Journal of Microbiology and Biotechnology
Vol. 4, No. 1, 63-67, 1994

Cometabolism in the Biodegradation of Benzene, Toluene, and *p*-Xylene Mixture by Isolated *Pseudomonas fluorescence* BE103

LIM, HYE-KYUNG, JANG-YOUNG LEE AND HAK-SUNG KIM*

Department of Biotechnology, Korea Advanced Institute of Science and Technology, 373-1 Kusung-Dong, Yusung-Gu, Taejon, 305-701, Korea

A microorganism showing degradative activity towards benzene, toluene and *p*-xylene (BTX) was isolated from an activated sewage sludge and was tentatively identified as *Pseudomonas fluorescence* BE103. This strain was found to utilize benzene and toluene as growth substrates, but to degrade *p*-xylene in the obligate presence of a growth substrate. The metabolic product resulted from the cometabolism of *p*-xylene was identified as 3,6-dimethylpyrocatechol by LC/MS analysis, and the metabolic pathway was analyzed to be similar to the tod pathway. From the kinetic studies done regarding BTX biodegradation using *Pseudomonas fluorescence* BE103, it was revealed that the cometabolism of *p*-xylene is significantly affected by the ratio of growth substrate concentration to biomass concentration, and that the cometabolism of *p*-xylene initiates only when this ratio was about 0.03.

Aromatic solvents such as benzene, toluene, and xylenes (collectively known as BTX) are widely used as solvents and fuel components in many industries, and have been classified as priority pollutants by the U.S. Environmental Protection Agency (9, 14, 15). Misuse, accidental spillage, and improper disposal of these toxic solvents have caused an extensive contamination of ground water and soil.

Much research has been focused on the biodegradation of these compounds in a natural environment, but most of these studies dealt with only a single compound. In reality, environmental contamination by a single compound is very rare, and BTX are usually released in the form of a mixture, making treatment difficult. A few studies have been carried out regarding the substrate interactions between BTX, to understand the behavior of BTX compounds in a natural environment and their similarities or differences in their biodegradation patterns. A BTX compound can inhibit the degradation of another by exerting toxicity, diauxie, catabolic repression, competitive inhibition for enzymes or depletion of electron acceptors. On the other hand, the presence of a BTX compound can also stimulate degradation of another BTX compound by inducing the required catabolic enzymes, and cometabolism is known to give such results (1-3, 8, 12). Cometabolism is defined as a microbial process through which a non-growth substrate is partially transformed in the presence of a growth substrate. A significant portion of the recalcitrant compounds in the environment, such as tricholoroethylene (TCE) and halogenated aromatic compounds, was reported to be degraded by cometabolism (13).

From a practical stand-point, employing a microorganism capable of degrading a BTX mixture is advantageous because of easy control and the high stability of biodegradation processes. However, little attention has been paid to the degradation of BTX mixture. We have focused on a method to degrade the BTX mixture, and we isolated a microorganism which showed a catabolic activity towards benzene, toluene and *p*-xylene mixture. This microorganism, identified as *Pseudomonas fluorescence*, was found to completely degrade benzene and toluene as the sole sources of carbon and energy, while *p*-xylene was cometabolized in the presence of growth substrates. Kinetic studies regarding the biodegradation of BTX mixture by the isolated microorganism were performed in a bioreactor developed in our laboratory.

MATERIALS AND METHODS

Materials

Benzene, toluene, *p*-xylene, and 2,5-dimethylphenol

*Corresponding author
Key words: biodegradation, benzene, toluene, *p*-xylene, cometabolism

were from the Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade. Silicon tubing (0.157 cm ID, 0.318 cm OD) was obtained from Dow Corning (Midland, MI).

Microorganisms

The soil samples obtained from an activated sewage sludge were incubated in a basal salt medium containing benzene as a sole source of carbon for enrichment. Strain BE103 was isolated from the enriched microorganisms by measuring the degradation activity towards benzene, toluene, and *p*-xylene in serum vials capped tightly with Teflon-coated rubber stopper. *Pseudomonas putida* F1 was a generous gift from Prof. D.T.Gibson (University of Iowa, IO). The composition of basal salts medium was: 5.8 g/L KH_2PO_4, 4.5 g/L K_2HPO_4, 2.0 g/L $(NH_4)_2SO_4$, 0.34 g/L $MgCl_2 \cdot 6H_2O$, 0.02 g/L $CaCl_2$, 0.002 g/L $FeSO_4 \cdot 7H_2O$, 0.0016 g/L $MnCl_2 \cdot 4H_2O$.

Identification of Microorganism

The isolated microorganism was identified by investigating its physiological and morphological characteristics according to the Bergey's Manual (10). Analysis of the fatty acid composition in the cell membrane was also conducted using the method reported earlier (11).

Biodegradation of BTX

Biodegradation of BTX was performed both in flasks with screw-capped side arm and in the bioreactor. Because of the high volatility and low solubility of BTX, the bioreactor developed in our laboratory was employed for the quantatitive analysis of the biodegradation (5). The bioreactor was a baffled, impeller-agitated type (KLF 2000, Bioengineering, Switzerland). A predetermined length of silicon tubing was installed at the bottom of the bioreactor, and the liquid solvent was circulated within the tubing at the flow rate of 30 mL/min from a solvent reservoir using a diaphragm pump. In this system, the liquid solvent diffused out of the tube wall into the culture broth, where the liquid solvent was aerobically degraded by microorganisms. The rate of solvent transfer through a silicon tubing into the bioreactor and the degradation rate were calculated as described (4). The rate of solvent mixture transfer was controlled by changing the composition of the sovent mixture in the solvent reservoir. The working volume of the bioreactor was l liter. The temperature, agitation speed, and aeration rate were controlled at 32°C, 600 rpm, and 1 L/min, respectively. All experiments were performed in duplicate.

Identification of Metabolic Product

The metabolic product formed from the cometabolism of *p*-xylene was identified using a high performance liquid chromatograph (HPLC) (LC9A, Shimadzu, Kyoto, Japan) and a liquid chromatograph/mass spectrometer (LC/MS). HPLC was performed on a ODS column (CLC-ODS(M), 25 cm, Shimadzu) using the acetonitril-water mixture (75 : 25) as mobile phase. The eluent flow rate was 1 mL/min. LC/MS analysis was conducted on the ODS column (CLC-ODS (M), 25cm, Shimadzu) linked to a Mass spectrometer detector (HP5989, Hewlett Packard Co., Palo Alto, CA) equipped with a particle beam interface (HP59980A). Mass spectra were obtained at an ionizing voltage of 70 eV and an accelerating voltage of 4 KV.

Analysis

The concentrations of benzene, toluene, and *p*-xylene were determined using a gas chromatograph (HP 5890 Model, Hewlett-Packard Co., Palo Alto, CA.) equipped with a flame ionization detector. A stainless column (6 ft×1/8 in.) packed with Silar 10C (80~100 mesh) was used. The temperatures of injector and detector were 200°C and 250°C, respectively. The oven temperature was initially maintained at 110°C for 2 min, raised 10°C/min and finally maintained at 220°C for 2 min. The flow rate of nitrogen as carrier gas was 15 mL/min. When determining the concentration of solvent in the exit gas of the bioreactor, the exit gas was collected using a home-made gas collector equipped with a Teflon-rubber stopper, and 100 μ L of the collected gas was injected into the gas chromatograph with a microsyringe. The concentrations of benzene, toluene, and *p*-xylene in the culture broth were determined after filtering the culture broth through a polycarbonate filter (0.45 μm pore size, Millipore). The dry cell weight of the filtered bacterium was determined by weighing it after it had been dried at 105°C for 24 hours.

RESULTS AND DISCUSSION

Identification and Metabolism of the Isolated Bacterium

A bacterium showing degradative activity towards benzene, toluene, and *p*-xylene (BTX) was isolated as described in Materials and Methods. Typical profiles of BTX degradation and cell growth are shown in Fig. 1. The biomass concentration was observed to increase as benzene, toluene, and *p*-xylene were simultaneously consumed when BTX were used as the sole sources of carbon and energy in the flask culture. Physiological tests were performed as shown in Table 1, and according to the Bergey's Manual of Systematic Bacteriology, the genus of this strain was tentatively determined to be *Pseudomonas*. In order to determine the species of the strain, the compositions of whole fatty acids were analyzed and compared with those of the standard library (Fig. 2). As a result, this bacterium was named *Pseudomonas fluorescence* BE103. Although *Pseudomonas fluorescence* BE103 exhibited degradative activity towa-

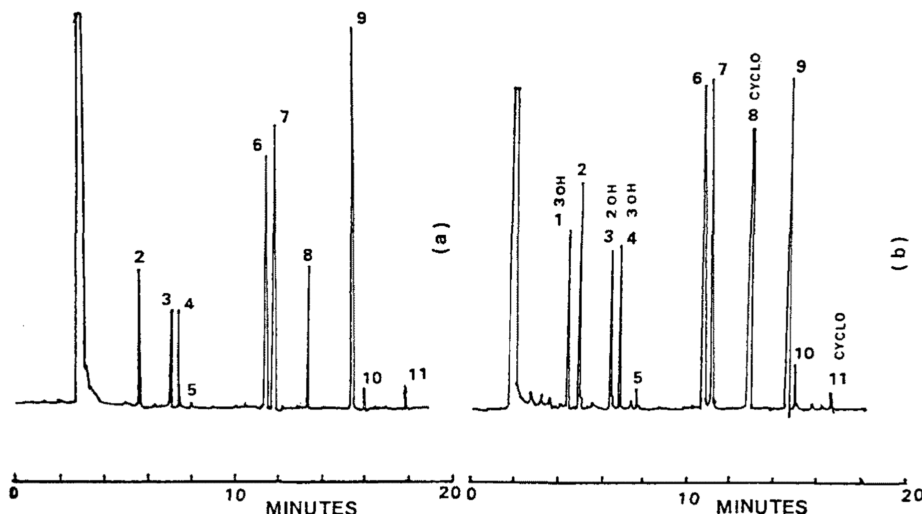

Fig. 1. Fatty acid prolfile of (a) *Pseudomonas* sp. BE103 (b) *Pseudomonas fluorescence.*

Table 1. Some physiological properties of the isolated strain

Gram	−
Oxidase	−
Voges-Proskauer	−
Indole	−
H_2S formation	−
ONPG	−
Urease	+
TDA	−
Denitrification	−
Gelatine Liquefaction	−
Lysine	−
Ornithine	−
Carbon source for Growth	
Glucose	+
Mannitol	−
Xylose	+
Malonate	+
Inositon	−
Sorbitol	−
Rhamnose	−
Sucrose	−
Lactose	−
Arabinose	+
Adonitol	−
Raffinose	−
Salicin	−
Arginine	−

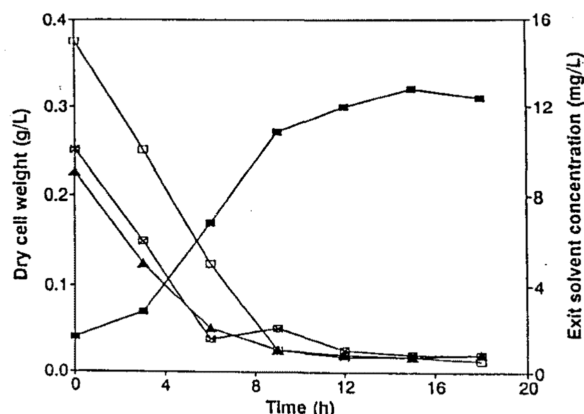

Fig. 2. Typical profile of BTX degradation and growth of the isolated microorganism.
(■) dry cell weight; (□) benzene; (▲) toluene ; (⊠) *p*-xylene.

rds benzene, toluene, and *p*-xylene, we had to investigate whether each solvent had been used by the isolated bacterium as a growth substrate. When benzene or toluene was used as the sole carbon and energy source, the compound was rapidly degraded with a significant increase in biomass concentration. However, when *p*-xylene was supplied as the sole carbon and energy source, no degradation of this compound was observed. After incubation of Pseudomonas fluorescence BE103 with *p*-xylene in the presence of toluene, we found that substantial amounts of a metabolic product had accumulated in the culture broth. This was detected at the retention time of 2.8 min during the HPLC analysis. The identity of the metabolic product was confirmed to be 3,6-dimethylpyrocatechol by LC /MS (Fig. 3). Gibson *et al* reported that *Pseudomonas* putida F1, which has the tod pathway, can utilize benzene and toluene as a growth substrate while *p*-xylene is only degraded by cometabolism in the presence of other growth substrates with an accumulation of 3,6-dimethylpyrocatechol as a metabolic intermediate (7). Analysis of metaobolic intermediate of *Pseudomonas*

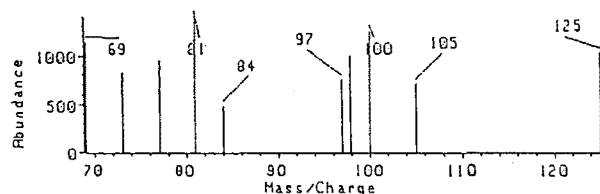

Fig. 3. EI mass spectrum of the metabolite from *p*-xylene.

putida BE103 produced from *p*-xylene and that of *Pseudomonas putida* F1 by HPLC confirmed that *Pseudomonas putida* BE103 cometabolizes *p*-xylene with formation of 3,6-dimethylpyrocatechol.

Cometabolism of *p*-Xylene

It is generally known that the rate and extent of the BTX mixture biodegradation are influenced by several factors such as pollutant concentration, active biomass concentration, availability of inorganic nutrients and electron acceptors, and microbial adaptation. Since *p*-xylene cometaoblism was revealed to depend on the presence of a growth substrate, it is thought that the rate of *p*-xylene cometabolism by *Pseudomonas fluorescence* BE103 may be closely related with the consumption rate of growth substrates (i.e. benzene or toluene). Thus, we investigated cometabolism of *p*-xylene by *Pseudomonas fluorescence* BE103 in the presence of benzene or toluene in the bioreactor. Fig 4a shows cometabolism of *p*-xylene in the presence of benzene when the transfer rates of benzene and *p*-xylene were 45 and 56.4 mg/L/h, respectively. Growth was observed without an apparent lag phase, and the exponential growth continued with the degradation of benzene. The concentration of *p*-xylene in the exit gas sharply decreased after a 3 hour lag and stayed at a constant level, which indicates that *p*-xylene was cometabolized at a constant rate as benzene was degraded for cell growth. 3,6-Dimethlypyrocatechol accumulated in the culture broth after the consumption of *p*-xylene was initiated. Since we could not succeed in purifying 3,6-dimethylpyrocatechol from the culture broth, peak intensity was used for the quantification of this compound. Tentatively, the degradation rates of benzene and *p*-xylene were determined to be 40 and 25.2 mg/L/h, respectively.

Fig.4b shows cometabolism of *p*-xylene in the presence of toluene by *Pseudomonas fluorescence* BE 103. The transfer rates of toluene and *p*-xylene were 100 and 120 mg/L/h, respectively. Cometabolism of *p*-xylene was initiated after a sufficient growth of microorganism was observed, and the rate of *p*-xylene cometabolism was maintained at constant level. At this point, the degradation rates of toluene and *p*-xylene were 96 and 85.8 mg/L/h, respectively.

We tried to investigate whether the cometabolism

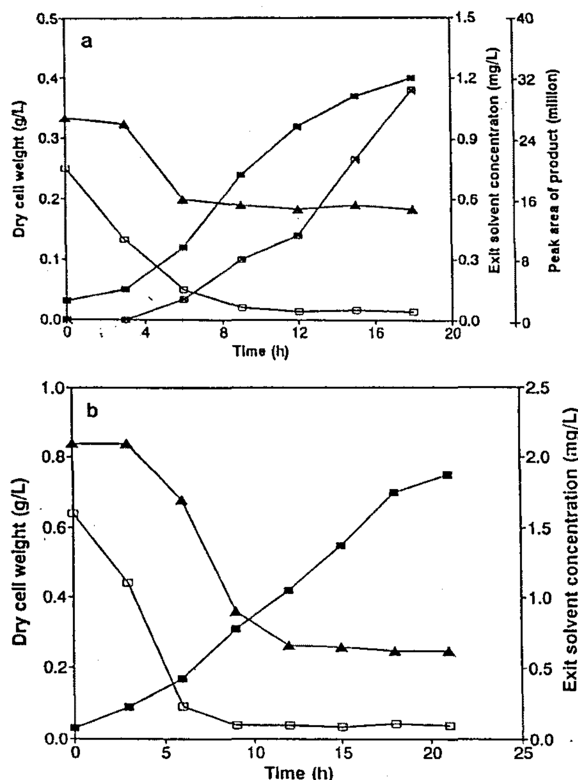

Fig. 4. Typical profile of *p*-xylene cometabolism in the presence of other growth substrates.
(a) in the presence of benzene: (■) dry cell weight; (□) benzene, (▲) *p*-xylene, (⊠) metabolic product from *p*-xylene. Transfer rates of benzene and p-xylene were fixed at 45 and 56.4 mg/L/h, respectively. (b) in the presence of toluene: (■) dry cell weight; (□) toluene; (▲) *p*-xylene. Transfer rates of toluene and *p*-xylene were fixed at 100 and 120 mg/L/h, repectively.

of *p*-xylene by *Pseudomonas fluorescence* BE103 can occur in the absence of growth substrate. The microorganism was cultivated in the bioreactor with a supply of toluene until the growth of microorganism became significant, and then by changing a solvent reservoir, *p*-xylene was supplied instead of toluene. However, the increase in the biomass and uptake of *p*-xylene were found to be negligible (data not shown). Thus, it is likely that *p*-xylene was not metabolized by *Pseudomonas fluorescence* BE103 when the growth substrate is absent. It has been reported that trichloroethylene is cometabolized by resting cells without a growth substrate, and this seem to be due to an endogenous metabolism of polyhydroxbutyrate reserved within the cells (6).

As in Fig. 4a and Fig. 4b, the consumption of *p*-xylene initiated after a significant amount of growth substrate was consumed, regardless of the growth substrate and the ratio of the transfer rates of growth substrate and *p*-xylene . If we assume that the degradation of the

Table 2. Effect of ratio of concentrations between growth substrate and biomass on the cometabolism of *p*-xylene at various transfer rates of growth substrate and *p*-xylene

Transfer rate (mg/L/h)	(Substrate/Biomass)[a]	The maximum rate of cometabolism of *p*-xylene (mg/L/h)
Ben: 45 Xyl: 56.4	0.0286	25.2
Tol: 100 Xyl: 120	0.0286	85.8
Tol: 114 Xyl : 30	0.0265	22.8
Ben: 115 Tol: 112 Xyl: 113	0.303	83.28

Ben=Benzene; Tol=Toluene; Xyl=*p*-Xylene.
[a] The ratio of growth substrate concentration to biomass conentration when cometabolism of *p*-xylene initiates.

growth substrate (benzene and toluene) and *p*-xylene are mediated through the same pathway, the effect of growth substrate on the cometabolism of *p*-xylene could be regarded as a competitive inhibition similar to the case of single enzyme kinetics. If this hypothesis is sound, higher concentration of growth substrate will inhibit the cometabolism of *p*-xylene, resulting in a lag period in the uptake of *p*-xylene as observed in Fig. 4a and Fig. 4b. However, as the growth substrate is utilized for growth, the concentration of growth substrate is lowered to a certain level, and then the uptake of *p*-xylene is initiated. In other words, the availability of growth substrate to microorganism may control the uptake of *p*-xylene, due to the higher affinity of the growth substrate to that of *p*-xylene. This availability of growth substrate to biomass could be expressed as the ratio of growth substrate concentration to biomass concentration, and the effect of this ratio on the cometabolism of *p*-xylene was investigated. *p*-Xylene was cometabolized at various transfer rates of growth substrate (benzene and/or toluene). As shown in Table 2, the uptake of *p*-xylene was found to occur when the ratio of growth substrate concentration to biomass concentration became about 0.03. This means that the cometabolism of *p*-xylene is initiated as the growth substrate begins to limit the microbial growth.

From the above results, it is likely that one of the most important parameters affecting the cometabolism of *p*-xylene is the ratio of growth substrate concentration to biomass concentration, which indicates the availability of growth substrate to a unit mass of microorganism.

REFERENCES

1. Alvarez, P.J.J. and Vogel, T.M. 1991. Substrate interactions of benzene, toluene, and *p*-xylene during microbial degradation by pure cultures and mixed culture aquifer slurries. *Appl. Environ. Microbiol.* **57**: 2981-2985.
2. Arvin, E., Jensen, B.K. and Gundersen, A.T. 1989. Substrate interactions during aerobic biodegradation of benzene. *Appl.Environ.Microbiol.* **55**: 3221-3225.
3. Bauer,J.E. and Capone, D.G. 1988. Effects of co-occuring aromatic hydrocarbons on degradation of individual polycyclic aromatic hydrocarbons in marine sediment slurries. *Appl. Environ. Microbiol.* **54**: 1649-1155.
4. Choi, Y.B., Lee, J.Y. and Kim, H.S. 1992. A novel bioreactor for the biodegradation of inhibitory aromatic solvents: Experimental Results and Mathematical Analysis. *Biotechnol. Bioeng.* **40**: 1403-1411.
5. Choi, Y.B., Lee, J.Y., Lim, H.K. and Kim, H.S. 1992. A novel method to supply the inhibitory aromatic compounds in aerobic biodegradation process. *Biotechnol. Tech.* **6**: 489-494.
6. Cohen, L.A. and McCarty, P.L. 1991. A cometabolic biotransformation model for halogenated aliphatic compounds exhibiting product toxicity. *Environ. Sci. Technol.* **25**: 1381-1387.
7. Gibson, D.T., Mahadevan,V. and Davey, J.F. 1974 Bacterial metabolism of para- and meta-xylene: Oxidation of the aromatic ring. *J. Bacteriol.* **119**: 930-936.
8. Goldsmith, J. and Balderson, R.K. 1988. Biodegradation and growth kinetics of enrichment isolates on benzene, toluene and xylene. *Water Sci. Technol.* **20**: 505-507.
9. Kobayashi, H., Rittmann, B.E. 1982. Microbial removal of hazardous organic compounds. *Environ. Sci. Technol.* **16**: 170-183.
10. Krieg, N.R. and Holt, J.C. 1984. Bergey's manual of systematic bacteriology **1**: 140.
11. Lindy, M. and Berger, T. 1985. Hewlett Packard GC Application Note 228-241.
12. Meyer, J.S., Marcus, M.D., and Bergman, H.L. 1984. Inhibitory interactions of aromatic organics during microbial degradation. *Environ. Toxicol. Chem.* **3**: 583-587.
13. Nelson, M.J., K., Montgomery, S.O., Mahaffey, W.R. and Pritchard, P.H. 1987. Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. *Appl. Environ. Microbiol.* **53**: 949-954.
14. Stensel, H.D., Reiber, S. 1983. Industrial wastewater treatment with a new biological fixed-film system. *Environ. Prog.* **2**: 110-114.
15. Truong, K.N., Blackbum, J.W. 1984. The stripping of organic chemicals in biological treatment processes. *Environ. Prog.* **2**: 143-152.

(Received September 29, 1993)