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# MICROBIAL ADAPTATION IN THE DEGRADATION OF PHENOL BY *Alcaligenes xylosoxidans Y234*

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**Abstract** – Microbial adaptation was performed to enhance the biological degradation of phenol. When *Alcaligenes xylosoxidans Y234* was adapted with benzene, it could degrade phenol of 1,000 ppm completely in 60 hours while phenol-adapted cell could not. This phenomenon was discussed in terms of intracellular enzyme activity and applied to the degradation of phenol in a packed-bed bioreactor.

*Key words*: *Adaptation, Phenol, Degradation, Enzyme, Packed-Bed*

## INTRODUCTION

Many aromatic compounds such as benzene, toluene, ethylbenzene, xylenes and phenol are the major products of petroleum and fine chemical industries. However, most of them are carcinogen or potential carcinogen and are classified as prior pollution materials by U.S. EPA (Environmental Protection Agency). Phenol is very toxic to biosphere and its odor and toxicity become much more serious when combined with halogens. Therefore, treatment to remove phenol in the effluent streams using microorganisms have been performed for several decades [Park and Kim, 1995; Worden and Donaldson, 1986; Yang and Humphrey, 1975; Zache and Rehm, 1989; Zilli et al., 1993].

In the study of biodegradation, many environmental factors such as pollutant concentration, viable biomass concentration, existence of inhibitor, temperature, pH, microbial competition and microbial adaptation must be considered [Arvin et al., 1989; Chang et al., 1993; Oh et al., 1994]. Among them, microbial adaptation has been widely studied because prior adaptation history significantly affects the degradation pattern [Rozich and Colvin, 1986]. Satsangee and Ghosh [1990] observed that the phenol degradation rate depends on the periods in which the culture was adapted to phenol. And Bauer and Capone [1988] reported that prior exposure to anthracene, naphthalene, phenanthrene and benzene resulted in enhanced naphthalene degradation while anthracene degradation was stimulated only by benzene and anthrathene preexposure. Shimp and Pfaender [1985] observed that the natural substrate such as amino acids, carbohydrates and fatty acids stimulated the degradation of m-cresol, m-aminophenol and p-chlorophenol. And there was an attempt to explain the mechanism of microbial adaptation. Hiepieper and DE Bont [1994] reported that *Pseudomonas putida S 12* was more tolerant to ethanol when preadapted to supersaturating concentration of toluene. And the reason was discussed in terms of change of cell membrane composition. But few report dealt the adpatation mechanism in the aspect of enzyme induction.

In this paper, novel microbial adaptation method to enhance the biological degradation of phenol is reported and microbial adaptation was discussed in terms of catechol 1,2-dioxygenase induction.

## MATERIALS AND METHODS

### 1. Microorganism

The microorganism, *Alcaligenes xylosoxidans Y234* (abbreviated as *Y234*), used in this study was isolated from crude oil contaminated soil. It can degrade benzene, toluene and phenol.

### 2. Mineral Medium

*Y234* was precultured at 30 °C in 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 $MgSO_4 \cdot 7H_2O$). After the cell was harvested, it was washed with distilled water several times. And about 20 mg of microorganisms were put into the 500 ml flask containing 200 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 $MgSO_4 \cdot 7H_2O$, 200 μl/L trace element and phenol). The trace element consists of 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.

### 3. Immobilization and Operating Conditions

Sodium alginate was dissolved in hot distilled water to make 5% solution. The microorganisms harvested from precultured solution by centrifugation (Hitachi, SCR 18B) were resuspended in the feeding solution and mixed with same amount of sodium alginic acid solution. This mixture was extruded through a thin needle into a 1 % $CaCl_2$ solution thus forming beads with a diameter of about 3 mm. After hardening for 1 hour in this solution, the beads were washed several times with distilled water. The column was 30 cm long and 2 cm inner diameter and 60 $cm^3$ volume of the column was packed with about 600 beads. The feeding solution contained 0.3 g/L $KH_2PO_4$, 2 g/L $(NH_4)_2SO_4$, 0.3 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2$ and phenol.

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The operation was conducted at 25 °C.

**4. Assays**

Phenol concentration was determining using a colorimetric method [Folsom et al., 1990]. In this assay, 2 ml of sample (below 25 ppm) was mixed with 100 μl of 2 N $NH_4OH$, 50 μl of 2% 4-aminoantipyrene and 50 μl of 8% $K_3Fe(CN)_6$. And the optical density of mixture was measured at 510 nm using spectrophotometer (UVIKON, Kontron Instrument). The optical density of microorganisms was determined at 660 nm. Catechol 1,2-dioxygenase activity was assayed as follows: 50 μl of 10 mM catechol and 50 μl of 0.5 g/L cell free extract were added into 2 ml phosphate buffer (60 mM, pH 7.0). Then the activity was assayed by measuring the rate of increase in absorbance at 255 nm in 1 min [Hamzah and Al-Baharna, 1994]. The total protein concentration in cell free extract was determined according to the Bradford method [Bradford, 1976] using a Bio-Rad protein assay kit with bovine albumin as a standard.

## RESULTS AND DISCUSSION

**1. Microbial Adaptation**

*Y234* can utilize benzene and toluene as a sole carbon and energy source. And as shown in Fig. 1, it can also degrade phenol. This is an advantage of *Y234* strain in treating wastewater containing phenol with benzene, toluene or both. But above 400 ppm, it could not degrade phenol. Thus the study to increase the maximum degradation concentration of phenol through microbial adaptation was conducted. To degrade one component easily, the microorganisms have been usually adapted with targeting component [Satsangee and Ghosh, 1990]. But in this study, adaptation with other components was conducted. When *Y234* was adapted with phenol, toluene or benzene, the adaptation result was somewhat different from what was expected. As shown in Fig. 2, when adapted with benzene, *Y234* could degrade higher concentration of phenol than any other case did. It could degrade 1,000 ppm of phenol completely in 60 hours.

Another effect of microbial adaptation was also investigated when other carbon source was fed with phenol. Glucose, the most available carbon source, was chosen. According to Satsangee and Ghosh [1990], the rate of phenol consumption was lower in the presence of glucose compared to the case where phenol was the only carbon source. These results indicated the interference of glucose in the uptake of phenol. But simultaneous consumption of both phenol and acetic acid was also observed when an acetate-adapted culture was used [Satsangee and Ghosh, 1990].

In this study, when glucose was added to the broth, the cell adapted with phenol, toluene or benzene could degrade two carbon sources, glucose and phenol, simultaneously (data is not shown). Among them, benzene-adapted cell showed the highest degradation rate of phenol as shown in Fig. 3. This results imply that adaptation with benzene makes the cell degrade phenol more easily even in the presence of more available carbon source.

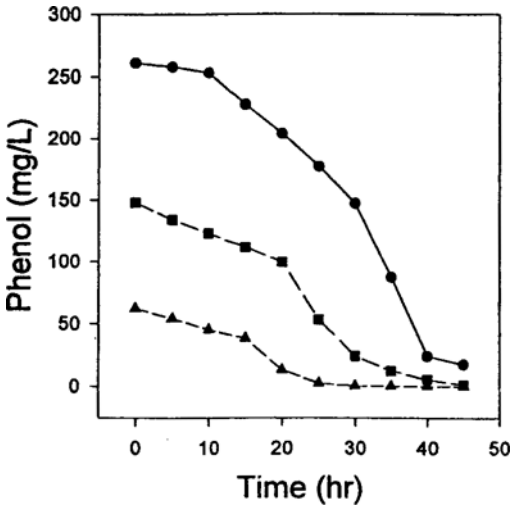


**Fig. 1. Time courses of phenol biodegradation.**

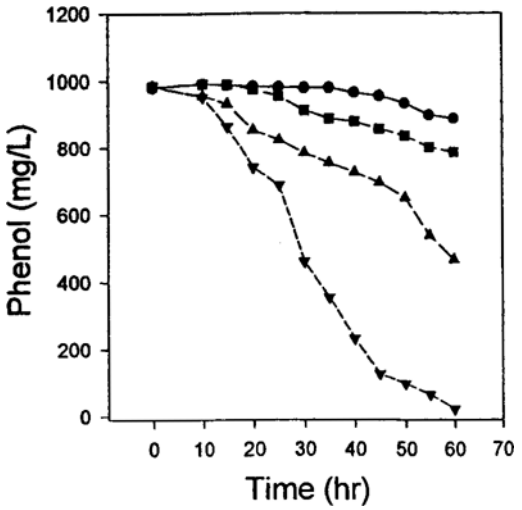


**Fig. 2. The effect of microbial adaptation on phenol degradation.**

●: non-adapted, ■: phenol-adapted, ▲: toluene-adapted, ▼: benzene-adapted

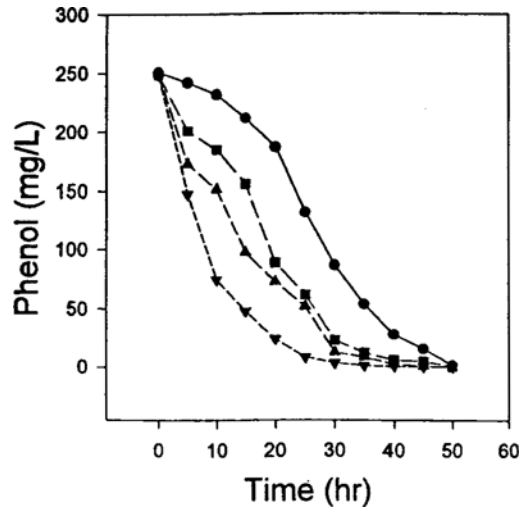


**Fig. 3. The effect of microbial adaptation on phenol degradation when glucose was fed with phenol.**

●: non-adapted, ■: phenol-adapted, ▲: toluene-adapted, ▼: benzene-adapted

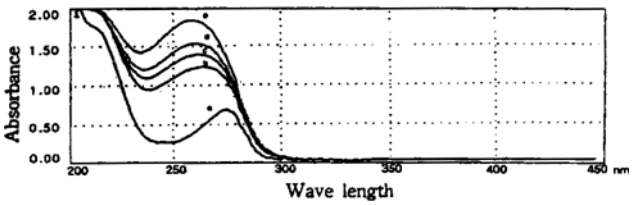


**Fig. 4. Ortho-ring cleavage by catehol 1,2-dioxygenase.**
a: 0 min, b: 5 min, c: 10 min, d: 15 min, e: 20 min

**Table 1. The comparison of catechol 1,2-dioxygenase activity[a] by substrates**

| Time[b] | Benzene | Toluene | Phenol | Glucose |
|---|---|---|---|---|
| 0% | 0.00 | 0.00 | 0.00 | 0.00 |
| 10% | 6.88 | 1.25 | 0.64 | 0.00 |
| 50% | 17.51 | 1.38 | 0.98 | 0.00 |
| 90% | 23.44 | 2.93 | 1.35 | 0.00 |

a: [ΔOD/min]×100, b: 0%; after 2 hours when degradation did not initiate, 10%; right after degradation initiated, 50%; when a half substrate was degraded, 90%; when most of substrate was degraded.

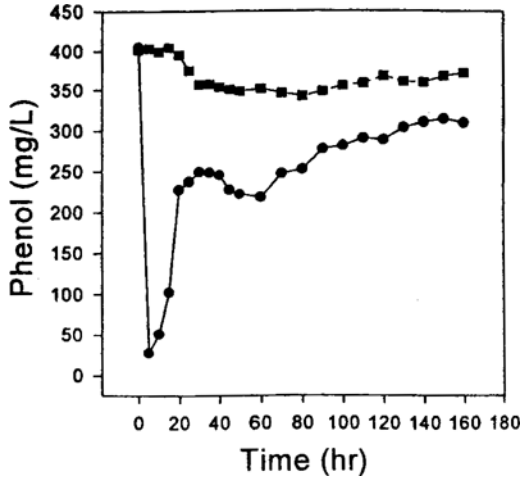


**Fig. 5. The effect of microbial adaptation with benzene in a packed-bed bioreactor.**
●: benzene-adapted, ■: non-adpated cell

## 2. Intracellular Enzyme Activity

There was an attempt to explain the mechanism of microbial adaptation. According to Hiepieper and Bont [1994], one of the key processes in the adaptation of some *Pseudomonas* strains, enabling them to tolerate organic solvents appears to be the isomerization of *cis*- into *trans*-unsaturated fatty acids of cell membrane. But few report dealt the adpatation mechanism in the aspect of enzyme induction.

To find out the reason why benzene is a more powerful adaptation substrate, the reaction of intracellular enzyme was investigated. When phenol, toluene or benzene was fed to *Y234*, each is known to be converted to carbon dioxide and water via catechol. Catechol is then cleaved to cis,cis-muconic acid or 2-hydroxymuconic semialdehyde either by ortho-cleavage pathway mediated by catechol 1,2-dioxygenase or meta-cleavage pathway mediated by catechol 2,3-dioxygenase [Hamzah and al-Baharna, 1994]. As shown in Fig. 4, when cell free extract was mixed with catechol, the specific UV peak shifted from 260

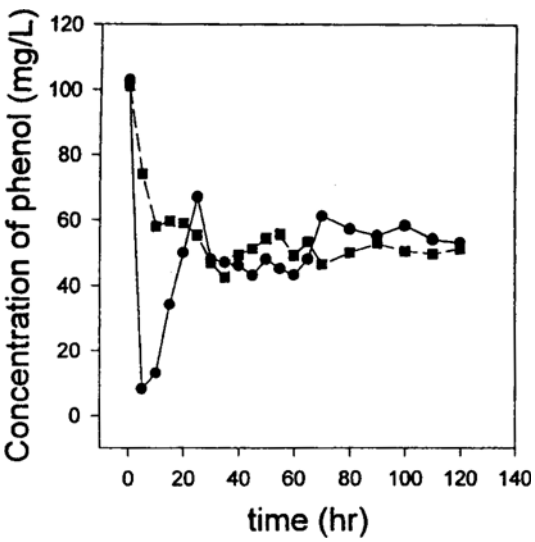


**Fig. 6. The effect of microbial adaptation with benzene when phenol was fed with glucose.**
●: benzene-adapted, ■: non-adapted

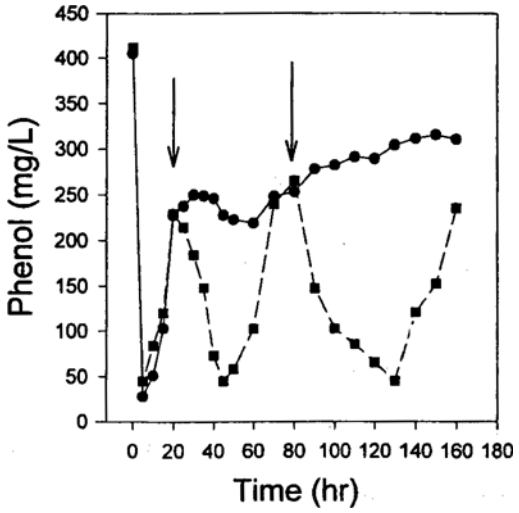


**Fig. 7. The addition effect of benzene on phenol degradation by benzene-adapted cell in a packed-bed bioreactor. Arrows represent the addition of benzene.**
●: without benzene addition, ■: with benzene addition

nm (catechol) to 250 nm(cis,cis-muconic acid). It showed that only ortho-cleavage was present in *Y234*. Since catechol 1,2-dioxygenase activity was induced in the cells grown on benzene, toluene or phenol but not in those grown on glucose, it is inducible. The level of catechol 1,2-dioxygenase of the cells induced by benzene was much higher than those induced by toluene or phenol as shown in Table 1.

Since the starting point of degradation was the same as the time of the enzyme induction, it was thought that catechol ring cleavage by catechol 1,2-dioxygenase may be a bottle-neck of phenol degrading process. Therefore benzene which enhanced the activity of catechol 1,2-dioxygenase made the degradation of phenol more readily.

## 3. Packed-Bed Bioreactor Operation

The result obtained from the above experiments was applied

to a packed-bed bioreactor. Fig. 5 shows that non-adapted cell could not degrade phenol but benzene-adapted cell did when phenol of 400 ppm was fed. But as the time went, the effluent concentration of phenol became high due to the loss of the enzyme activity. When the mixture of glucose (400 ppm) and phenol (100 ppm) was fed, the similar result was obtained as shown in Fig. 6. In both cases, it was thought that the catechol 1,2-dioxygenase activity was decreased gradually in the absence of benzene. Therefore benzene was fed intermittently to the bioreactor. Fig. 7 shows that when benzene of about 100 ppm was fed intermittently for 2 hours during the phenol degradation, the removal efficiency was much improved.

From the results obtained in this study, when the wastewater containing phenol is to be treated, microbial adaptation process with benzene is recommended. When other aromatic compounds such as benzene and toluene are fed with phenol, the degradation efficiency of phenol can be increased.

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