Korean J. Chem. Eng., **19**(5), 780-782 (2002)
SHORT COMMUNICATION

Analysis of Microbial Adaptation at Enzyme Level for Enhancing Biodegradation Rate of BTX

Sung-Ho Yeom*,** and Young-Je Yoo*,†**

*School of Chemical Engineering, **EnzyBiotech Inc., Technology Incubation Network,
Seoul National University, Seoul 151-742, Korea
(*Received 22 November 2001 • accepted 15 May 2002*)

Abstract−Catechol was found to be a common intermediate in the degradation of benzene and toluene by *Alcaligenes xylosoxidans* Y234, and the ring cleavage of the catechol mediated by catechol 1,2-dioxygenase was a rate-determining step. Since benzene induced higher level of catechol 1,2-dioxygenase than toluene, the cells pre-adapted to benzene showed higher degradation rate of benzene and toluene. The degradation rate of *m*-xylene was also increased significantly when benzene-adapted cells were inoculated. *m*-Xylene was metabolized via 3-methyl catechol which was effectively cleaved by catechol 1,2-dioxygenase.

Key words: BTX, Microbial Adaptation, Enzyme Induction, Catechol 1,2-Dioxygenase, Biodegradation

INTRODUCTION

Microbial adaptation has been widely studied because prior adaptation history significantly affects the degradation pattern. Satsangee and Ghosh [1990] observed that the phenol degradation rate depends on the periods in which the culture was adapted to phenol. Bauer and Capone [1988] reported that prior exposure to anthracene, naphthalene, phenanthrene and benzene results in enhanced naphthalene degradation, while anthracene degradation was stimulated only by pre-exposure to benzene and anthracene. Shimp and Pfaender [1985] observed that the natural substrate classes such as amino acids, carbohydrates and fatty acids stimulated the degradation of m-cresol, m-aminophenol and p-chlorophenol.

There was a novel explanation for the mechanism of microbial adaptation. Heipieper and Bont [1994] reported that *Pseudomonas putida* S12 was more tolerant to ethanol when pre-adapted to supersaturating concentration of toluene; the reason was discussed in terms of change of cell membrane composition. However, for benzene, toluene and xylene (BTX) degradation, since enzymes are inducible [Hamzah and Al-Baharna, 1994], microbial adaptation can be easily discussed in terms of enzyme induction. Nonetheless, few have dealt with the microbial adaptation of BTX quantitatively in the aspect of a key enzyme induction which controls overall catabolic reaction rate.

In this communication, the microbial adaptation was analyzed quantitatively by measuring the key enzyme activity, and the results deduced from the mechanism of microbial adaptation were applied to increase the biological degradation rate of BTX.

MATERIALS AND METHODS

1. Microorganism and Mineral Medium

Alcaligenes xylosoxidans Y234 was used in this study and the mineral medium was well described in the previous study [Yeom et al., 1998].

2. Assays

The procedures of measuring BTX concentration and cell mass were already described in the previous study [Yeom et al., 1998]. The presence of catechol or 3-methyl catechol was colorimetrically identified by both Arnow method [Arnow, 1937; Waite and Tanzer, 1981] and amino-antipyrine method [Folsom et al., 1990]. Catechol 1,2-dioxygenase (or 3-methyl catechol 1,2-dioxygenase) activity was assayed as follows. The cells were harvested and broken by sonication. A mixture of 50 µl of 10 mM catechol (or 3-methyl catechol) and 50 µl of 0.5 g/L cell-free extract was added into 2 mL phosphate buffer (60 mM, pH 7.0). The activity was then determined by measuring the rate of increase in absorbance at 255 nm in 1 min [Hamzah and Al-baharna, 1994; Yeom et al., 1997]. The total protein concentration in cell-free extract was determined according to Bradford method by using a Bio-Rad protein assay kit with bovine albumin as a standard.

RESULTS AND DISCUSSION

1. Enhancing Degradation of Benzene and Toluene

As a microorganism is adapted to a pollutant, the degradation rate becomes higher [Satsangee and Ghosh, 1990]. So if the key mechanism of microbial adaptation is revealed, it can be used to accelerate the degradation rate of the pollutant. For adaptation purposes, microorganisms are usually adapted to the pollutant to be treated [Satsangee and Ghosh, 1990]. As expected, benzene- or toluene-adapted cells degraded benzene or toluene, respectively, without adaptation time and highly enhanced degradation rate (data not shown). A new concept of microbial adaptation, a cross-adaptation (adapted to benzene to degrade toluene and vice versa), was also tried to find a method to increase degradation rate of benzene and toluene. As shown in Fig. 1, benzene-adapted cells could also degrade toluene faster than toluene-adapted cells. The benzene and toluene degradation rates of benzene-adapted cells were about twice

†To whom correspondence should be addressed.
E-mail: yjyoo@snu.ac.kr

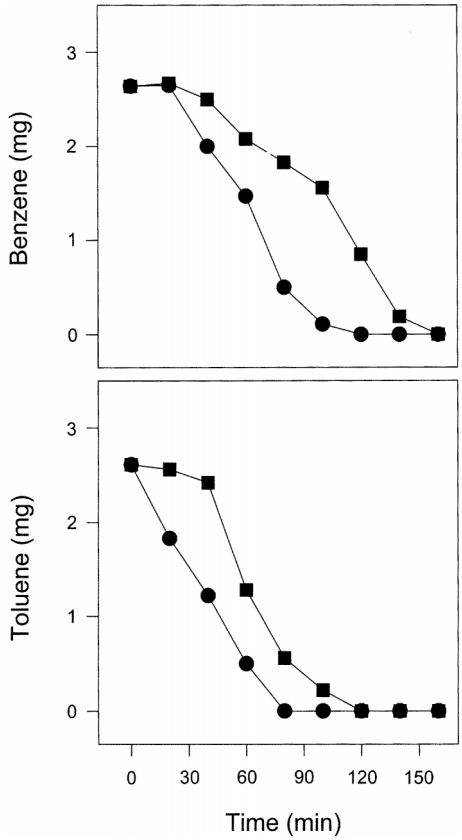

Fig. 1. The effect of cross-adaptation on the degradation of benzene and toluene.
●: benzene-adapted cells, ■: toluene-adapted cells

as high as those of toluene-adapted cells (2.72 mg/hr vs. 1.05 mg/hr for benzene degradation; 2.25 mg/hr vs. 1.53 mg/hr for toluene degradation). To reveal the reason why benzene-adapted cells are better in the degradation of benzene and toluene, the enzyme level induced by benzene or toluene was investigated.

Since many enzymes are involved in the degradation of benzene and toluene [Duetz et al., 1994], it is almost impossible to check out the activities of all the relevant enzymes. Instead, we decided to concentrate on the most important enzyme which would control the degradation rates of benzene and toluene. If a step is rate-determining or bottleneck in a series of reactions, it controls the overall reaction rates. In other words, the reaction rate is increased if the bottleneck is removed. The identification of intermediates is often used to reveal the rate-determining step. The studies concerned microbial phenol degradation; catechol accumulation in culture media was observed during phenol degradation, and so it was suggested that the reaction catalyzed by catechol oxygenase was the rate-determining step [Evans, 1947; Fujita et al., 1993]. In this study, catechol was detected in the culture broth during cultivation of *Alcaligenes xylosoxidans* Y234 in the presence of benzene or toluene. Therefore, catechol was a common metabolite in the degradation of benzene and toluene [Duetz et al., 1994] and catechol ring cleavage step was rate-determining.

Since catechol ring cleavage was suggested to be a rate-determining step, it is important to find out the way to increase its rate. First, a spectrophotometric study was performed to know how the

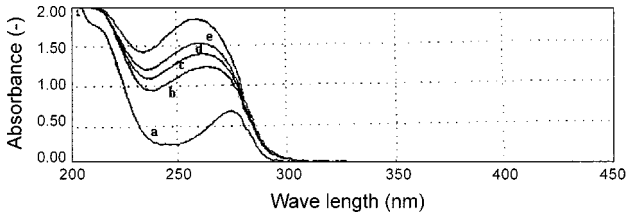

Fig. 2. Ortho-ring cleavage by catechol 1,2-dioxygenase from cell-free extract of *Alcaligenes xylosoxidans* Y234.
a: 0 min, b: 5 min, c: 10 min, d: 15 min, e: 20 min

catechol ring cleavage occurs. Catechol was mixed with cell-free extract from benzene- or toluene-grown cells and the absorbance change was measured in the range of 200 to 400 nm by using a spectrophotometer. As shown in Fig. 2, a peak shift from 260 nm (catechol) to 250 nm (cis,cis-muconic acid) was observed, which indicated that the catechol ring cleavage occurred by catechol 1,2-dioxygenase (ortho-cleavage) not by catechol 2,3-dioxygenase (meta-cleavage). If there was a catechol 2,3-dioxygenase activity, a peak would appear at 375 nm [Hamzah and Al-Bahama, 1994]. The metabolic pathways of benzene and toluene were briefly suggested as shown in Fig. 3. Since catechol 1,2-dioxygenase is induced in cells grown on aromatic compounds such as benzene, toluene, *m*-xylene, phenol and benzoic acid but not in those grown on glucose (Table 1), the enzyme is inducible. The fact that benzene-grown cells inducing higher level of catechol 1,2-dioxygenase degraded benzene and toluene much faster than the toluene-adapted cells as mentioned above can be another evidence of catechol ring cleavage step being

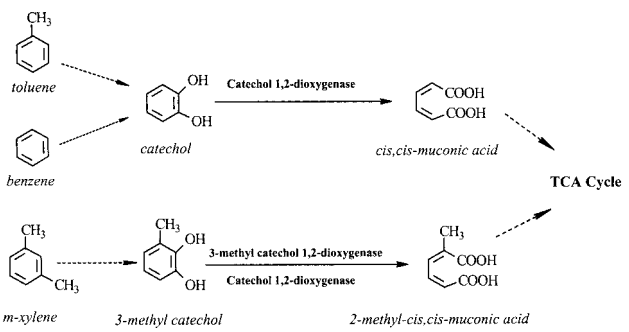

Fig. 3. Simplified metabolic pathways of BTX by *Alcaligenes xylosoxidans* Y234.

Table 1. The induction of catechol 1,2-dioxygenase[a] by various aromatic compounds

Time[b]	Benzene	Toluene	*m*-Xylene	Phenol	Benzoic acid	Glucose
0 %	0.00	0.00	0.00	0.00	0.00	0.00
10 %	2.75	0.46	0.02	0.26	0.62	0.00
50 %	7.00	0.63	0.03	0.39	1.22	0.00
90 %	9.38	1.24	0.05	0.54	1.71	0.00

[a][ΔOD/mg-protein·min]
[b]0%: When degradation did not begin; 10%: right after degradation began; 50%: when half of the substrate was degraded; 90%: when most of the substrate was degraded.

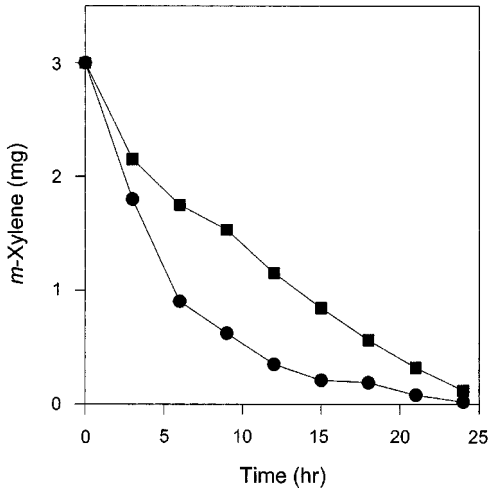

Fig. 4. The effect of microbial adaptation on the degradation of *m*-xylene.
●: benzene-adapted cells, ■: toluene-adapted cells

rate-determining.

In brief, the uptake of benzene induced high level of catechol 1,2-dioxygenase, mitigating the bottleneck of catechol ring cleavage, and this led to rapid degradation of benzene and toluene.

2. Enhancing Degradation of *m*-Xylene

Alcaligenes xylosoxidans Y234 could degrade *m*-xylene but it took more than 3 days to consume 3 mg of *m*-xylene with 2 days of adaptation period [Yeom et al., 1998]. When the benzene- or toluene-adapted cells were inoculated, *m*-xylene was rapidly degraded within 24 hours as shown in Fig. 4. The benzene-adapted cells degraded *m*-xylene faster than toluene-adapted cells, which may have been related to the difference of induction levels of catechol 1,2-dioxygenase as will be discussed later. *m*-Xylene was metabolized via 3-methyl catechol which was detected in the culture medium when the non-adapted cells were inoculated. On the contrary, when benzene- or toluene-adapted cells were used, the intermediate was not detected. From the results, the metabolic pathway of *m*-xylene was briefly suggested in Fig. 3. Interestingly, the cell-free extract from cells grown on benzene (inducing catechol 1,2-dioxygenase) could cleave 3-methyl catechol efficiently as well as catechol as shown in Table 2. However, the cell-free extract from cells grown on *m*-xylene (inducing 3-methyl catechol 1,2-dioxygenase) could poorly cleave 3-methyl catechol and almost not catechol. It was believed that catechol 1,2-dioxygenase has relatively broad specificity to degrade both catechol and 3-methyl catechol while 3-methyl catechol 1,2-dioxygenase has relatively narrow specificity to degrade only 3-methyl catechol. Since *m*-xylene induced very low level of 3-methyl catechol 1,2-dioxygenase, the degradation rate of *m*-xylene was very low.

Table 2. The ring cleavage[a] by catechol 1,2-dioxygeanse and 3-methyl catechol 1,2-dioxygenase

Substrate	Cell-free extract[b]		
	Benzene	Toluene	*m*-Xylene
Catechol	9.38	1.24	0.05
3-Methyl catechol	4.46	0.70	0.30

[a][ΔOD/mg-protein·min]

[b]Cell-free extract from the cells grown on

In brief, when the cells were pre-adapted to benzene inducing high level of catechol 1,2-dioxygenase, the degradation of *m*-xylene was accelerated.

Though this research supplies useful theoretical and experimental information to increase the degradation rates of BTX, further study is required to clarify the exact action of enzymes on BTX degradation and apply the principles found in this study to the degradation of other aromatic pollutants.

REFERENCES

Arnow, L. E., "Colorimetric Determination of the Components of 3-4-dihydroxy-phenylalanine-tyrosine Mixture," *J. Biol. Chem.*, **118**, 531 (1937).

Bauer, J. E. and Capone, D. G., "Effects of Co-occurring Aromatic Hydrocarbons on Degradation of Individual Polycyclic Aromatic Hydrocarbons in Marine Sediment Slurries," *Appl. Environ. Microbiol.*, **54**, 1649 (1988).

Duetz, W. A., de Jong, C., Williams, P. A. and van Andel, J. G., "Competition in Chemostat Culture Between *Pseudomonas* Strains that Use Different Pathways for the Degradation of Toluene," *Appl. Environ. Microbiol.*, **60**, 2858 (1994).

Evans, W. C., "Oxidation of Phenol and Benzoic Acid by Some Soil Bacteria," *J. Biochem.*, **41**, 373 (1947).

Folsom, B. R., Chapman, P. J. and Pritchard, P. H., "Phenol and Trichloroethylene Degradation by *Pseudomonas cepacia* G4: Kinetics and Interactions Between Substrates," *Appl. Environ. Microbiol.*, **56**, 1279 (1990).

Fujita, M., Ike, M. and Kamiya, T., "Accelerated Phenol Removal by Amplifying the Gene Expression with a Recombinant Plasmid Encoding Catechol 2,3-oxygenase," *Wat. Res.*, **27**, 91 (1993).

Hamzah, R. Y. and Al-Baharna, B. S., "Catechol Ring-cleavage in *Pseudomonas cepacia*: the Simultaneous Induction of Ortho and Meta Pathways," *Appl. Microbiol. Biotechnol.*, **41**, 250 (1994).

Heipieper, H. J. and de Bont, J. A. M., "Adaptation of *Pseudomonas putida* S12 to Ethanol and Toluene at the Level of Fatty Acid Composition of Membranes," *Appl. Environ. Microbiol.*, **60**, 4440 (1994).

Satsangee, R. and Ghosh, P., "Anaerobic Degradation of Phenol Using an Acclimated Mixed Culture," *Appl. Microbiol. Biotechnol.*, **34**, 127 (1990).

Shimp, R. J. and Pfaender, F. K., "Influence of Easily Degradable Naturally Occurring Carbon Substrates on Biodegradation of Monosubstituted Phenol by Aquatic Bacteria," *Appl. Environ. Microbiol.*, **49**, 394 (1985).

Waite, J. H. and Tanzer, M. L., "Specific Colorimetric Detection of o-Diphenols and 3,4-Dihydroxyphenylalanine-containing Peptides," *Anal. Biochem.*, **111**, 131 (1981).

Yeom, S. H., Kim, S. H., Yoo, Y. J. and Yoo, I. S., "Microbial Adaptation in the Degradation of Phenol by *Alcaligenes xylosoxidans* Y234," *Korean. J. Chem. Eng.*, **14**, 37 (1997).

Yeom, S. H., Lee, J. H. and Yoo, Y. J., "Cometabolic Removal of Xylene Isomers by *Alcaligenes Xylosoxidans* Y234," *J. Microbiol. Biotechnol.*, **8**, 222 (1998).