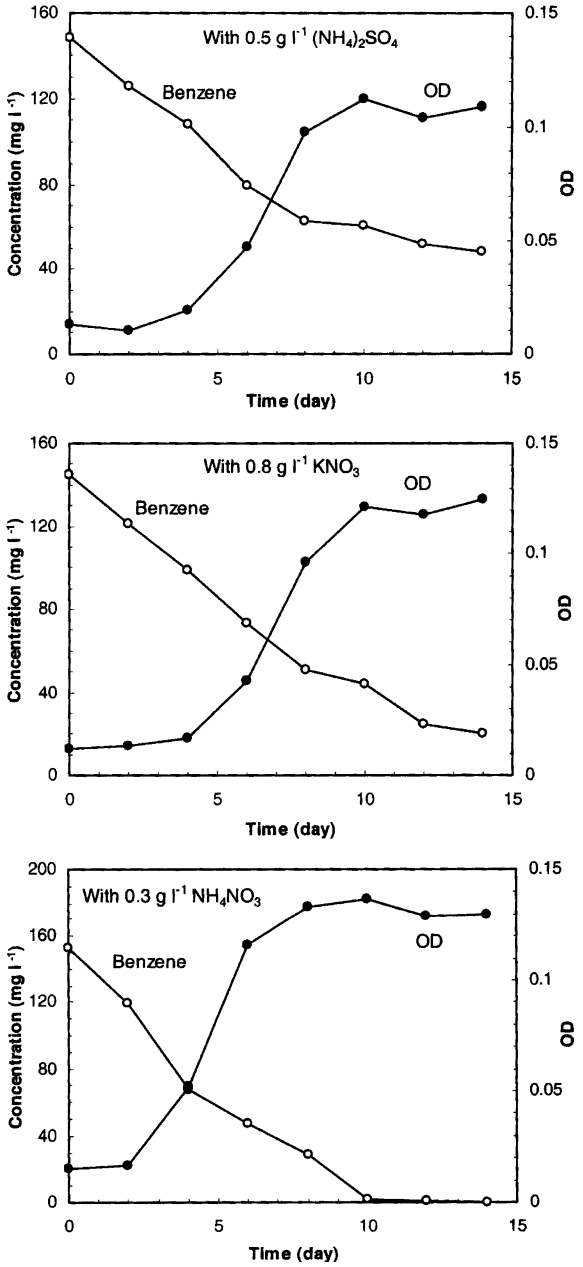

Figure 3. Effect of nitrate and ammonia on benzene biodegradation by the coculture of *P. putida* and *P. fluorescens* in serum bottles.

the medium was depleted. This experiment clearly suggested that 'aerobic denitrification' (Wilson & Bouwer 1997) took place and helped benzene biodegradation under oxygen-limited condition when nitrate was present in the medium. It would be thus beneficial to include nitrate for the bioremediation of BTEX-contaminated water when oxygen is limited.

Figure 4 shows the effect of NH_4NO_3 concentration in the medium on benzene biodegradation. Without NH_4NO_3 as a nitrogen source with nitrate also as an alternative electron acceptor, benzene biodegradation was poor. However, cells degraded benzene almost the same when NH_4NO_3 was given in the concentration range of 100–3,000 mg l^{-1}. The biodegradation efficiency was low at 5000 mg l^{-1} NH_4NO_3, indicating cell toxicity at this high NH_4NO_3 level.

Carbon mass balance for benzene and toluene biodegradation

Carbon mass balance using [^{14}C]-benzene and [^{14}C]-toluene as substrates after 4-week incubation is shown in Table 2. Since the original radiolabeled benzene and toluene were dissolved and stored in methanol and these stock solutions were used for the mass balance study for benzene and toluene, the coculture was first tested for the ability to utilize methanol as a substrate. These microorganisms did not grow on methanol at the concentration (80 μM) used in this study and at this concentration methanol did not inhibit benzene and toluene mineralization.

As shown in Table 2, 9–13% and 6–15% of the applied radioactivity for benzene and toluene, respectively, were recovered as biomass, and 57–65% for benzene and 59–71% for toluene were evolved as $^{14}CO_2$, indicating that most organic

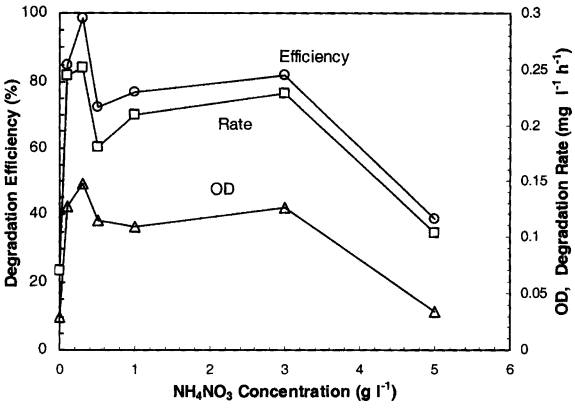

Figure 4. Effects of NH_4NO_3 on benzene biodegradation by the coculture grown on benzene (150 mg l^{-1}) as the sole carbon source in serum bottles.

Table 2. Biodegradation of [^{14}C]-benzene and [^{14}C]-toluene by the coculture of *P. putida* and *P. fluorescens* from a liquid-continuous fibrous-bed bioreactor, in serum bottles with NH_4NO_3 as the nitrogen source

Substrate	Initial concentration (mg l^{-1})	^{14}C balance after an incubation time of 4 weeks (as % of initial total count)				
		Recovered from biomass	Recovered from liquid medium	Trapped in KOH (CO_2)	Sorbed to stopper	Total recovery
Benzene	100	8.9 (11.0)[a]	9.6 (11.8)	62.6 (77.2)	0.1	81.2 (100)
	200	12.7 (15.0)	7.1 (8.4)	64.9 (76.6)	0.3	85.0 (100)
	500	11.6 (13.6)	16.4 (19.3)	57.0 (67.1)	0.3	85.3 (100)
Toluene	100	6.4 (7.6)	16.5 (19.3)	61.4 (72.8)	0.2	84.5 (100)
	200	9.8 (11.0)	8.5 (9.5)	71.0 (79.5)	0.2	90.5 (100)
	500	15.3 (18.1)	10.3 (12.2)	58.8 (69.7)	0.8	85.2 (100)
Controls without organisms:						
Benzene	100	–	84.6 (95.5)	1.5 (1.7)	2.5 (2.8)	88.6 (100)
	200	–	84.8 (96.1)	1.5 (1.7)	1.9 (2.2)	88.2 (100)
	500	–	87.9 (96.2)	1.5 (1.6)	2.0 (2.2)	91.4 (100)

[a] Values in parentheses are based on the assumption of 100% total recovery.

carbon was mineralized. Up to 17 % remained in the culture medium as ^{14}C-labeled residues. The amounts of benzene and toluene extractable from the stoppers were generally below 1%. The overall recovery of radioactivity in the cultures was 81–91%. The less than 100% recovery was not unusual and could be caused by sorption to the glass bottle or leakage. At higher concentrations, significant amounts of benzene and toluene also might be present in the headspace (gas-phase).

About 7–17% of the total ^{14}C remained in the liquid medium. Since the control experiment with microorganisms showed complete degradation of benzene and toluene, these ^{14}C most likely would be $^{14}CO_2$ produced from biodegradation and dissolved as carbonic acid in the medium. Analysis of the residues in the liquid medium by either GC or MEKC did not reveal any identifiable metabolites. Several common intermediary metabolites identified for benzene and toluene degradation by other *Pseudomonas* species, including phenol, catechol, and benzoic acid, were tested on GC and MEKC but were not found in this study. It is thus concluded that the radioactivity remaining in the liquid culture media, except for biomass, was mainly due to the production of carbonic acid, as also evidenced by a slight pH decrease from 7 to 6.4 (pK_{a1} value for carbonic acid is 6.35) as well as due to the formation of the organic soluble microbial products, which all microorganisms release (Namkung & Rittmann 1985). It is thus clear that the coculture of these two *Pseudomonas* species previously acclimated in the fibrous-bed bioreactor was able to completely mineralize various concentrations of benzene or toluene as a sole carbon source, using NH_4NO_3 (500 mg l^{-1}) as a nitrogen source with nitrate also as an alternative electron acceptor, under limited oxygen conditions.

Conclusions

BTEX biodegradation has been studied extensively, both in the subsurface and in aboveground bioreactors for the pump-and-treat applications. Although aerobic biodegradation is relatively rapid, most traditional bioreactors result in partial stripping losses to the air stream due to aeration rather than biodegradation, causing another aspect of environmental problems (i.e., transferring pollutants from one phase to another). On the other hand, even though anaerobic conditions often exist in natural ecosystems, in leachate plumes emanating from contaminated sites, and in immobilized cell bioreactors after oxygen depletion by aerobic microorganisms, the anaerobic biodegradation of BTEX, in general, is considered relatively slow. In aerobic *in situ* bioremediation of BTEX, oxygen can be supplied

by air sparging, by using pure oxygen, hydrogen peroxide, ozone, or by soil venting or air flooding, and by colloidal dispersion of air (colloidal gas aphrons) in a surfactant matrix. However, depending upon the temperature of the groundwater, only 8–12 mg l^{-1} of dissolved oxygen can be achieved by air sparging, and incomplete transfer of oxygen into water may reduce this even further. On the other hand, concentrations of 40–50 mg l^{-1} of dissolved oxygen can be achieved with pure oxygen, but pure oxygen is expensive, may bubble out of solution before the microorganisms can use it, and may be an explosion hazard if handled improperly. In many cases, the major limitation on aerobic biodegradation in the subsurface is the low solubility of oxygen into water. Therefore, even though aerobic biorestoration has been shown to be effective for many fuel spills, success is often limited by the inability to provide sufficient oxygen to the contaminated intervals. This problem, however, can be overcome by using H_2O_2 as an oxygen source to provide greater quantities of oxygen or by adding nitrate as an alternative electron acceptor to allow efficient biodegradation under hypoxic conditions.

The coculture of *P. putida* and *P. fluorecens* was able to completely degrade all BTEX compounds under both aerobic and hypoxic conditions. Carbon mass balance using ^{14}C-labeled benzene and toluene confirmed that the degradation of BTEX by this coculture resulted in complete mineralization to CO_2 and H_2O with no detectable intermediary metabolites left in the culture media. However, benzene at 500 mg l^{-1}, *o*-xylene at 600 mg l^{-1}, and toluene at 1000 mg l^{-1} were toxic to the cells. Since the solubilities of B, T, and *o*-X are significantly higher than the concentration levels toxic to cells, it could be a problem to use these cells to treat high-concentration industrial waste streams. This toxicity problem can be partially overcome by using immobilized cells since cells can maintain their degradation abilities even at a concentration level much higher than those toxic to cell growth. Also, cells may be adapted to tolerate higher BTEX concentrations in an immobilized cell bioreactor such as a fibrous-bed bioreactor (Shim 1997; Shim & Yang 1999). The coculture thus would have very promising applications in treating BTEX-contaminated industrial wastewater and groundwater.

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