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# Cometabolic Removal of Xylene Isomers by *Alcaligenes xylosoxidans* Y234

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**Abstract** The characteristics of cometabolic removal of xylenes by *Alcaligenes xylosoxidans* Y234 were investigated. *m*-Xylene was found to be degraded while *o*- and *p*-xylene were biotransformed into cresols in the presence of benzene or toluene. A lower level of benzene was required than that of toluene to remove the same amount of xylenes, which suggested benzene was a more effective primary substrate than toluene. *o*-Xylene was found to be the most toxic to *Alcaligenes xylosoxidans* Y234 followed by *p*-xylene and *m*-xylene. Rates of cell decay during cometabolic removal of *o*-, *m*-, or *p*-xylene were decreased by up to 76% when benzene-adapted cells were inoculated. Xylenes were removed efficiently using benzene-adapted cells.

**Key words:** Xylenes, benzene, toluene, cometabolism, microbial adaptation, *Alcaligenes*

BTX (benzene, toluene, and *o*-, *m*-, and *p*-xylene) compounds are an important family of pollutants that are components of gasoline and aviation fuels, and are widely used in industrial syntheses [19, 21]. Since BTX are carcinogenic and neurotoxic, they are classified as priority pollutants and regulated by the Environmental Protection Agency in the U.S.A. [16, 22]. But BTX compounds frequently enter soil, sediments, and groundwater because of leakage from underground storage tanks and pipelines, accidental spills, improper waste disposal practices, and leaching landfills [21]. Therefore, for several decades, many researches have tried to remove BTX from the environment [9, 15, 24]. In general, xylenes are known to be more recalcitrant than benzene and toluene, and *o*-xylene is reported to be the most recalcitrant of the BTX compounds, which suggests the importance of the position of the methyl substituent on the aromatic ring in the microbial degradation [4].

The individual compounds constituting BTX mixtures are easily degraded by the microorganisms of soil and surface water. However, since they are usually produced and discharged into the environment from a factory, it has been emphasized that BTX should be degraded simultaneously. But most of the laboratory investigations concerning the biodegradation of BTX have been focused on one of the individual compounds. Although each BTX compound can be mineralized by natural microorganisms, the five components of BTX mixtures cannot be successfully mineralized simultaneously [14]. Therefore, substrate interactions are important for understanding the behavior of BTX compounds in the environment and the similarity or differences in their biodegradation. The presence of a given BTX compound can stimulate the degradation of another BTX compound by inducing the required catabolic enzymes. Arvin *et al.* [3] found that benzene degradation was enhanced in the presence of either toluene or *o*-xylene. Alvarez and Vogel [2] observed enhanced degradation of benzene and *p*-xylene using *Pseudomonas* sp. in the presence of toluene. Another beneficial substrate interaction would be a BTX compound acting as a primary substrate and stimulating microbial growth, which could enhance cometabolism of another BTX compound. On the other hand, a BTX compound could inhibit the degradation of another by exerting toxicity, diauxy, catabolic repression, competitive inhibition for enzymes, or depletion of electron acceptors [2].

To remove BTX from the environment, a simple and reliable method is to use a microorganism capable of degrading BTX simultaneously. Both aerobic and anaerobic bacteria have been shown to degrade BTX compounds, but most of the studies on bacterial degradation of BTX have used microbial consortia in an activated sludge or aquifer where microorganisms have various abilities [2, 10]. Although Yadav and Reddy [21] reported that

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*Phanerochaete chrysosporium*, a type of fungi, degraded benzene, toluene, ethylbenzene, and xylenes (BTEX), there are few reports on screening bacteria degrading BTX simultaneously. Fungi causing bulking in activated sludge is difficult to control and inadequate in the wastewater treatment process [20]. An attempt to use genetically modified bacteria to increase the degradation range of a single organism was made [14]. They constructed a hybrid pathway in a *Pseudomonas putida* which mineralized components of a benzene, toluene, and *p*-xylene mixture simultaneously. But the safety of genetically modified microorganism in the environment has still been in doubt and most of the genetically engineered microorganisms are unable to compete with indigenous microorganisms. Cometabolic removal, as an alternative method, can be considered for the simultaneous removal of BTX. Although there is some dispute on the definition of cometabolism, it is generally accepted that cometabolism is a biotransformation of an organic compound (secondary substrate) only in the presence of a primary substrate, which is the compound that supports cell growth [7]. However, Alexander [1] insisted that the term can also be used to describe the case where the compound is biotransformed in the absence of a second substrate. Cometabolism has been considered a useful process in the detoxification process [6, 13]. The addition of a hydroxyl group to an aromatic or aliphatic molecule often makes it less harmful and makes it metabolized by other microorganisms which cannot degrade it in the original form [1, 18]. Therefore, much research on cometabolism has been conducted [1, 5, 17]. Chang *et al.* [5] and Oh *et al.* [17] observed that *p*-xylene was cometabolically removed by cultures in the presence of benzene and/or toluene. The metabolic intermediates of *p*-xylene, dimethyl aromatic compounds with a hydroxyl group, accumulated in the medium. Oh *et al.* [17] also reported that cometabolic removal of *p*-xylene reduced the cell yields of both benzene and toluene. Chang *et al.* [5] observed that cometabolism increased cell decay rates, and the observed yield of *Pseudomonas* sp. B1 decreased in the presence of *p*-xylene. That is, cometabolic removal can be conducted at the expense of cell mass.

In this study, the characteristics of cometabolic removal of xylenes by *Alcaligenes xylosoxidans* Y234 in the presence of benzene or toluene were investigated and an efficient method to remove xylenes is suggested.

## MATERIALS AND METHODS

### Microorganism

*Alcaligenes xylosoxidans* Y234 isolated from oil-contaminated soil was used in this study. It can degrade benzene, toluene, *m*-xylene, and phenol [22].

### Mineral Medium

*Alcaligenes xylosoxidans* Y234 was precultured at 30°C in a 500-ml flask containing 200 ml of medium (10 g/l glucose, 5 g/l yeast extract, 5 g/l $(NH_4)_2SO_4$, 5 g/l $KH_2PO_4$, and 1 g/l $MgSO_4 \cdot 7H_2O$). The cells were harvested and washed several times with distilled water. 2 mg of microorganisms were put into the 120 ml serum bottle closed with silicon rubber septa and an aluminum crimp cap. The serum bottle contained 20 ml of medium (5 g/l $K_2HPO_4$, 4.5 g/l $KH_2PO_4$, 2 g/l $(NH_4)_2SO_4$, 0.3 g/l $MgSO_4 \cdot 7H_2O$, and 200 µl/l trace element). The trace element contains 16.2 g/l $FeCl_3 \cdot 6H_2O$, 10.2 g/l $CaCl_2 \cdot 2H_2O$, 0.22 g/l $CoCl_2 \cdot 6H_2O$, 0.15 g/l $CuSO_4 \cdot 5H_2O$, 0.13 g/l $CrCl_3 \cdot 6H_2O$, 0.09 g/l $NiCl_3 \cdot 6H_2O$, and 40.0 g/l citric acid. The main experiments were conducted at 30°C in a shaking incubator with 150 rpm.

### Assays

The concentration of BTX was analyzed by head space analysis. After 500 µl of head space was withdrawn by gas-tight syringe, it was injected into the GC (HP 5890 II) equipped with FID detector. HP-1 column was used and GC operation conditions were: 150°C injection port, 100°C oven, and 250°C detection port temperature. Since GC cannot separate *m*-xylene and *p*-xylene, they were measured together. Two controls without microorganism were also assayed to compensate for abiotic loss. The optical density of the microorganisms was measured at 660 nm using a spectrophotometer (UVIKON, Kontron Instrument). For the analysis of cometabolic products by xylenes, the cells were removed by centrifugation and the supernatant was scanned using a spectrophotometer from 200 nm to 400 nm. The supernatant was also extracted with two volumes of ethyl acetate. The extract was filtrated to remove undissolved matters (e.g., cell debris) and dried in a vacuum dry oven at 30°C. The extract was redissolved in acetone and analyzed using GC-Mass (GC: HP 5890 II, Ultra 1 column, Mass: JEOL, JMS-AX505WA).

## RESULTS AND DISCUSSION

### Effects of Benzene and Toluene on Xylene Degradation

*Alcaligenes xylosoxidans* Y234 was found to degrade benzene and toluene [22] but it degraded only *m*-xylene out of the three xylene isomers with very low degradation rates, as shown in Fig. 1. Since there were reports on the cometabolic removal of *p*-xylene [5, 17], the possibility of cometabolism of xylenes was checked. When each xylene was fed with benzene or toluene to *Alcaligenes xylosoxidans* Y234, xylenes began to disappear as soon as the degradation of benzene was initiated; while in the case of toluene feeding, they began to

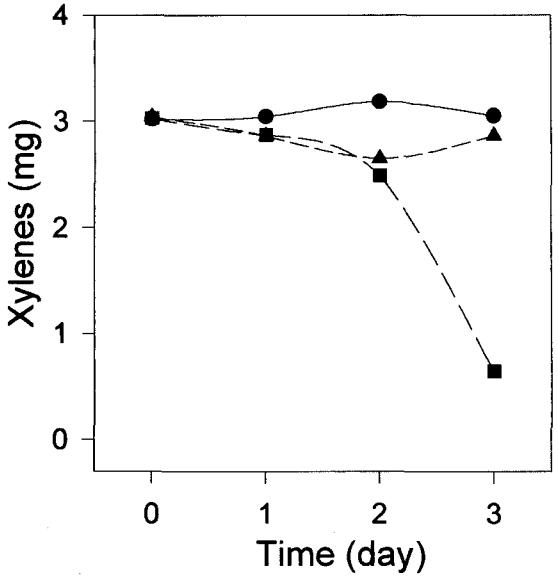


**Fig. 1.** The time courses of xylenes degradation by *Alcaligenes xylosoxidans* Y234.
●: *o*-xylene; ■: *m*-xylene; ▲: *p*-xylene.

disappear after 80% of toluene was degraded, as shown in Figs. 2 and 3. Although xylenes disappeared, we cannot say that they were degraded by microorganisms. To check whether xylenes were degraded or biotransformed into other organic compounds, the cells were removed from the culture broth by centrifugation and the supernatant was scanned using a UV spectrophotometer. As shown in Fig. 4, a peak was detected at about 260 nm, in the case of *o*- and *p*-xylene, which implied that they were biotransformed into aromatic compounds having a hydroxyl group [17]. However, no peaks appeared in the case of *m*-xylene, which implied that *m*-xylene was not biotransformed but degraded. To find out the structure of the products from xylenes, ethyl acetate was added to the supernatant to extract cometabolic products. After ethyl acetate was completely evaporated, the residue was redissolved in acetone and analyzed by GC-Mass. As shown in Fig. 5, *o*- and *p*-xylenes were biotransformed into *o*- and *p*-cresols (MW=108), respectively. Since *Alcaligenes xylosoxidans* Y234 could not further oxidize cresols, it excreted them into the broth. In the case of *m*-xylene, there was no detectable residue after the ethyl acetate was evaporated. The biotransformation of xylenes into cresols suggested that the degradation of xylene was initiated not by benzene ring attack but by the oxidation of methyl substituent [24]. The microbial oxidation of xylene may be initiated by the oxidation of one of the methyl group or by direct oxidation of the aromatic nucleus [4, 8, 11]. They reported that 3,6-dimethyl pyrocatechol or α,α'-dimethyl-cis,cis-muconic acid was produced from *p*-xylene by the direct oxidation of the aromatic nucleus and excreted to

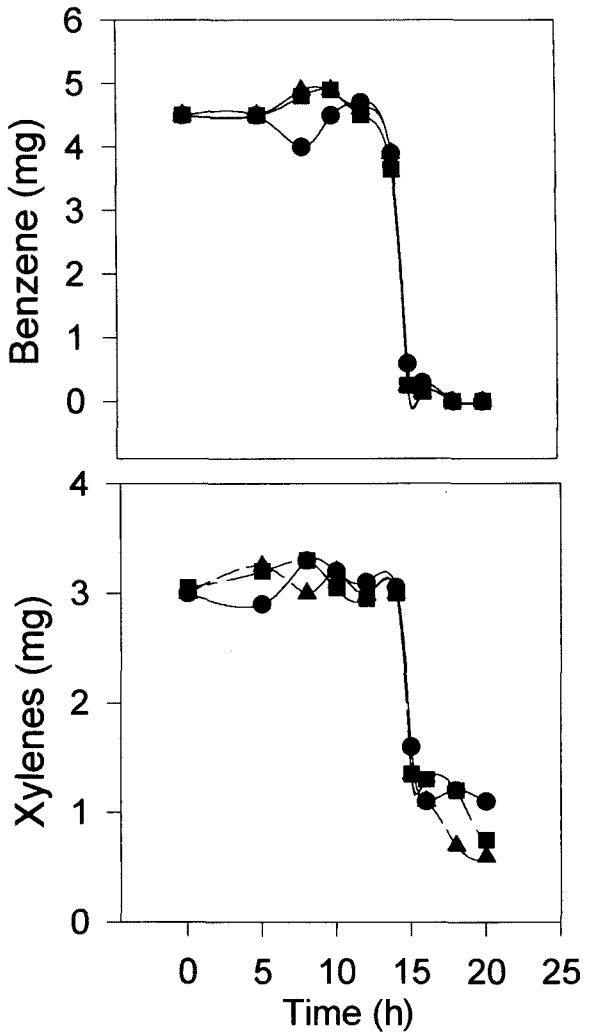


**Fig. 2.** The effect of benzene on the cometabolic removal of xylenes.
●: *o*-xylene; ■: *m*-xylene; ▲: *p*-xylene.

the broth, while *p*-toluic acid and 4-methylcatechol were produced transiently from *p*-xylene by the oxidation of a methyl substituent and then mineralized. But there are few reports that xylene was biotransformed into cresols. From these results it can be said that benzene or toluene accelerated the degradation of *m*-xylene and biotransformation of *o*- and *p*-xylene into cresols.

In this study, *o*- and *p*-xylene were cometabolized but *m*-xylene degraded rapidly in the presence of benzene or toluene. That is, the degradation rate of m-xylene was dramatically enhanced by the action of benzene or toluene. In the strict definition of cometabolism, such a phenomenon is not cometabolism because *m*-xylene was not biotransformed. But since the degradation of *m*-xylene was mainly due to the broad specificity of enzymes induced by benzene or toluene, it can be referred to as cometabolism.

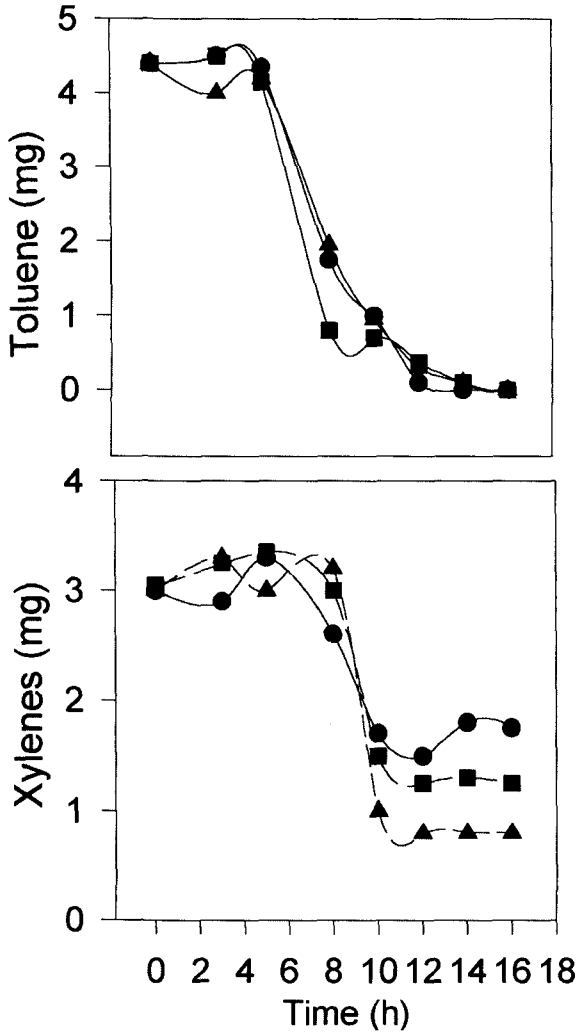


**Fig. 3.** The effect of toluene on the cometabolic removal of xylenes.
●: *o*-xylene; ■: *m*-xylene; ▲: *p*-xylene.

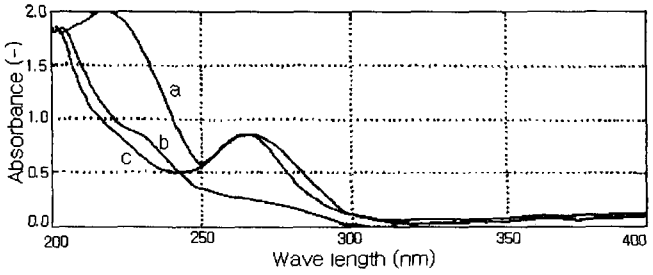


**Fig. 4.** UV spectra of intermediates in broth.
(a) *o*-xylene; (b) *m*-xylene; (c) *p*-xylene.

## Comparison of Benzene and Toluene Ability in the Removal of Xylenes

As pointed out, benzene or toluene was required as a primary substrate to remove xylenes cometabolically. Therefore, it is important to investigate the effect of benzene or toluene level on the cometabolism of xylenes

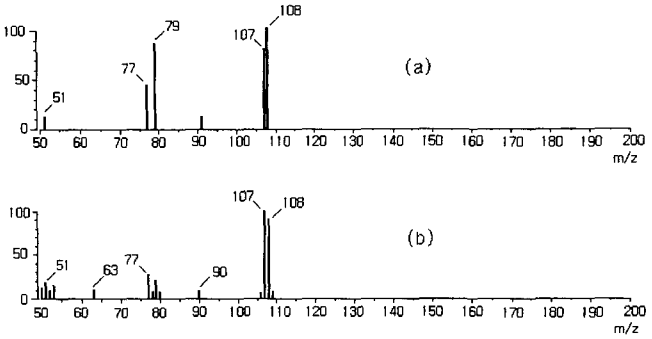


**Fig. 5.** GC-Mass spectra of cometabolic products from xylenes.
(a) from *o*-xylene; (b) from *p*-xylene.

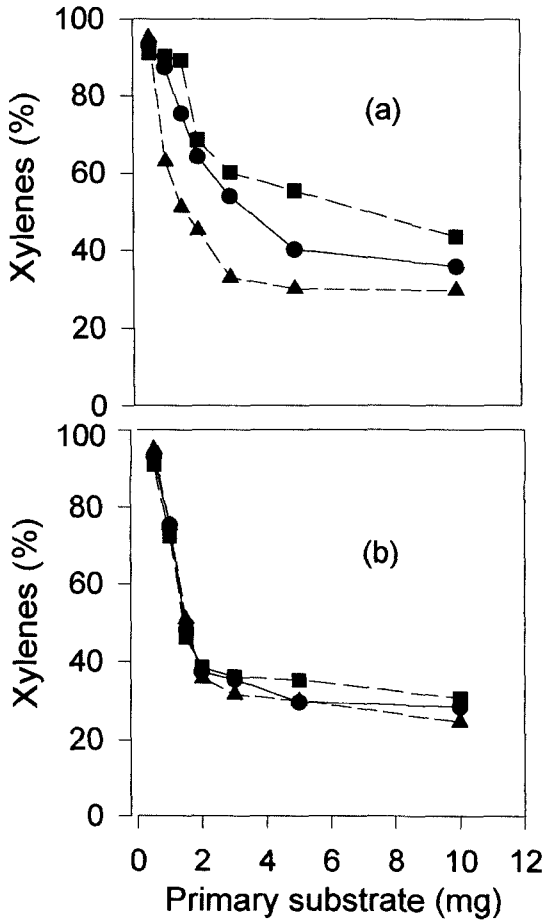


**Fig. 6.** The effect of benzene or toluene level on the cometabolic removal of xylenes.
(a) toluene; ●: *o*-xylene; ■: *m*-xylene; ▲: *p*-xylene. (b) benzene; ●: *o*-xylene; ■: *m*-xylene; ▲: *p*-xylene.

(secondary substrate). Benzene or toluene was added by increasing the level from 0.5 mg to 10 mg to the medium containing 3 mg of xylene. As shown in Fig. 6, when 2 mg of benzene was fed, the removal efficiency of the three xylene isomers was not much different from one another; that is, 62.6% for *o*-xylene, 61.5% for *m*-xylene, and 64.4% for *p*-xylene. Although benzene level

was increased to 10 mg, the removal efficiency did not increase above 75%. For the removal of 1.5 mg xylene, the required benzene level was about 1.5 mg in all cases. In the case of toluene, when 2.0 mg of toluene was added, 35.8% of *o*-xylene, 31.5% of *m*-xylene, and 54.8% of *p*-xylene were removed, respectively. When 10 mg of toluene was added to the medium, the removal efficiencies differed greatly, that is, 54.2% for *o*-xylene, 57.5% for *m*-xylene, and 70.3% for *p*-xylene. The required toluene levels for the removal of 1.5 mg of *o*-, *m*-, and *p*-xylenes were about 6.0 mg, 3.0 mg, and 2.5 mg, respectively. A lower level of benzene was required than the toluene level to remove the same amount of xylene. These results suggest three facts. First, benzene is a better primary substrate than toluene in the cometabolic removal of xylenes. Second, the biotransformation of *p*-xylene required a smaller amount of toluene than the biotransformation of *o*-xylene and the biodegradation of *m*-xylene, while the required benzene level was the same. Third, BTX could be removed simultaneously by *Alcaligenes xylosoxidans* Y234. *Alcaligenes xylosoxidans* Y234 was cultured in the presence of benzene, toluene, and three isomers of xylenes. Cell mass was decreased significantly before toluene degradation and then increased until benzene and toluene were depleted as shown in Fig. 7. The removal of *p*-xylene and *m*-xylene was followed by that of *o*-xylene. The absorbance of cell broth at 260 nm was increased due to the biotransformation of *o*- and *p*-xylene into cresols.

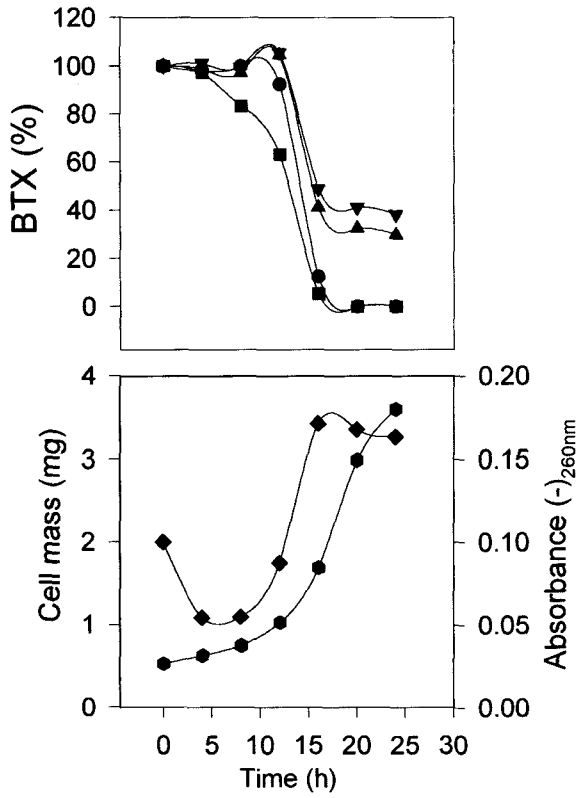


**Fig. 7.** The simultaneous BTX removal by *Alcaligenes xylosoxidans* Y234.
●: benzene (5 mg); ■: toluene (5 mg); ▲: *o*-xylene (3 mg); ▼: *m*- + *p*-xylene (3 mg + 3 mg); ◆: cell mass; ●: absorbance

### Effect of Microbial Adaptation

According to Oh *et al.* [17] and Chang *et al.* [5], cometabolic removal of *p*-xylene reduced the cell yields on both benzene and toluene due to cell decay by the toxicity of xylenes. The same results were obtained in this study as shown in Fig. 8. The cell yield on benzene or toluene decreased as the ratio of benzene or toluene to xylenes decreased. The results also showed that *o*-xylene was the most toxic followed by *p*- and *m*-xylene.

In the case in which BTX are discharged simultaneously, they will be removed simultaneously. But when only

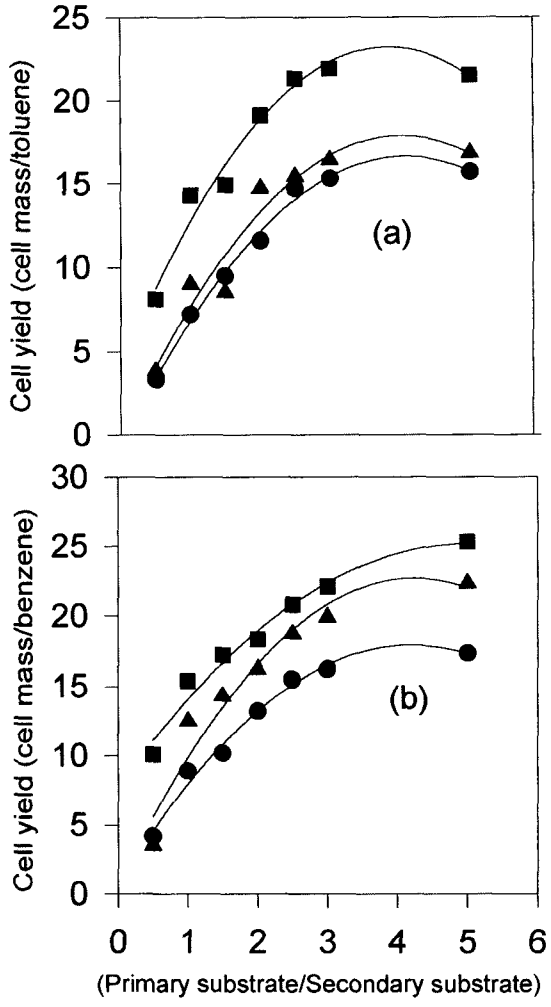


**Fig. 8.** The effect of xylenes on the cell yield on benzene or toluene.
(a) toluene; ●: *o*-xylene; ■: *m*-xylene; ▲: *p*-xylene. (b) benzene; ●: *o*-xylene; ■: *m*-xylene; ▲: *p*-xylene.

xylenes are discharged, a primary substrate is required both to remove xylene and to sustain cell mass. But it is undesirable to add high levels of benzene or toluene continuously for the removal of xylenes. Therefore, a method to remove xylenes without adding benzene or toluene was considered. Yeom *et al.* [23] reported that microbial adaptation is a good method to increase the degradation rate of BTX and discussed the mechanism of microbial adaptation in respect to enzyme induction. Another explanation of microbial adaptation has also been presented. Hiepier and Bont [12] reported that *Pseudomonas putida* S12 was more tolerant to ethanol when preadapted to toluene. The reason was discussed in terms of cell membrane composition change, which reduced cell membrane damage. From these points of view, microbial adaptation was thought to be a good method to remove xylenes without adding benzene or toluene, with less cell decay.

The experimentally measured endogenous decay rate of *Alcaligenes xylosoxidans* Y234 was 0.67 $day^{-1}$. The maximum decay rates of *o*-, *m*-, and *p*-xylene were 2.42, 1.87, and 2.30 $day^{-1}$, respectively, at 12 ppm of xylenes as shown in Table 1 and the values did not change up to 50 ppm of xylenes. Table 1 also shows that the cell decay rate by xylenes could be reduced by microbial adaptation. When the cells were adapted to benzene, the decay rate by *p*-xylene was decreased to as much as 76.3% but the cell decay could not be prevented entirely. The removal of xylenes by adapted cells was investigated quantitatively by measuring the decayed cell mass during the removal of 1 mg xylene. Nonadapted, benzene-adapted, or toluene-adapted cells were inoculated to the medium containing xylenes as a carbon source. The cell capacities, defined as the amount of removed substrate divided by the decayed cell mass, of nonadapted cell to *o*-, *m*-, and *p*-xylene were 0.00, 0.03, and 0.00, respectively. This meant that nonadapted cells were only decayed without removing xylenes. But the capacities of benzene-adapted cells to *o*-, *m*-, and *p*-xylene were 2.50, 12.14, and 3.03, respectively, while those of toluene-adapted cells were 0.80, 9.09, and 1.20, respectively, as shown in Table 2. These results show that xylenes could be removed effectively using benzene-adapted cells instead of adding benzene or toluene.

**Table 1.** Maximum cell decay rate by xylenes ($day^{-1}$).

| | Benzene-adapted | Toluene-adapted | Nonadapted |
|---|---|---|---|
| *o*-xylene | 0.91 | 1.27 | 2.42 |
| *m*-xylene | 0.70 | 0.74 | 1.87 |
| *p*-xylene | 0.56 | 1.01 | 2.30 |

adaptation: after 90% of toluene or benzene was degraded, adapted cells were transferred to the new medium containing only xylene as the sole carbon source.

**Table 2.** The amount of removed xylenes during 1 mg of cell decay[a].

| | Benzene-adapted cell | Toluene-adapted cell | Nonadapted cell |
|---|---|---|---|
| *o*-xylene | 2.50 | 0.80 | 0.00 |
| *m*-xylene | 12.14 | 9.09 | 0.03 |
| *p*-xylene | 3.03 | 1.20 | 0.00 |

[a](mg-xylene disappeared/mg-cell decayed).

## REFERENCES


1. Alexander, M. 1994. *Biodegradation and Bioremediation*, pp. 196–225, Academic Press, New York.
2. Alvarez, P. J. J. and T. M. Vogel. 1991. Substrate interactions of benzene, toluene, and para-xylene during microbial degradation by pure cultures and mixed culture aquifer slurries. *Appl. Environ. Microbiol.* **57:** 2981–2985.
3. Arvin, E., B. K. Jensen, and A. T. Gundersen. 1989. Substrate interaction during aerobic biodegradation of benzene. *Appl. Environ. Microbiol.* **55:** 3221–3225.
4. Baggi, G., P. Barbieri, E. Galli, and S. Tollari. 1987. Isolation of a *Pseudomonas stutzeri* strain that degrades *o*-xylene. *Appl. Environ. Microbiol.* **53:** 2129–2132.
5. Chang, M. K., C. V. Thomas, and S. C. Craig. 1993. Kinetics of competitive inhibition and cometabolism in the biodegradation of benzene, toluene, and *p*-xylene by two *Pseudomonas* isolates. *Biotechol. Bioeng.* **41:** 1057–1065.
6. Cohen, A. L. and P. L. McCarty. 1991. A cometabolic transformation model for halogenated aliphatic compounds exhibiting product toxicity. *Environ. Sci. Technol.* **25:** 1387–1393.
7. Dalton, H. and D. I. Stirling. 1982. Co-metabolism. *Phil. Trans. R. Soc. Lond. Ser. B*, **297:** 481–496.
8. Davey, J. F. and Gibson, D. T. 1974. Bacterial metabolism of para- and meta-xylene: Oxidation of a methyl substituent. *J. Bacteriol.* **119:** 923–929.
9. Duetz, W. A., DE C. Jong, P. A. Williams, and VAN J. G. Andel. 1994. Competition in chemostat culture between *Pseudomonas* strains that use different pathways for the degradation of toluene. *Appl. Environ. Microbiol.* **60:** 2858–2863.
10. Edwards, E. A., L. E. Wills, M. Reinhard, and D. Grbic-Galic. 1992. Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions. *Appl. Environ. Microbiol.* **58:** 794–800.
11. Gibson, D. T., V. Mahadevan, and J. F. Davey. 1974. Bacterial metabolism of para- and meta-xylene: Oxidation of the aromatic ring. *J. Bacteriol.* **119:** 930–936.
12. Heipieper, H. J. and DE J. A. M. Bont. 1994. Adaptation of *Pseudomonas putida* S12 to ethanol and toluene at the level of fatty acid composition of membranes. *Appl. Environ. Microbiol.* **60:** 4440–4444.
13. Landa, A. S., E. M. Pipkema, J. Weijma, A. A. C. M. Beenackers, J. Dolfing, and D. B. Janssen. 1994. Cometabolic degradation of trichloroethylene by *Pseudomonas*

*cepacia* G4 in a chemostat with toluene as the primary substrate. *Appl. Environ. Microbiol.* **60:** 3368–3374.
14. Lee, J. Y., K. H. Jung, S. H. Choi, and H. S. Kim. 1995. Combination of the tod and tol pathways in redesigning a metabolic route of *Pseudomonas putida* for the mineralization of a benzene, toluene, and *p*-xylene mixture. *Appl. Environ. Microbiol.* **61:** 2211–2217.
15. Marr, E. K. and R. W. Stone. 1960. Bacterial oxidation of benzene. *J. Bacteriol.* **81:** 425–430.
16. Norbert, G. S. C. 1995. Chemical contamination of the environment: Sources, types, and fate of synthetic organic chemicals. pp. 27–74. *In Microbial Transformation and Degradation of Toxic Organic Chemicals.* Wiley-Liss Inc., New York.
17. Oh, Y. S., Z. Shareefdeen, C. B. Basil, and B. Richared. 1994. Interactions between benzene, toluene, and *p*-xylene (BTX) during their biodegradation. *Biotechnol. Bioeng.* **44:** 533–538.
18. Owen, W. J. 1989. *Herbicides and Plant Metabolism,* pp. 1171–1198. Cambridge Univ. Press. Cambridege, UK.
19. Smith, M. R. 1990. The biodegradation of aromatic hydrocarbons by bacteria. *Biodegradation* **1:** 191–206.
20. Tchobanoglous, G. 1979. *Wastewater Engineering,* pp. 520–522. McGRAW-Hill, Boston.
21. Yadav, J. S. and C. A. Reddy. 1993. Degradation of benzene, toluene, ethylbenzene, and xylenes (BTEX) by the lignin-degrading basidiomycete *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **59:** 756–762.
22. Yeom, S. H., S. H. Kim, Y. J. Yoo, and I. S. Yoo. 1997. Microbial adaptation in the degradation of phenol by *Alcaligenes xylosoxidans* Y234. *Kor. J. Chem. Eng.* **14:** 37–40.
23. Yeom, S. H., Y. J. Yoo, and J. W. Lee. 1997. The importance of microbial adaptation in the degradation of BTX. pp. 683–693. *In Global Environmental Biotechnology.* Elsevier Science B. V.
24. Zylstra, G. J. and D. T. Gibson. 1991. *Aromatic Hydrocarbon Degradation: A Molecular Approach. Genetic Engineering,* pp. 183–203. Plenum Press, New York.