Characterization of BTX-degrading Bacteria and Identification of Substrate Interactions during Their Degradation

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From several industrial wastewaters, 14 bacterial strains which degrade benzene, toluene, o-xylene, m-xylene, or p-xylene (BTX) were obtained. These strains were characterized as to their species composition and the substrate range, kinetic parameters and the substrate interactions were investigated. Although BTX components have a similar chemical structure, isolated strains showed different substrate ranges and kinetic parameters. None of the strains could degrade all of BTX components and most of them showed an inhibition (Haldane) kinetics on BTX. BTX mixtures were removed under inhibitory substrate interactions with variation in the intensity of inhibition. For a complete degradation of BTX, a defined mixed culture containing three different types of pathways was constructed and all of the BTX components were simultaneously degraded with the total removal rate of 225.69 mg/g biomass/h. Judging from the results, the obtained mixed culture seems to be useful for the treatment of BTX-contaminated wastewater or groundwater as well as for the removal of BTX from the contaminated air stream.

Key words: Aromatic hydrocarbons, biodegradation, bioremediation, BTX

Aromatic hydrocarbons are ubiquitous in the environment. Indeed, next to glucosyl residues, the benzene ring is the most widely distributed unit of chemical structure in nature (8). Benzene, toluene, and the xylene isomers (hereafter referred to as BTX) are among the most hazardous chemicals and this classification is based on their substantial toxicities and on the carcinogenic potential of the benzene component (9). BTX hydrocarbons are substituents of gasolines with production figures in the order of millions of tons per year (21). These compounds are widely used as fuels and industrial solvents and are among the most frequently identified substances in polluted soils and groundwaters (16).

BTX-contaminated soils or aquifers may be restored *in situ* by bioremediation techniques. Biodegradation of BTX during bioremediation process involves the simultaneous metabolism of structurally related hydrocarbon substrates. The pathways of individual BTX components are well known (13, 22), but substrate interactions make treatment outcome less predictable for mixtures (1, 7, 20). Substrate interactions, such as competition, sparing or cometabolism are highly relevant to the success and to the kinetics of bioremediation process.

As an initial step for the application of BTX-degrading bacteria in bioremediation of BTX-contaminated soils or aquifers, BTX-degrading bacteria were isolated and their biodegradation kinetics and substrate interactions were investigated.

Materials and Methods

Enrichment and isolation of BTX degraders

The bacterial strains used in this study were isolated from wastewaters obtained from industrial areas such as Kuro, Yeochon, Puchon, and Dongduchon. One-milliliter portions of the wastewater were suspended in a mineral salts base (MSB) medium (24). As the only source of carbon and energy, a mixture of BTX was added, at an initial concentration of 25 mg/L each. Erlenmeyer flasks with a 9:1 airspace/liquid ratio were closed with Teflon-wrapped butyl rubber stoppers (Bellco Glass Inc., USA) and were incubated with rotary shaking (200 rpm) at 25'C. The flasks were aerated daily and BTX was added as needed. Enrichments that developed substantial turbidity were serially transferred until stable consortia developed. Members of the consortia were isolated by spreading onto MSB solidified with 2% Noble agar (Difco) and incubated in enclosures saturated with each component of

BTX. Isolates which showed high ability to metabolize each of BTX were selected for further study. Using fatty acid methyl ester (FAME) analysis (5), the isolates were identified.

Determination of biodegradation kinetics

The utilization of BTX and its individual components by the isolated strains was measured as follows. Cells grown on MSB liquid medium with BTX were harvested by centrifugation (8,000×g, 20 min) and the resulting cell pellet was resuspended in fresh MSB medium. Ten-milliliter aliquots of this suspension were placed into 160 ml serum bottles (Wheaton Glass Co., USA) closed with Teflon-faced gray butyl rubber septa (Wheaton) and aluminum seals. Initial cell concentrations for yield and kinetic determinations were in the range of 0.1 to 0.3 mg dry biomass/L and 0.6 to 2.2 g dry biomass/L, respectively.

Biodegradation of 16 combinations of benzene, toluene, *o*-xylene, *m*-xylene, and *p*-xylene were tested : (i) benzene alone; (ii) toluene alone; (iii) *o*-xylene alone; (iv) *m*-xylene alone; (v) *p*-xylene alone; (vi) benzene with toluene; (vii) benzene with *o*-xylene; (viii) benzene with *m*-xylene; (ix) benzene with *p*-xylene; (x) toluene with *o*-xylene; (xi) toluene with *m*-xylene; (xii) toluene with *p*-xylene; (xiii) *o*-xylene with *m*-xylene; (xiv) *o*-xylene with *p*-xylene; (xv) *o*-xylene with *m*-xylene and *p*-xylene; (xvi) benzene with toluene and xylene isomers. For the measurement of single solvent removal, seven different concentrations of each solvent were added to the serum bottles via a 10 μl Microliter syringe (Hamilton Co., USA) to attain a concentration range of 40 to 450 mg/L. Similarly, various concentrations of each solvent were added to the serum bottles to measure the removal of solvent mixtures. In case of BTX removal by defined mixed cultures, suspensions of each strain in MSB medium were prepared. The biomass concentrations in each of the microbial suspensions were adjusted to be identical by appropriate dilutions based on the protein measurement (6). Subsequently, identical volumes from each of the prepared suspensions were combined and 10 ml of the combined cell suspension was placed into serum bottles, and then 1 μl each of BTX were added. The serum bottles were then incubated on a rotary shaker (200 rpm) at 25°C. Utilization of BTX components was monitored by taking 100 μl gas samples from the headspace of the serum bottles using a gas-tight syringe (Hamilton Co.) and the content determined by gas chromatography (Hewlett-Packard Model 5890 Series II Plus, USA) equipped with a flame ionization detector and a 0.53 mm diameter × 30 m

length capillary column (Hewlett-Packard HP-1). Operating conditions were : injector temp., 150°C; oven temp., 100°C; detector temp., 250°C; and nitrogen gas flow rate, 10 ml/min. All experiments were performed in duplicate and abiotic losses of solvent vapors were subtracted by sampling an identical but uninoculated bottle.

Increase in microbial biomass was followed by withdrawing, also by syringe, 100 μl amount of suspension. Biomass was measured using a dye-binding method of Bradford (6). Coomassie blue reagent concentrate was purchased from Bio-Rad Co. and bovine serum albumin (Sigma) served as standard. A factor of 2 was used for converting protein content to total cell biomass (22).

From each run, the change in BTX concentration was monitored in order to determine the specific removal rate (r) of BTX component at the initial solvent concentration of the run. The specific removal rate is defined as the amount of BTX removed per unit amount of protein per hour. Upon the plotting of r values versus [S] (BTX concentrations), if the indication was that r increases with [S] and eventually reaches a constant value, the data were regressed to the Michaelis-Menten expression.

$$r = \frac{r_{max} \cdot [S]}{K_m + [S]}$$

When the r versus [S] plot indicated that r drops at high [S] values, the data were regressed to the Haldane (2) expression.

$$r = \frac{r_{max}}{1 + \dfrac{k_m}{[S]} + \dfrac{[S]}{k_{ES}}}$$

Results and Discussion

Isolation of BTX degraders

Stable consortia that degrade BTX were obtained after 6 to 8 weeks of enrichment. Among these, consortium enriched from the wastewater originated from the Kuro Industrial Complex showed the highest BTX degradation activity; therefore, individual strains were isolated from the consortium. A total of 14 strains were isolated and their growth characteristics on BTX and identification profiles are presented in Table 1. Ten of the isolates were identified as *Pseudomonas* and the others as *Aureobacterium, Acinetobacter* and *Micrococcus*. All the isolates which showed degradation of *m*-xylene and *p*-xylene could also degrade toluene, but not benzene. This is well known phenomenon of the *tol* BTX degradation pathway (25). Two strains, BTXO

Table 1. Identification and growth characteristics of the isolated BTX – degrading bacterial strains

Strains	Identification[a]	Growth characteristics[b]				
		Benzene	Toluene	o-Xylene	m-Xylene	p-Xylene
BTXO1	*Aureobacterium esteroaromaticum*	+	+	*[c]	*	*
BTXO2	*Pseudomonas pseudoalcaligenes*		+		+	+
BTXO3	*Acinetobacter johnsonii*	+	+	*	*	*
BTXO4	*Pseudomonas pseudoalcaligenes*		+	–	+	+
BTXO5	*Pseudomonas pseudoalcaligenes*		+		+	+
BTXO6	*Pseudomonas pseudoalcaligenes*		+		+	+
BTXO7	*Aureobacterium saperdae*	–	+		+	+
BTXO8	*Pseudomonas pseudoalcaligenes*		+	–	+	+
BTXO9	*Pseudomonas alcaligenes*			+		
BTXO10	*Pseudomonas stutzeri*	+	+	+		
BTXO11	*Pseudomonas stutzeri*	+	+	+	–	
BTXO12	*Pseudomonas alcaligenes*	+	+	+	#[d]	#
BTXO13	*Pseudomonas stutzeri*		+		+	+
BTXO14	*Pseudomonas kristinae*		+		+	+

[a] Identification based on the fatty acid methyl ester analysis. [b] Utilization of given substrates is represented by either + or – . [c] transformation in the presence of benzene and/or toluene. [d] transformation in the presence of o-xylene.

1 and BTXO3, which showed benzene and toluene degradation, could not degrade xylene isomers, but transformed them in the presence of benzene and/ or toluene as cosubstrates. This is a typical characteristics of the strains harboring the *tod* pathway and it is known that the transformation produces a dead-end product (12). Interestingly, there were 4 strains (BTXO9-BTXO12) which degraded o-xylene. Two of them were *Pseudomonas stutzeri* and the other two were *Pseudomonas alcaligenes*. So far, there have been only a few studies on bacterial degradation of o-xylene due to the closeness of the two methyl groups. Baggi *et al.* (3) isolated a strain of *P. stutzeri* which utilized o-xylene and toluene as growth substrates, and Fredrickson *et al.* (10, 11) reported *Sphingomonas* sp. strain F199 which degraded all the isomers of xylene. BTXO12 which degraded benzene, toluene and o-xylene, and also transformed m- and p-xylene in the presence of o-xylene (Table 1) seems to be a different strain from previously isolated o-xylene degraders. Further study on the o-xylene degradation pathway of this microorganism is in progress.

Utilization of individual BTX components

As shown in Table 1, individual bacterial strains had a limited substrate range and were unable to degrade all the BTX components. Benzene is known to be degraded through a direct ring attack, and methylbenzenes such as toluene and xylenes have been shown to be oxidized by either ring attack or methyl group hydroxylation (4). However, m- and p-xylenes are known to be completely degraded by certain *Pseudomonas* strains containing the TOL plasmid by initial oxidation of one of the

two methyl groups (23). Therefore, there should be several types of bacterial strains, one which oxidizes benzene or toluene through initial ring attack and another which contains the *tol* pathway for complete biodegradation of m-, p-xylene, along with a third pathway for o-xylene degradation. Lee *et al.* (15) constructed a hybrid strain which had the *tod* and the *tol* pathways for degradation of BTX using a single bacterial strain. However, the hybrid strain was unable to degrade o-xylene and it is doubtful to construct a hybrid strain possessing the third pathway for degradation of o-xylene. Furthermore, there are still some controversies regarding the release of genetically engineered microorganisms into the environment. Using various strains responsible for each of the BTX degradation pathway could be another way of approaching this problem. For that purpose, it is necessary to evaluate the substrate interactions of BTX and their degradation kinetics.

Strains BTXO3 and BTXO12 showed the highest degradation activity for benzene and o-xylene, respectively, and the degradation patterns are shown in Fig. 1. There were no appreciable lag period in both cases, but it took less time (0.5 h) for the complete removal of benzene than o-xylene. For toluene, m-xylene and p-xylene degradation, BTXO2, BTXO5 and BTXO13 strains showed similar levels of activities. Subsequently these three strains were chosen for kinetic study of toluene, m-xylene and p-xylene degradation. Values of the specific removal rate (r, the amount of BTX removed per g protein per h) obtained from the strain BTXO13 were plotted versus the corresponding toluene concentration values as shown
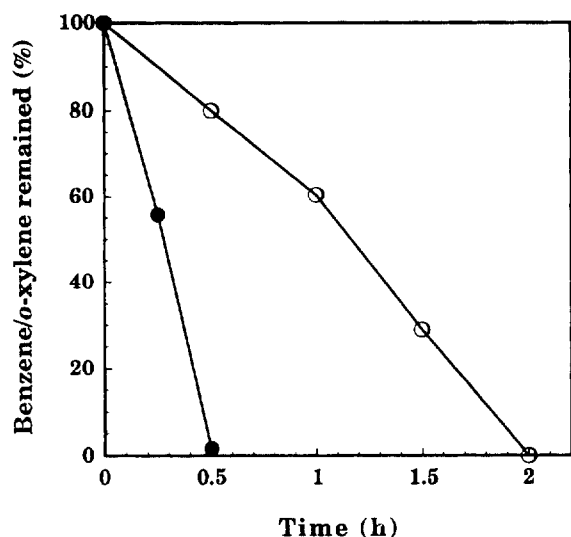
Fig. 1. Removal of benzene by the strain BTXO3 (closed circles), and o-xylene by the strain BTXO12 (open circles). Initial concentrations of both substrates and biomass were 132 mg/L and 1.2 g/L, respectively. Remaining solvents were analyzed by headspace analysis using a gas chromatograph. An identical but uninoculated sample showed no loss of solvents during the incubation period.

in Fig. 2. Toluene utilization pattern by this strain showed no inhibitory effect up to 4.2 mM suggesting a Michaelis-Menten kinetics and the data were regressed to the Lineweaver-Burk equation (inset of Fig. 2). From this, the values of K_m, r_{max} and the yield coefficient (Y) were estimated as 0.62,

147.1, and 0.44, respectively. However, utilization of m-xylene by BTXO13 indicated a drop in r at high concentration of m-xylene ($\geq$ 2.5 mM), and the data were regressed to the Haldane (or Andrews) kinetic model (2). BTX utilization kinetics by other strains were investigated in the similar way, and the calculated growth yield coefficients (Y) and kinetic parameters of each strain for BTX components are summarized in Table 2. Growth yields on BTX were in the range of 0.42 to 0.59, and these were similar to the yields on BTX obtained in other studies (7, 20). For benzene degradation, substrate affinity (inversely relates to K_m value) of BTXO3 was two times higher than that of BTXO12, and also BTXO3 showed almost five times higher r_{max} than BTXO12. These results indicate that BTXO3 is much better in benzene degradation than BTXO12. K_m values obtained from toluene degradation were found to be in the range of 0.34 to 0.62 mM, and again BTXO3 was found to have the highest value of r_{max}. For strains degrading m- and p-xylene (BTXO2, BTXO5, BTXO13), BTXO5 showed the lowest K_m values for both m- and p-xylene, and other strains did not show any significant difference in the values of K_m. BTXO2 and BTXO5 showed r_{max} of around 300 h^{-1}, and BTXO13 showed lower r_{max} than the other two strains.

Utilization of BTX mixtures

Table 2. Kinetic parameters and growth yield coefficients on BTX obtained from isolated strains

Substrates	Strain	BTXO2	BTXO3	BTXO5	BTXO12	BTXO13
Benzene	K_m[a]	[d]	0.26(H[e])		0.53(M[f])	--
	r_{max}[b]	--	526.3	--	109.9	--
	Y[c]	--	0.44	--	0.49	--
Toluene	K_m	0.39(H)	0.36(H)	0.34(H)	0.44(H)	0.62(M)
	r_{max}	135.1	714.3	131.6	144.9	147.1
	Y	0.59	0.54	0.51	0.42	0.44
o-Xylene	K_m				1.01(H)	
	r_{max}	--		--	149.3	--
	Y				0.49	
m-Xylene	K_m	0.50(H)		0.17(H)		0.38(H)
	r_{max}	384.6	--	277.8	--	153.8
	Y	0.56		0.48	--	0.51
p-Xylene	K_m	0.50(H)		0.24(H)		0.50(H)
	r_{max}	357.1	--	303.0		185.2
	Y	0.48		0.46		0.53

[a] Michaelis-Menten constant (mM).
[b] Maximum specific removal rate (h^{-1}).
[c] Growth yield coefficient.
[d] No utilization observed.
[e] Haldane kinetics.
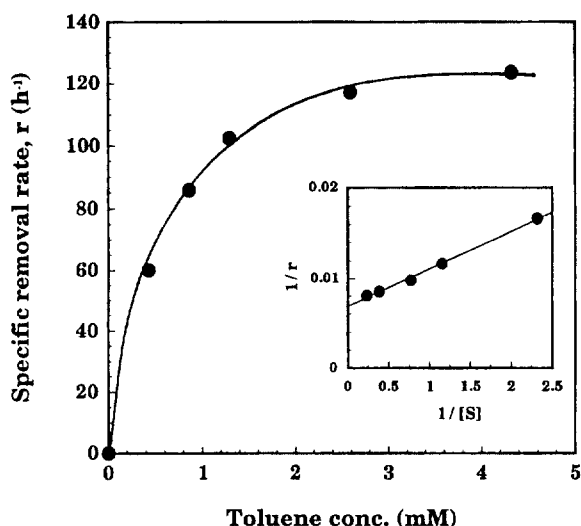[f] Michaelis-Menten kinetics.

Fig. 2. Kinetic parameters of the strain BTXO13 on toluene. Toluene utilization followed Michaelis-Menten kinetics and K_m of 0.62 mM and a maximum specific removal rate of 147.1 h^{-1} were estimated from the Lineweaver-Burk plot (inset). The reaction was performed at 30°C with initial biomass of 1.63 g/L.

The removal of toluene by BTXO13 either in the absence or in the presence of m- and p-xylene is shown in Fig. 3a. As the concentration of xylenes increases from 1:0 to 1:3 (toluene : xylenes), toluene removal was decreased proportionally. The removal of m- and p-xylene by BTXO13 in the presence of toluene was also inhibited (Fig. 3b), but the intensity of inhibition turned out to be much less severe than that of toluene degradation by xylenes. Experiments using BTXO2 and BTXO5 gave very similar results (data not shown). The observed phenomenon in this study was not contradictory to the previous report on the degradation of toluene and xylenes by $P.$ $putida$ strain harboring the tol pathway (17). Degradation of benzene or toluene by BTXO3 was also inhibited by the presence of other BTX components (data not shown). Comparable data were presented in other studies in which strains having the tod pathway were used for degradation of benzene and toluene (7, 20). Compared to other interactions among BTX, o-xylene degradation by BTXO12 was severely inhibited in the presence of toluene or benzene. The extent of inhibition was found to be almost 90% when the competing substrates were added at a ratio of 1:3 (o-xylene : benzene or toluene).

Substrate interactions during BTX degradation by the isolates are summarized in terms of specific removal rates in the presence or absence of other BTX components (Table 3). None of the isolated strains could degrade all of BTX components; therefore, a defined mixed cultures of the isolates were needed for complete degradation of BTX. For this purpose, we used three defined mixed cultures, BTXO2-BTXO3-BTXO12, BTXO3-BTXO5-BTXO12, and BTXO3-BTXO12-BTXO13. Fig. 4 shows degradation of BTX by the mixed culture, BTXO2-BTXO3-BTXO12. In this case, toluene, m- and p-xylenes and benzene were degraded simultaneously without lag phases, except o-xylene which started to be degraded only after most of other compounds were degraded. When compared to other mixed cul-
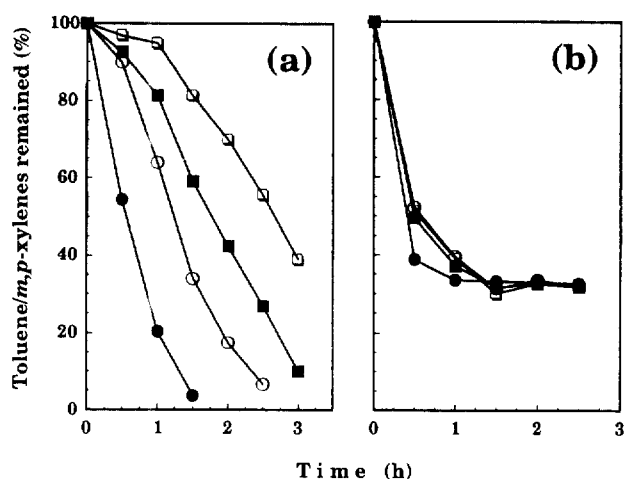

Fig. 3. Substrate interactions between toluene and m-, p-xylenes during their degradation by the strain BTXO13. (a) Removal of toluene with varying amounts of xylenes. The initial concentration of toluene was fixed at 86.5 mg/L, and the ratios between toluene : xylenes were varied from 1 : 0 (●), 1 : 1 (○), 1 : 2 (■), to 1 : 3 (□). (b) Removal of m-, p-xylenes with varying amounts of toluene. The combined initial concentration of m-, p-xylenes was fixed at 86.5 mg/L, and the ratios between xylenes : toluene were varied from 1 : 0 (●), 1 : 1 (○), 1 : 2 (■), to 1 : 3 (□).

ture combinations, BTXO2-BTXO3-BTXO12 combination was found to be the best mixed culture for degradation of BTX. The removal rates of benzene, toluene, m-, p-xylene, and o-xylene by BTXO2-BTXO3-BTXO12 were 47.75, 98.35, 67.06, and 12. 53 mg/g biomass/h, respectively, and the total removal rate was 225.69 mg/g/h. The obtained specific removal rate was higher than that reported by Lee et $al.$ (15) using a hybrid strain of $P.$ $putida$ which had the tod and the tol pathway for BTX degradation. Furthermore, the mixed culture of BTXO2-BTXO3-BTXO12 was able to degrade o-xylene which was not degraded by the hybrid strain. These results suggest that combining several strains which are responsible for the removal of each BTX component is more useful for a complete

Table 3. Specific removal rates (h^{-1}) for BTX compound in the presence or absence of competing substrates[a]

Compounds[a]	BTXO3	Compounds[a]	BTXO2	BTXO5	BTXO13	Compounds[a]	BTXO12
B[b] only	345.2 (0)[c]	T only	96.9 (0)[c]	197.4 (0)[c]	84.4 (0.5)[c]	o-X[d] only	46.6 (0)[c]
B + T[e](1:1)	208.8 (0)	T + mp-X(1:1)	73.5 (0.5)	147.3 (1.5)	51.2 (0.5)	o-X + B(1:1)	19.9 (0)
B + T(1:2)	186.1 (0.5)	T + mp-X(1:2)	56.4 (0.5)	117.6 (1.5)	37.9 (1)	o-X + B(1:2)	7.8 (0)
B + T(1:3)	175.8 (1)	T + mp-X(1:3)	49.5 (0.5)	109.5 (1.5)	29.7 (1)	o-X + B(1:3)	5.2 (0)
T only[d]	425.3 (0)	mp-X only[e]	124.7 (0)	174.4 (0)	130.8 (0)	o-X only[a]	46.6 (0)
T + B(1:1)	350.8 (0)	mp-X + T(1:1)	139.2 (0)	150.6 (0)	102.2 (0)	o-X + T(1:1)	27.5 (0)
T + B(1:2)	292.8 (0)	mp-X + T(1:2)	121.7 (0)	148.9 (0.5)	107.9 (0)	o-X + T(1:2)	16.1 (0)
T + B(1:3)	262.7 (0)	mp-X + T(1:3)	108.9 (0)	125.2 (0.5)	104.3 (0)	o-X + T(1:3)	6.4 (0)

[a] Initial concentration of each compound was 86.5 mg/L. [b] B = Benzene.
[c] Numbers in parenthes are lag period (h). [d] X = Xylene. [e] T = Toluene.
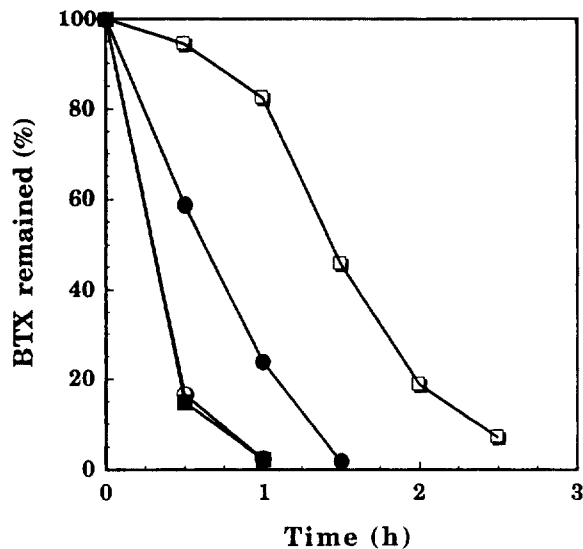
Fig. 4. Simultaneous removal of benzene (●), toluene (○), *m*-, *p*-xylenes (■), and o-xylene (□) by the constructed mixed culture consisting of the BTXO2-BTXO3-BTXO12 strains. Each solvent was added at the concentration of 86.5 mg/L, and the combined initial biomass was 1.22 g/L.

degradation of mixed substrates when substrate interactions of BTX are evaluated. The use of two bacterial strains with different metabolic pathways was reported in a limited number of studies. For the removal of a mixture of acetone and naphthalene by a biotrickling filter, Kirchner *et al.* (14) used a mixture of two strains, an *Aureobacterium* sp. and a *Rhodococcus* sp. In this case, naphthalene was removed only by the *Aureobacterium* sp. and acetone only by *Rhodococcus* sp. Both bacterial strains were immobilized on the carrier material and successfully removed acetone and naphthalene simultaneously. Oh and Bartha (18) used a mixture of two bacterial consortia, one of which was responsible for the removal of chlorobenzene and the other for the removal of o-dichlorobenzene. The authors achieved a successful removal of chlorobenzene/o-dichlorobenzene mixture using a biotrickling filter during biofiltration process. Recently, a defined mixed culture of two bacterial strains was developed, one of which uses the *tod* pathway and the other which uses the *tol* pathway for BTX degradation, and it was shown that the mixed culture successfully degraded benzene, toluene, and *p*-xylene mixture at the same time (19).

In this study, we obtained stable mixed cultures containing three different types of pathways for BTX degradation. From this, the degradation of benzene, toluene, and all of xylene isomers was possible. Judging from our results, the obtained mixed cultures might be useful for the treatment of BTX-

contaminated wastewater or groundwater as well as for the removal of BTX from the contaminated air stream. For these purposes, we are currently developing a fluidized bed reactor and/or biofiltration system using the mixed culture developed in this study.

Acknowledgments

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