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OVERCOMING THE INHIBITION EFFECTS OF METAL IONS IN THE DEGRADATION OF BENZENE AND TOLUENE BY *ALCALIGENES XYLOSOXIDANS Y234*

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Abstract – The effects of metal ions on the biodegradation of benzene and toluene were investigated. Among 12 tested metal ions, Cu^{2+}, Ni^{2+}, Co^{2+} and A_g^{+} inhibited the degradation of benzene and toluene severely by *Alcaligenes xylosoxidans Y234*. Cu^{2+} was found to inhibit catechol 1,2-dioxygenase in the degradation of benzene and toluene. Co^{2+} and Ni^{2+} were supposed to inhibit benzoate 1,2-dioxygenase, while Ag^{+} was supposed to inhibit benzaldehyde dehydrogenase in the degradation of toluene. The inhibition effect caused by these metal ions could be overcome both by microbial adaptation and by adding specific aromatic compounds to the broth.

Key words : Metal Ion, Benzene, Toluene, Biodegradation, Inhibition

INTRODUCTION

Aromatic compounds such as benzene, toluene, ethylbenzene, xylenes and phenol are the major products of petroleum and fine chemical industries. But most of them are regarded as carcinogen or potential carcinogen and classified as prior pollution materials by EPA(Environmental Protection Agency) because of their toxicity to environment and human health. Since benzene and toluene have relatively higher solubility in water than any other hydrocarbon and tend to migrate from contaminated soils into aquifers, if discharged, drinking water can be contaminated [Oh et al., 1994]. Therefore many researches have been conducted to remove them from soil and wastewater [Alvarez and Vogel, 1991; Arvin et al., 1989; Lee et al., 1995]. Recently, biological treatment using microorganisms has been extensively explored instead of physical or chemical treatment because it does not produce secondary effluent problems [Satsangee and Ghosh, 1990].

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 [Alexander, 1994; Chang et al., 1993; Oh et al., 1994]. Metal ions including heavy metal ions play an important role with trace level in the action of enzyme as inhibitor or stimulator to the activity of microorganisms [Gadd and Griffiths, 1978; Gottschalk, 1985]. And heavy metal contamination from both natural (erosion, fires, leaching, volcanic activity and microbial transformation) and anthropogenic (industrial waste, dumping of sewage, burning of fossil fuels, etc.) sources also results in the accumulation of heavy metals in the environment [Chun and Genthner, 1996]. Therefore, the effects of metal ions on the biodegradation of pollutants are also to be considered. Many researches have been conducted on the effects of metal ions on cell physiology and degradation of pollutants by microorganism. In the degradation of benzene and toluene, many enzymes such as benzene oxygenase, toluene oxidase, benzyl alcohol dehydrogenase, benzaldehyde dehydrogenase, benzoate 1, 2-dioxygenase, catechol 1,2-dioxygenase and catechol 2,3-dioxygenase are known to be involved. Of them, catechol 1,2-dioxygenase and catechol 2,3-dioxygenase are relatively well studied [Hamzah and Al-Baharna, 1994; Nakazawa and Nakazawa, 1970; Nazaki, 1970]. According to Hughes and Poole, most dioxygenases appear to contain a metal as their cofactor and the metal may be iron in both monooxygenase and dioxygenase; certain monooxygenase contain copper. Gibson et al. found that Fe^{2+}, Fe^{3+} and Ca^{2+} increased the activity of benzene oxygenase while Mg^{2+} and Zn^{2+} inhibited that enzyme completely. Yeh et al. observed that the activity of toluene dioxygenase was enhanced by Fe^{2+} and Fe^{3+} while it was inhibited by Cu^{2+} and Zn^{2+}. Nakazawa and Nakazawa reported that catechol 1,2-dioxygenase was inhibited by Hg^{2+}, Ag^{+} and Cu^{2+}. Chun and Genthner investigated the effect of added Cd^{2+}, Cu^{2+}, Cr^{4+} and Hg^{2+} on the metabolism in anaerobic bacterial consortia which degrade 2-chlorophenol, 3-chlorobenzoate, phenol and benzoate. According to them, heavy metal ions extended acclimation periods and reduced dechlorination or biodegradation rate.

In this study, the effects of metal ions on the degradation of benzene and toluene by *Alcaligenes xylosoxidans Y234* were investigated by considering the relationship between enzymes and metal ions. And a strategy to overcome the inhibition effect by metal ions were suggested.

MATERIALS AND METHOD

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 m-xylene, phenol

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and so on.

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 2 mg of microorganisms were put into the 120 ml serum bottle closed with silicon rubber septa and 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 $(NH_4)_2SO_4$ and 0.3 g/L $MgSO_4 \cdot 7H_2O$)

3. Metal Ion

12 metal ions of 5×10^{-2} mol/L were prepared by dissolving following compounds; $FeSO_4 \cdot 7H_2O$, $Fe_2(SO_4)_3 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, $CrCl_2 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, $ZnCl_2$, $NiCl_2 \cdot 6H_2O$, $CdCl_2 \cdot H_2O$, $AlCl_3 \cdot 6H_2O$ and $AgNO_3$. To find out the effect of metal ion on the degradation of benzene and toluene, 100 μl of metal solution was added to the bottle which didn't contain any trace element.

4. Assays

The concentration of benzene and toluene 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. The used column was HP-1 and GC operation conditions were: 150°C injection port, 100°C oven and 250°C detection port temperature. Two controls not containing microorganism were also assayed to compensate abiotic loss. The optical density of microorganisms was determined at 660 nm using spectrophotometer (UNIKON, Kontron Instrument). The concentration of metal ions was measured by ICP (Per- kin Elmer, UK). 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. The Effect of Metal Ions on the Degradation of Benzene and Toluene

To investigate the effect of metal ions on the degradation of benzene and toluene, 12 metal ions were tested. As shown in Table 1, Fe^{2+}, Fe^{3+}, Mg^{2+} and Ca^{2+} increased the degradation rate of toluene by about 20% in comparison with the control which did not contain any metal ion. But there was no helpful metal ion in the degradation of benzene. The reason why degradation rate of benzene was lower than that of toluene was that the adaptation time of *Y234* to benzene was much longer than that of *Y234* to toluene.

Benzene and toluene were not degraded at all in the presence of Cu^{2+}, Ni^{2+}, Co^{2+} or A_g^+. And the cell mass was significantly decreased during incubation in the presence of these inhibitory metal ions since *Y234* did not utilize benzene and toluene as a substrate. But in the case of Cu^{2+}, the decrease of cell mass was very slight. The uptake level of these four metal ions by *Y234* were very low when compared with those of other tested metal ions as can be seen in Table 2. Therefore, it is thought that if an inhibitory metal ion is fed to microorganism, it neither degrade substrate nor uptake the metal ion.

Table 1. The effects of metal ions on the degradation of benzene and toluene

Metal ion	Benzene		Toluene	
	Rate[a]	Final cell mass (O.D.)	Rate[a]	Final cell mass (O.D.)
Control[b]	0.2272	0.3686	0.3125	0.3872
Fe^{2+}	0.2000	0.4152	0.3333	0.4203
Fe^{3+}	0.2083	0.3750	0.3846	0.3925
Al^+	0.2174	0.3124	0.3225	0.3291
Zn^{2+}	0.1851	0.3233	0.3225	0.3289
Mg^{2+}	0.2083	0.3313	0.3333	0.3517
Cr^{2+}	0.0000	0.3260	0.2777	0.2873
Cd^{2+}	0.1851	0.3361	0.2941	0.3129
Ca^{2+}	0.2083	0.3354	0.3333	0.3361
Ni^{2+}	0.0000	0.1474	0.0000	0.1378
Co^{2+}	0.0000	0.1433	0.0000	0.1386
Ag^+	0.0000	0.1671	0.0000	0.1693
Cu^{2+}	0.0000	0.2878	0.0000	0.2634

a: degradation rate (mg-substrate/hr), b : no metal ions were added to the medium.
initial cell mass (O.D.): 0.3012
initial benzene or toluene: 5 mg, 100 μl of metal solution was added.

Table 2. The uptake level of metal ions by *Alcaligenes xylosoxidans Y234* (after 48 hours' incubation)

Metal ion*	Uptake (%)	
	Benzene	Toluene
Fe^{2+}	100	100
Fe^{3+}	100	100
Al^+	93.1	91.23
Zn^{2+}	100	100
Mg^{2+}	100	95.43
Cr^{2+}	100	92.89
Cd^{2+}	100	98.78
Ca^{2+}	97.3	95.53
Ni^{2+}	14.5	12.34
Co^{2+}	29.1	30.62
Ag^+	17.12	15.15
Cu^{2+}	31.2	28.54

*: initial metal ion of 0.25 mmol/L.

2. Analysis of Catabolic Pathway

To find out the enzyme which is affected by metal ion, the catabolic pathway must be revealed. The catabolic pathways of benzene and toluene have been extensively studied by many research groups [Duetz et al., 1994; Gibson et al., 1968; Zylstra and Gibson, 1991]. Since the investigation on biochemical properties of *Y234* was not performed yet, the catabolic pathway of benzene and toluene by *Y234* was assumed on the basis of published papers and indirect evidences. According to previous study, benzene was metabolized to carbon dioxide and water via catechol [Gibson et al., 1968] while toluene was metabolized via catechol or catechol derivatives such as protocatechuic acid or 3-methyl catechol [Duetz et al., 1994; Zylstra and Gibson, 1991]. Five catabolic pathways are known in the degradation of toluene [Duetz et al., 1994; Zylstra and Gibson, 1991]. In three cata-

bolic pathways by *Pseudomonas cepacia, Pseudomonas picketii* PK01 and *Pseudomonas mendocina* KR, o-, m- and p-cresol were found as intermediates, respectively. And these cresols were transformed to 3-methyl catechol or protocatechate. In these cases, catechol 2,3-dioxygenase was involved in the aromatic ring fission. In fourth catabolic pathway by *Pseudomonas putida* F1, toluene was transformed to cis-toluene dihydrodiol and then to 3-methyl catechol. In the fifth catabolic pathway by *Pseudomonas putida* mt-2, toluene was transformed to catechol via benzyl alcohol, benzaldehyde and benzoic acid.

When benzene or toluene was fed excessively to *Y234*, catechol was found in the medium in this study, which was checked by colorimetric method [Waite and Tanzer, 1981]. And the fact that *Y234* cannot degrade o-, m- and p-cresol and that catechol was cleaved by catechol 1,2-dioxygenase as reported elsewhere [Yeom et al., 1997] also implies that catechol was the common metabolite during the degradation of benzene and toluene. When 3-methyl catechol was mixed with cell-free extract of *Y234* culture broth, only small amount of 3-methyl catechol was cleaved at very low rate while catechol was cleaved very quickly (data not shown). And *Y234* degraded all of the aromatic metabolites well found in the degradation of toluene by *Pseudomonas putida* mt-2. These indirect evidences confirmed that *Y234* degraded toluene through the same catabolic pathway as that of *P. putida* mt-2. Fig. 1 shows the assumed catabolic pathway.

3. Analysis of Inhibitory Effect by Metal Ion on Catabolic Pathway

To find out the catabolic step affected by the metal ions in the degradation of toluene, the degradation of four aromatic intermediates mentioned above was checked in the presence of four metal ions. As shown in Table 3, in the presence of Co^{2+} or Ni^{2+} *Y234* did not grow on benzyl alcohol, benzaldehyde, benzoic acid but grew on catechol. It suggests that the catabolic step from benzoic acid to catechol (by benzoate 1,2-dioxygenase) was inhibited by Co^{2+} or Ni^{2+}. As the same logic, Ag^{+} inhibited the step from benzaldehyde to benzoic acid (by benzaldehyde dehydrogenase). When Cu^{2+} was present in the medium, *Y234* did not grow on any aromatic compounds tested. Therefore, Cu^{2+} was thought to inhibit the step from catechol to cis,cis-muconic acid (by catechol 1,2-dioxygenase) or lower catabolic step. As

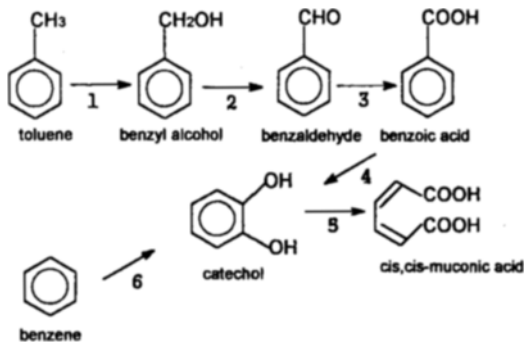

Fig. 1. The suggested catabolic pathway of *Alcaligenes xylosoxidans Y234* in the degradation of benzene and toluene.
1: toluene oxidase, 2: benzyl alcohol dehydrogenase, 3: benzaldehyde dehydrogenase, 4: benzoate 1,2-dioxygenase, 5: catechol 1,2-dioxygenase, 6: benzene oxygenase.

Table 3. The effect of metal ions on the degradation of aromatic intermediates[a]

Substrate		Co^{2+}	Ni^{2+}	Cu^{2+}	Ag^{+}
Benzyl alcohol	14 hr	0.2064	0.1931	0.2521	0.2378
	24 hr	0.1889	0.1739	0.1865	0.2265
Benzyl aldehyde	15 hr	0.2211	0.2222	0.1930	0.1897
	24 hr	0.1999	0.2072	0.1788	0.1795
Benzoic acid	15 hr	0.1954	0.1666	0.2314	0.4932
	24 hr	0.1757	0.1561	0.2925	0.7669
Catechol	15 hr	0.5403	0.5467	0.1433	0.7157
	24 hr	0.7721	0.7589	0.1756	0.6934

a: cell mass (O.D.) after 24 hours' incubation
initial cell mass (O.D.): 0.3017

Table 4. The effect of metal ion on the activity of catechol 1,2-dioxygenase[a]

Metal ion	Catechol 1,2-dioxygenase
Control[b]	10.12
Fe^{2+}	13.75
Fe^{3+}	7.35
Ni^{2+}	6.84
Co^{2+}	6.98
Ag^{+}	7.12
Cu^{2+}	0.01

a: 50 μl of metal solution was added to cell free extract and incubated for 10 minutes. (ΔOD/min)×100
b: no metal ions were added to the broth.

shown in Table 4, Cu^{2+} inhibited catechol 1,2-dioxygenase completely but Ni^{2+}, Co^{2+}, and Ag^{+} did not affect the activity of catechol 1,2-dioxygenase significantly.

But when it comes to benzene, the explanation mentioned above is no longer valid since the catabolic pathway of benzene is different that of toluene. Benzene is known to converted to catechol by benzene oxygenase and then cleaved either by catechol 1,2-dioxygenase or catechol 2,3-dioxygenase. In the case of *Y234*, catechol, whether it was produced from benzene or toluene, was cleaved by catechol 1,2-dioxygenase [Yeom et al., 1997]. According to Nakazawa and Nakazawa, Cu^{2+} is an inhibitor to catechol 1,2-dioxygenase, which is the same result from this study. But Hamzah and Al-Baharna showed Ag^{+} is an inhibitor to catechol 1,2-dioxygenase from *Pseudomonas cepacia*, which is different from the results obtained in this study. Therefore, it can be said that catechol 1,2-dioxygenase from *Y234* has different property from that of *Pseudomonas cepacia*. Gibson et al. reported Mg^{2+} and Zn^{2+} inhibited benzene oxygenase completely, which would inhibit the degradation of benzene. But in this study, Mg^{2+} and Zn^{2+} did not affect the degradation of benzene significantly.

4. Method to Overcome the Inhibition of Metal Ion

As mentioned above, four metal ions inhibited the degradation of benzene and toluene by *Y234*. But the steps affected by these metal ions were supposed to be different from one another in the degradation of toluene. To overcome the inhibition effects caused by metal ions in the degradation of benzene and toluene, many methods can be applied. For example, coculture involving the microorganisms which have excellent ability to remove metals ions may be a available method. And combining

physical or chemical process such as precipitation, ion exchange, membrane separation and filtration also can be a good method.

In this study, enzymatic adaptation method was considered to overcome the inhibition effect caused by metal ions. The logic is that if one inhibitory metal ion to enzyme A is present, a microorganism which already have enough enzyme A through microbial adaptation is inoculated or a compound inducing enzyme A is added to offset the inhibition effect.

At least four enzymes (benzyl alcohol dehydrogenase, benzaldehyde dehydrogenase and benzoate 1,2-dioxygenase and catechol 1,2-dioxygenase) are needed to degrade benzyl alcohol. In other words, whether benzyl alcohol is degraded, four enzymes are already induced. As a same logic, to degrade benzaldehyde, three enzymes (benzaldehyde dehydrogenase, benzoate 1,2-dioxygenase and catechol 1,2-dioxygenase) are required. And to degrade benzoic acid, two enzymes are required while to degrade catechol only one enzyme, catechol 1,2-dioxygenase is required. Therefore, to degrade toluene in the presence of Co^{2+} or Ni^{2+} which inhibits the benzoate 1,2-dioxygenase, benzyl alcohol, benzaldehyde and benzoic acid can be used as microbial adaptation reagent to induce benzoate 1,2-dioxygenase to a high level. As a same logic, to degrade toluene in the presence of Ag^{+}, benzyl alcohol and benzyl aldehyde can be used. To degrade toluene in the presence of Cu^{2+}, all of four aromatic compounds can be used. To impliment this idea, microbial adaptation were conducted. As shown in Table 5, when *Y234* was adapted to benzyl alcohol, toluene was degraded in the presence of Co^{2+}, Ni^{2+}, Cu^{2+} or Ag^{+} in 24 hours while adapted to catechol, only small amount of toluene was degraded in the presence of Co^{2+} or Ni^{2+}. When *Y234* was adapted to any other aromatic compound tested in this experiment, toluene was degraded well in the presence of Cu^{2+}. Though *Y234* could degrade toluene in the presence of any tested four metal ion when adapted to benzyl alcohol or benzaldehyde, in real process, benzyl alcohol may be a more available adaptation reagent since benzaldehyde is more toxic and volatile than benzyl alcohol and the inhibition concentration of benzaldehyde to *Y234* was about 1,000 ppm while that of benzyl alcohol was about 2,000 ppm (data not shown). After 48 hours' incubation, toluene was degraded completely for all cases.

Instead of microbial adaptation, adding of aromatic compound to the medium was tested. When benzyl alcohol was added to the broth instead of inoculating benzyl alcohol-adapted cell, toluene was not degraded in the presence of any four metal ion (data not shown). The reason was thought to be that the inhibition effect by metal ion was more powerful than the stimulating effect by benzyl alcohol. Therefore, amount of metal ion added was lowered from 100 μl to 20 μl. As shown in Fig. 2, toluene was degraded as expected. From these results, the strategy to reduce inhibition effect by metal ion can be suggested as follows : when the concentration of inhibitory metal ion was low, aromatic compounds which induces the target enzyme is added to the broth. And in the case of high concentration of inhibitory metal ions, the adapted cell can be used to overcome the inhibition effect.

But when it comes to benzene, the results are very different. As shown in Table 6, there is no general rule. The microbial adaptation reagent to overcome the inhibition effect by metal ion must be chosen case by case. For example, to degrade benzene in the presence of Ni^{2+}, *Y234* must be adapted to benzaldehyde while in that of Co^{2+}, *Y234* must be adapted to catechol. But the suggested strategy to overcome inhibition effect by metal ions are also available in the degradation of benzene as shown in Fig. 3.

Of four inhibitory metal ions, the inhibition effect by Cu^{2+} which inhibits catechol 1,2-dioxygenase, one of the common enzymes in the degradation of benzene and toluene, could be overcome relatively easily by the suggested method. And the inhibition effect by Ni^{2+} in the degradation of benzene and toluene was the most refractory to overcome.

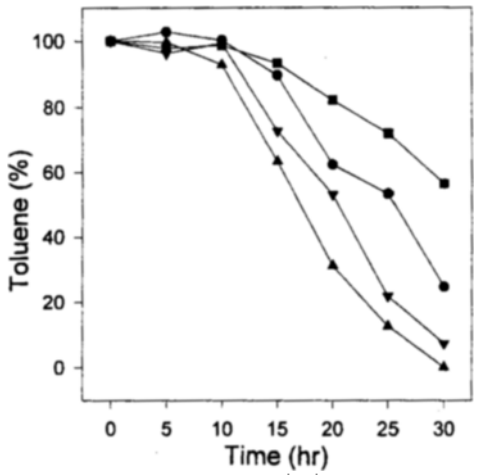

Fig. 2. The effect of adding of aromatic compound on the degradation of toluene in the presence of inhibitory metal ions.
■: Ni^{2+}, ●: Co^{2+}, ▲: Cu^{2+}, ▼: Ag^{+}

Table 5. The effect of microbial adaptation to the aromatic compounds on the degradation of toluene in the presence of metal ions (%)[a]

Adaptation substrate		Co^{2+}	Ni^{2+}	Cu^{2+}	Ag^{+}
Benzyl alcohol	24 hr	88.8	63.2	93.6	83.7
	48 hr	100	100	100	100
Benzyl aldehyde	24 hr	91.9	73.7	91.5	93.9
	48 hr	100	100	100	100
Benzoic acid	24 hr	42.1	26.6	100	66.3
	48 hr	100	100	100	100
Catechol	24 hr	37.7	36.2	100	43.7
	48 hr	100	100	100	100

a: 5 mg of toluene was fed to the broth.

Table 6. The effect of aromatic compound on the degradation of benzene in the presence of metal ions (%)[a]

Substrate		Co^{2+}	Ni^{2+}	Cu^{2+}	Ag^{+}
Benzyl alcohol	24 hr	10.8	0.0	12.9	95.0
	48 hr	13.1	95.6	100	100
Benzyl aldehyde	24 hr	11.2	9.8	4.5	19.7
	48 hr	89.6	100	100	96.2
Benzoic acid	24 hr	4.9	0.0	44.1	19.8
	48 hr	69.3	4.8	100	95.1
Catechol	24 hr	29.8	0.0	44.6	16.8
	48 hr	100	0.0	100	100

a: 5 mg of benzene was fed to the broth.

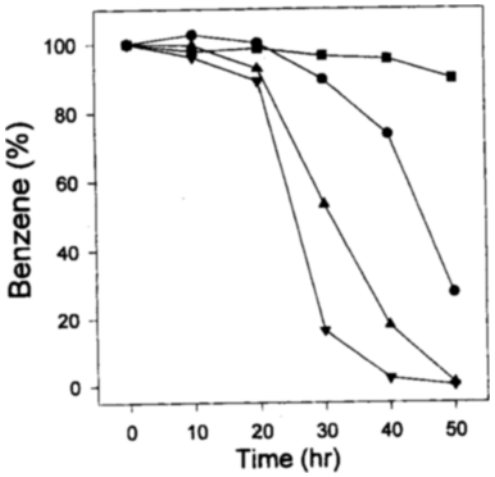

Fig. 3. The effect of adding of aromatic compound on the degradation of benzene in the presence of inhibitory metal ions. ■: Ni^{2+}, ●: Co^{2+}, ▲: Cu^{2+}, ▼: Ag^{+}

Table 7. The effect of microbial adaptation on the uptake level of metal ion (%) by *Alcaligenes xylosoxidans Y234*

Substrate		Co^{2+}	Ni^{2+}	Cu^{2+}	Ag^{+}
Benzyl alcohol	Benzene	39.66	17.20	70.77	55.41
	Toluene	33.69	13.90	75.83	53.33
Benzyl aldehyde	Benzene	76.94	14.69	80.97	56.79
	Toluene	72.34	14.83	79.10	57.1
Benzoic acid	Benzene	66.80	16.59	81.71	61.03
	Toluene	62.97	15.14	83.59	56.31
Catechol	Benzene	58.29	15.07	81.71	50.53
	Toluene	51.54	18.33	78.91	49.67

initial metal ion concentration: 0.25 mmol/L, after 48 hours' incubation.

Table 7 shows the uptake level of metal ion by *Y234* when microbial adapted-*Y234* was inoculated. When compared with the results from Table 3, the uptake level was much higher. But the uptake level of Ni^{2+} was still low.

In this paper, the effects of metal ions on the degradation of benzene and toluene are reported. Though further study must be performed to elucidate the exact relationship between metal ions and degradation of benzene and toluene, the results from this study can be applied to treat wastewater containing both aromatic compounds and metal ions.

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