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Substrate Utilization Patterns During BTEX Biodegradation by an *o*-Xylene-Degrading Bacterium *Ralstonia* sp. PHS1

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Abstract The biodegradation of BTEX components (benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, and *p*-xylene) individually and in mixtures was investigated using the *o*-xylene-degrading thermo-tolerant bacterium *Ralstonia* sp. strain PHS1, which utilizes benzene, toluene, ethylbenzene, or *o*-xylene as its sole carbon source. The results showed that as a single substrate for growth, benzene was superior to both toluene and ethylbenzene. While growth inhibition was severe at higher *o*-xylene concentrations, no inhibition was observed (up to 100 mg l^{-1}) with ethylbenzene. In mixtures of BTEX compounds, the PHS1 culture was shown to degrade all six BTEX components and the degradation rates were in the order of benzene, toluene, *o*-xylene, ethylbenzene, and *m*- and *p*-xylene. *m*-Xylene and *p*-xylene were found to be co-metabolized by this microorganism in the presence of the growth-supporting BTEX compounds. In binary mixtures containing the growth substrates (benzene, toluene, ethylbenzene, and *o*-xylene), PHS1 degraded each BTEX compound faster when it was alone than when it was a component of a BTEX mixture, although the degree of inhibition varied according to the substrates in the mixtures. *p*-Xylene was shown to be the most potent inhibitor of BTEX biodegradation in binary mixtures. On the other hand, the degradation rates of the non-growth substrates (*m*-xylene and *p*-xylene) were significantly enhanced by the addition of growth substrates. The substrate utilization patterns between PHS1 and other microorganisms were also examined.

Key words: BTEX, *o*-xylene, biodegradation, *Ralstonia* sp., substrate interactions

BTEX compounds (benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, and *p*-xylene) are important pollutants due to their toxic properties and widespread occurrence. They are major aromatic components in many petroleum products, and are often found in groundwater as a result of leaks from underground storage tanks, pipelines, waste disposal practices, inadvertent spills, and leaching from landfills. Compared to other fuel components, these compounds are relatively water soluble, which enables them to migrate to the subsurface and contaminate drinking water supplies. Moreover, since BTEX compounds typically exist as a complex in contaminated sites, and the biodegradation of one component can be inhibited by the other compounds in the mixture [12, 35], substrate interactions are important for understanding the behavior of BTEX compounds in the environment and the environmental applications. However, little is known regarding the biodegradation of mixtures of all six BTEX components and, particularly, the effect of other BTEX components on *o*-xylene biodegradation.

Most of the prior BTEX biodegradation studies used either a bacterial consortium from sewage sludge and indigenous soil or from groundwater microorganisms [17, 31, 40]. However, the results from these studies are contradictory [38]. Several pure cultures have also been investigated, but they are usually unable to degrade all BTEX compounds simultaneously and efficiently [6, 10, 14, 23]. Moreover, most previous substrate interactions studies have focused on only three BTEX components, i.e. benzene, toluene, and *p*-xylene [1, 3, 9, 33]. This may in part be attributed to the abundance of microorganisms that can degrade *p*-xylene, when compared to *o*- and *m*-xylene, in soils. Recently, Deeb and Alvarez-Cohen [16] investigated the substrate interactions of all six BTEX components (benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, and *p*-xylene) individually and in mixtures, using two mixed consortia and one pure culture derived from a gasoline-contaminated aquifer, and reported some interesting results. However, the microorganisms used in their study were unable to utilize the xylenes as growth substrates, and a significant reduction in the cell density was observed after repeated additions of *o*-xylene and *m*-xylene.

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Although a large number of bacteria that are able to utilize *m*- and *p*-xylene are known, only a few microorganisms have been reported to grow on *o*-xylene: a *Pseudomonas stutzeri* [5] and a *Ralstonia* sp. PHS1 [29] among the Gram-negative microorganisms, and a *Corynebacterim* C125 [37] and three *Rhodococcus* bacteria [7, 19, 24] among the Gram-positive bacteria. This suggests that the presence of a methyl group in the *ortho* position makes compound degradation difficult in bacteria. In some cases, only the partial oxidation of *o*-xylene concomitant with the metabolism of hydrocarbons has been observed [18, 34]. Since *o*-xylene is the most intractable of the BTEX compounds, its biodegradation in relation to other aromatic substances is an important issue. Nevertheless, there is a paucity of reports on the biodegradation patterns and substrate interactions of BTEX compounds in a pure culture of *o*-xylene-degrading microorganisms. A thermotolerant bacterium *Ralstonia* sp. strain PHS1 (PHS1), which is able to utilize benzene, toluene, ethylbenzene, or *o*-xylene as the sole carbon source, has been previously isolated [29]. The objectives of this study were to investigate the biodegradation of BTEX compounds by this novel *o*-xylene-degrading microorganism and to identify both the negative and positive substrate interactions in BTEX degradations.

MATERIALS AND METHODS

Organism and Cultivation Conditions

A BTEX-degrading bacterium, *Ralstonia* sp. strain PHS1, was isolated from a hot spring in Pohang, Korea. For all experiments, a mineral salts medium (MSM) was used as the carbon-free medium [29] with the pH of the medium adjusted to 7.2 with NaOH. The inocula were placed in 160-ml serum bottles with a 9:1 airspace/liquid ratio containing the carbon sources. The bottles were sealed with a Teflon-coated rubber stopper (Bellco Glass, Vineland, NJ, U.S.A.) and aluminum crimps to minimize water and solvent evaporation. The bottles were incubated in a rotary shaker (42°C, 150 rpm).

The aqueous concentrations of the aromatic compounds in the two-phase systems were calculated using the following equation:

$$M=S_L V_L+S_G V_G=S_L\left[1+\left(\frac{H}{RT}\right)\left(\frac{V_G}{V_L}\right)\right],$$

where M is the total mass of the BTEX substrates added, S_G and S_L are the substrate concentrations in the gas phase and in the liquid phase, respectively, H is the Henry's law constant, R is the gas constant, T is the temperature, and V_G and V_L are the volumes of the gas and liquid phases, respectively. The Henry's law constants, which were obtained from Mackay and Shiu [30], were corrected for an incubation temperature of 42°C using a temperature regression equation reported by Ashworth *et al.* [4].

Analytical Methods

The cell concentrations were measured as the optical density at 660 nm (OD_{660}) with a spectrophotometer and were correlated to a biomass concentration. The OD-mass correlation was linear over the cell concentration with 1.0 OD_{660}=485 mg/l. The BTEX concentrations were measured by taking 150-µl samples of the culture bottle headspace using a gas-tight syringe (Hamilton Co., Reno, NV, U.S.A.) and determining the solvent vapor content with a gas chromatograph (Model 680 D Young-In, Korea) equipped with a capillary HP-1 column (30-m length, 0.2-mm inner diameter, 0.33-µm film thickness) and a flame ionization detector. The operating conditions were 230°C at the injector, 80°C in the oven, and 250°C at the detector. The carrier gas was N_2. Typical GC peak elution times for the individual BTEX compounds in minutes were benzene, 2.5; toluene, 3.4; ethylbenzene, 4.7; *m*-/*p*-xylene (unresolved), 4.9; *o*-xylene, 5.3. The BTEX concentrations in the control bottles (without microorganism) had decreased by 5-15% at the end of each batch study.

Preparation of Washed-Cell Suspension

Strain *PHS1* was grown overnight on 2 g of L-glutamate per liter. The cells were harvested by centrifugation at 6,000 rpm for 10 min at 4°C, and washed twice in 0.1 M potassium phosphate buffer (pH 7.0). The cell pellet was concentrated in MSM so the OD_{660} was equivalent to 5.0 for all experiments.

Culture Growth on Individual BTEX

Experiments examining the growth on the individual BTEX components were conducted in 160-ml serum bottles with various initial substrate concentrations and 10% (v/v) of the inoculate grown on the same test compounds. For each batch study, the individual growth substrates were injected directly into the bottle from the stock solution to give a desired substrate concentration. Liquid samples were removed from the bottles for the OD_{660} measurements and then returned to prevent a change of medium volume.

Substrate Interactions in Mixtures

Prior to inoculation, the required concentrations of the compounds tested were added to the 160-ml serum bottles, which were then sealed with Teflon-coated rubber stoppers. The bottles were then incubated under experimental conditions for at least 3 h to equilibrate the hydrocarbons between the gas and liquid phases. The incubations were initiated by adding the concentrated, washed-cell suspension. The cells were injected into the sealed bottles through the stoppers to give an initial OD_{660} of 0.5. Gas samples (150 µl) were withdrawn from the sealed bottles throughout the

experiment to monitor the concentrations of the aromatics remaining in the gas phase. This was considered to be a good method of measuring the concentrations since degradation in the liquid phase forces the equilibrium to shift from the gas phase into the liquid phase. The cell concentrations were large enough, so that they did not undergo substantial increases.

RESULTS AND DISCUSSION

Cell Growth on Individual BTEX

The effects of the individual BTEX compounds on growth were examined by calculating the specific growth rate (μ) at various substrate concentrations up to 120 mg/l. For a given substrate concentration in the liquid-phase, m was estimated by plotting the OD_{660} versus time on a semi-logarithmic scale. The best-fit line was determined by linear regression.

The four aromatics are each alone able to act as the sole carbon and energy sources for the strain PHS1 (Fig. 1). The highest growth rate was observed for benzene, and a small degree of substrate inhibition in benzene and toluene biodegradation was observed in the concentration ranges tested. However, in the case of *o*-xylene, the growth rates were markedly reduced when the *o*-xylene concentration exceeded 10 mg/l. Cell growth was almost ceased at 90 mg/l, showing a typical substrate-inhibition pattern. Strong substrate inhibition observed for *o*-xylene is probably due to the toxicity of *o*-xylene [13, 27]. On the other hand, cell growth on ethylbenzene increased with increasing concentration and reached a maximum without exhibiting substrate inhibition up to 100 mg/l. In addition, the apparent half-saturation coefficient (K_s) for ethylbenzene was much higher than that of either benzene or toluene. This appears to be the reason for its lower degradation rate in BTEX mixtures, as shown below.

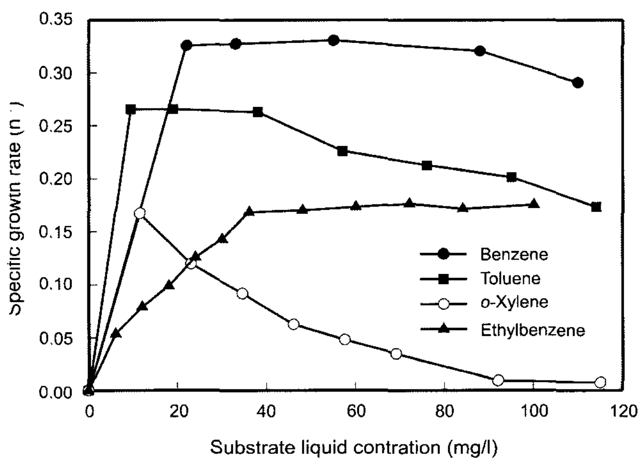

Fig. 1. Effects of the initial individual BTEX concentrations on the specific growth rate of PHS1.
(●) benzene, (■) toluene, (▲) ethylbenzene, (○) *o*-xylene.

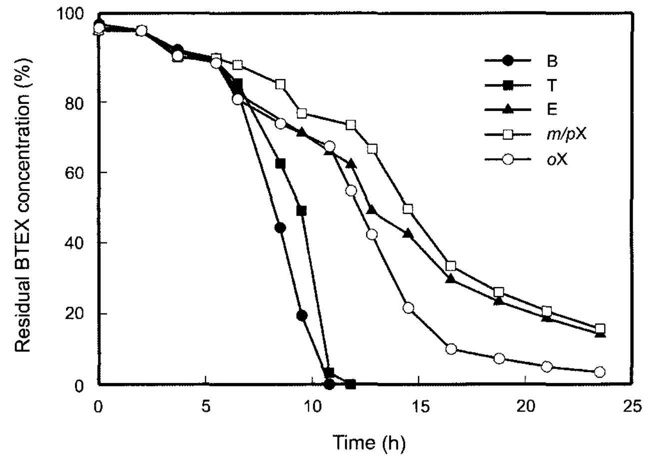

Fig. 2. Time courses of BTEX mixture degradation by PHS1.
(●) benzene, (■) toluene, (○) *o*-xylene, (▲) ethylbenzene, (□) *m*- and *p*-xylene. Initial substrate concentration: 10 mg/l each of BTEX compounds; initial cell density: 240 mg/l.

Degradation of BTEX Compounds in Mixtures

The *Ralstonia* sp. strain PHS1 was isolated from the hot spring sample, because of its ability to grow in MSM containing the BTEX compounds at 50°C [29]. PHS1 was able to grow on benzene, toluene, and *o*-xylene as the sole carbon source, but not on *m*- and *p*-xylene. Therefore, PHS1 was further examined to determine if it could grow in comprehensive mixtures of BTEX compounds. As shown in Fig. 2, all six BTEX compounds were degraded when the cells were grown in the BTEX compound mixture, suggesting that *m*- and *p*-xylene are co-metabolized in the presence of other growth-supporting BTEX compounds. Both benzene and toluene degraded rapidly at similar rates, while *o*-xylene and ethylbenzene degradations delayed until most of the benzene and toluene had been degraded. This suggests that PHS1 preferentially degrades benzene and toluene. The degradation order of the BTEX mixture was benzene>toluene>*o*-xylene>ethylbenzene>*m*- and *p*-xylene.

Substrate Interactions in Binary Mixtures of Growth Substrates

The effects of one BTEX compound on the degradation of other BTEX compounds were evaluated by comparing the degradation rate of the substrate alone with that of binary mixtures. As can be seen in Fig. 3, negative substrate interactions were observed during the degradation of the growth substrates (benzene, toluene, ethylbenzene, and *o*-xylene). In binary mixtures of these compounds, the individual BTEX degradation rate was reduced in the dual substrates, compared with its degradation rate as a single substrate. For example, ethylbenzene inhibited benzene,

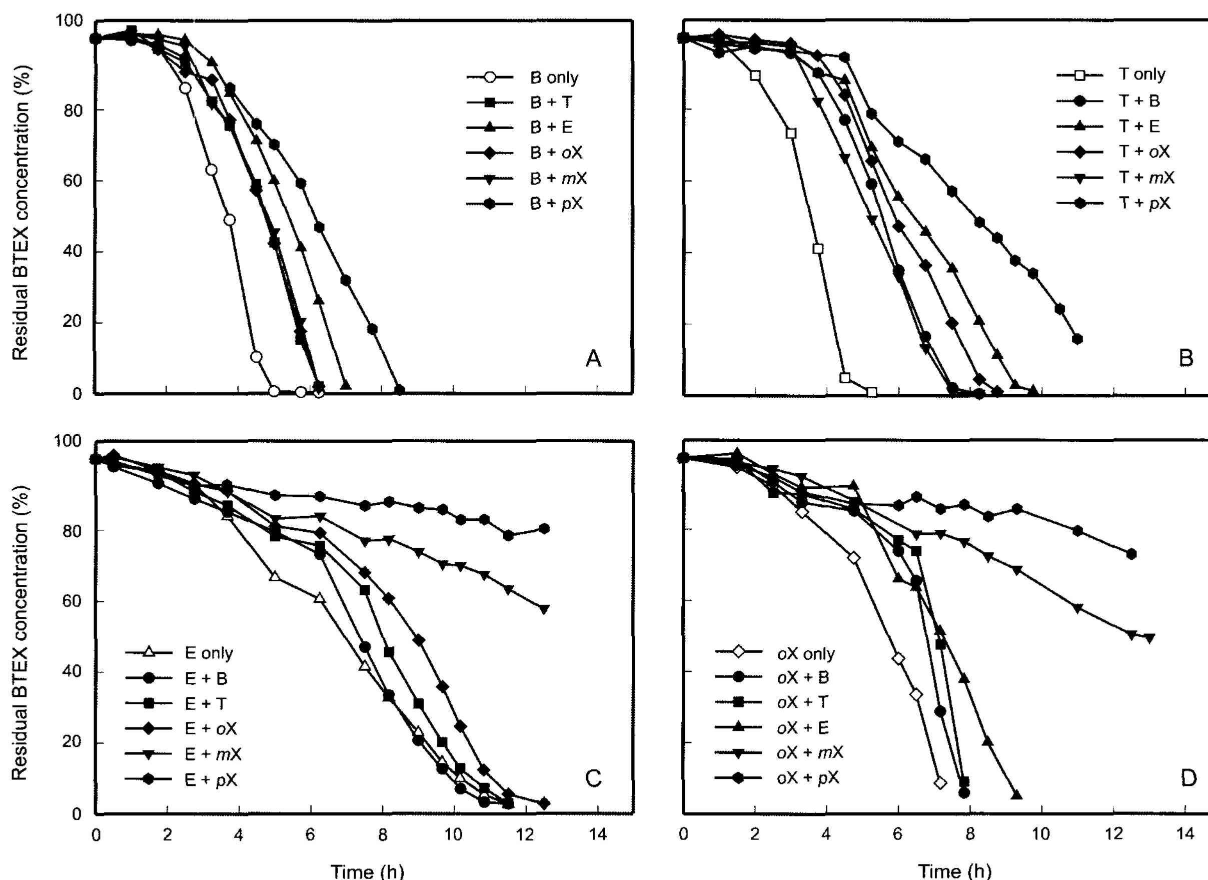

Fig. 3. Time courses of individual BTEX degradation in binary mixtures.
In the case of benzene and toluene mixtures, the initial substrate concentration was 30 mg/l for each BTEX compound, and in the case of *o*-xylene and ethylbenzene mixtures, the initial substrate concentration of each BTEX compound was 20 mg/l. Residual substrate concentration in the reaction mixture was determined for benzene (A), toluene (B), ethylbenzene (C), and *o*-xylene (D) and expressed as the percentage of the initial substrate concentration.

toluene, and *o*-xylene degradations. However, the effect of non-growth-supporting substrates (*m*- and *p*-xylene) on BTEX biodegradation was rather unusual. *p*-Xylene appeared to be the most potent inhibitor of BTEX degradation, and the degradation rates of all binary mixtures of BTEX compounds were retarded significantly by its presence. On the other hand, the presence of *m*-xylene had little inhibitory effect on the degradation of mixtures with either benzene or toluene, whereas the presence of *m*-xylene with either ethylbenzene or *o*-xylene strongly inhibited their degradation. When compared to *m*-xylene and *p*-xylene, *o*-xylene had little inhibitory effect on benzene, toluene, and ethylbenzene degradations. The inhibitory effects of *o*-xylene were similar to those of both benzene and toluene.

Substrate Interactions in Binary Mixtures of Non-Growth Substrates

The effects of the other BTEX compounds on the degradation of non-growth substrates (*m*-xylene and *p*-xylene) were also examined (Fig. 4). These experiments were evaluated by measuring the disappearance of *m*-xylene or *p*-xylene in the gas phase when one of the growth substrates was present in the binary mixtures. As can be seen in Fig. 4, *m*- and *p*-xylene degradations were not observed in the absence of the growth substrates. However, after adding the growth-supporting BTEX compounds, the degradation rates were significantly enhanced, indicating positive substrate interactions between *m*- and *p*-xylene and the other BTEX compounds. During these experiments, *m*- and *p*-xylene rapidly disappeared while the growth substrate remained in the mixtures and the subsidence of the *m*- and *p*-xylene degradation rates was observed after depleting the growth substrates. In addition, the transformation of *m*- and *p*-xylene by PHS1 resulted in the formation of brownish compounds, which did not disappear with further incubation. The results in Fig. 2 and Fig. 4 support the co-metabolic transformation of the *m*- and *p*-xylene by the PHS1 cultures. *p*-Xylene was found to be more difficult to degrade than *m*-xylene. Moreover, *p*-xylene did not degrade at a measurable rate when *o*-xylene was used as a growth substrate.

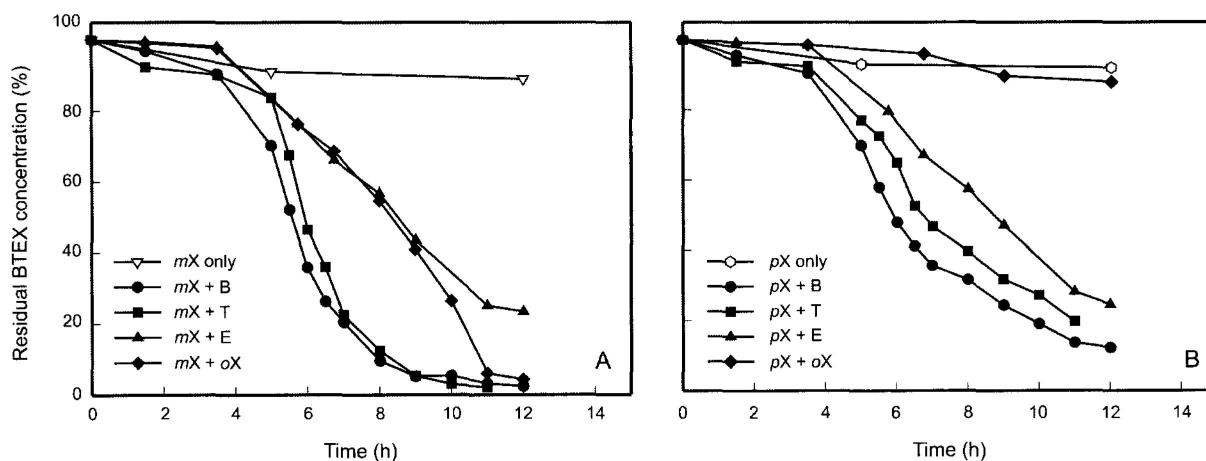

Fig. 4. Degradation of non-growth substrates (*m*-xylene and *p*-xylene) in the presence of growth substrates.
The initial substrate concentration: 10 mg/l each of *m*-xylene and *p*-xylene and 30 mg/l each of benzene, toluene, ethylbenzene, or *o*-xylene. Residual *m*-xylene (A) and *p*-xylene (B) concentrations in the reaction mixture were determined and expressed as the percentage of the initial substrate concentration.

Comparison with Other BTEX-Degrading Microorganisms

Previous studies [1, 3, 9, 16, 33] have shown that there are complicated interaction patterns among the aromatic hydrocarbons, despite the similarities in their chemical properties and structures. *Ralstonia* sp. PHS1 degrades BTEX compounds via a meta-cleavage pathway of catechol [29], which is similar to the degradation of phenol or its derivatives [2, 8, 11, 15, 25, 32, 36, 39]. However, significant differences in the substrate utilization patterns can be observed (Table 1), when the results of the present study are compared with those reported earlier. As reported before, PHS1 utilizes benzene, toluene, ethylbenzene, and o-xylene as the sole carbon and energy sources [29]. Although most BTEX-degrading microorganisms grow faster on toluene than benzene [9, 33, 35] and benzene is more toxic than toluene [13, 27], PHS1 utilized benzene more efficiently than toluene (Fig. 1). In addition, *p*-xylene was shown to be the strongest inhibitor of BTEX biodegradation by PHS1 whereas the most potent inhibitor of BTX degradation in binary mixtures for *R. rhodochrous* was ethylbenzene [16].

Although PHS1 is unable to utilize *m*-xylene or *p*-xylene as the sole carbon source [29], the results shown in Fig. 2 and Fig. 4 suggest that *m*- and *p*-xylene can be utilized by PHS1 in the presence of growth-supporting BTEX compounds (benzene, toluene, ethylbenzene, or *o*-xylene). The partial oxidation of *p*-xylene, leading to intermediates that are not utilized for growth, has been reported for *o*-xylene-degrading bacteria such as *P. putida* [20], *P. stutzeri* [6], and *Nocardia corallina* [20]. Incubating the PHS1 cultures in *m*- and *p*-xylene resulted in the formation of brownish compounds, which did not disappear with further incubation. These results indicate that *m*- and *p*-xylene are not completely degraded through the *o*-xylene catabolic route. Moreover, the relative position of the methyl groups on the aromatic ring play an important role in determining biodegradability of xylene. In addition, *m*- and *p*-xylene degradations in the presence of a growth substrate (benzene, toluene, ethylbenzene, or *o*-xylene) suggest that the nonspecific monooxygenase activity necessary for *m*- and *p*-xylene dissimilation may be induced by the presence

Table 1. Substrate utilization patterns of the BTEX-degrading microorganisms[a].

Microorganism	Growth rate or growth substrate	Degradation rate in BTEX mixtures	Most potent inhibitor	Co-metabolic degradation	Reference
Isolates from gasoline-contaminated soil	T>B>*p*X	T>B>*p*X>*o*X	NA[b]	*o*X	[18]
Pseudomonas sp. strain B1	T>B	NA[b]	NA[b]	*p*X	[8]
Pseudomonas sp. strain X1	T>*p*X (no growth on benzene)	NA[b]	NA[b]	NA[b]	[8]
Pseudomonas sp. strain PPO1	T>B	T>B>*p*X	NA[b]	*p*X	[29]
R. rhodochrous	T, B, E	NA[b]	E	*o*X, *m*X, *p*X	[13]
P. putida strain F1	T>B	T>B	NA[b]	NA[b]	[32]
Ralstonia sp. strain PHS1	B>T>*o*X>E	B>T>*o*X>E>*m*X, *p*X	*p*X	*m*X, *p*X	This study

[a]Abbreviations: B, benzene; T, toluene; E, ethylbenzene; *o*X, *o*-xylene; *m*X, *m*-xylene; *p*X, *p*-xylene.
[b]Information not available.

of growth substrates and partially oxidizes *m*- and *p*-xylene, permitting the co-metabolism of these compounds. A similar mechanism of the co-metabolic degradation of BTEX compounds has been reported [6, 20, 22, 26].

In a binary mixture system, some similarities and differences were found between our results and previous studies. For example, the presence of toluene has been shown to inhibit benzene degradation in mixtures [9, 33], and *p*-xylene inhibits benzene and toluene degradations [1, 33]. On the other hand, the effects of xylenes were significantly different. Although Deeb and Alvarez-Cohen [16] reported that the presence of xylenes had little or no effect on benzene, toluene, and ethylbenzene degradation, we observed in the present study a strong inhibitory effect of xylene isomers on the degradation of benzene, toluene, and ethylbenzene. Interestingly, the inhibitory effect of *m*-xylene on BTEX degradation was different depending on the nature of the compounds in the binary mixtures. The presence of *m*-xylene had a slight inhibitory effect on benzene and toluene degradation, whereas *m*-xylene strongly inhibited ethylbenzene and *o*-xylene degradation. The lower inhibitory effect of *m*-xylene on benzene and toluene degradation may be due to the preferential utilization of benzene and toluene by PHS1.

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