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Influence of phenol on biodegradation of *p*-nitrophenol by freely suspended and immobilized *Nocardioides* sp. NSP41

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Abstract

The effect of the presence of an alternate toxic compound (phenol) on the *p*-nitrophenol (PNP)-degrading activity of freely suspended and calcium alginate immobilized *Nocardioides* sp. NSP41 was investigated. In the single substrate experiments, when the concentration of phenol and PNP was increased to 1400 mg l^{-1} and 400 mg l^{-1}, respectively, the initial cell concentrations in the freely suspended cell culture should be higher than 1.5 g dry cell weight l^{-1} for complete degradation. In the simultaneous degradation experiment, when the initial concentration of phenol was increased from 100 to 400 mg l^{-1}, the specific PNP degradation rate at the concentration of 200 mg l^{-1} was decreased from 0.028 to 0.021 h^{-1}. A freely suspended cell culture with a high initial cell concentration resulted in a high volumetric degradation rate, suggesting the potential use of immobilized cells for simultaneous degradation. In the immobilized cell cultures, although simultaneous degradation of PNP and phenol was maintained, the specific PNP and phenol degradation rate decreased. However, a high volumetric PNP and phenol degradation rate could be achieved by immobilization because of the high cell concentration. Furthermore, when the immobilized cells were reused in the simultaneous degradation of PNP and phenol, they did not lose their PNP- and phenol-degrading activity for 12 times in semi-continuous cultures. Taken together, the use of immobilized *Nocardioides* sp. NSP41 for the simultaneous degradation of PNP and phenol at high concentrations is quite feasible because of the high volumetric PNP and phenol degradation rate and the reusability of immobilized cells.

Abbreviations: DCW – dry cell weight (mg l^{-1}), 4-NC – 4-nitrocatechol, PNP – *p*-nitrophenol, q – specific degradation rate (h^{-1}), r – volumetric degradation rate (mg l^{-1} h^{-1}), Y – biomass yield (g g^{-1})

Introduction

Nitroaromatic compounds have been used as dyes, pesticides, and explosives. Among these nitroaromatic compounds, *p*-nitrophenol (PNP), a major metabolite resulting from the microbial degradation of parathion (Stutz 1966; Nelson 1982) or methyl parathion (Rani and Lalithakumari 1994), widely occurs as an environmental pollutant during the production of dyes, pesticides, and pharmaceuticals (Popov 1965). Because of its toxicity, PNP is listed as U.S. Environmental Protection Agency priority pollutant (Keith and Telliard 1979). Thus, nitrophenols resulting from industrial activities should be eliminated before they enter the environment.

An attractive method of eliminating PNP is biodegradation to convert it to such essentially harmless compounds as CO_2 and H_2O. This biological technology is also a desirable method for cleaning up contaminated subsurface and ground water (Thomas and Ward 1989). When immobilized cells are used, the efficacy of biodegradation is often improved (Dwyer et al. 1986; Westmeier and Rehm 1987; Keweloh et

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al. 1989; O'Reilly and Crawford 1989; Balfanz and Rehm 1991; Ferschl et al. 1991; Menke and Rhem 1992; Rhee et al. 1996).

In polluted environments, organic pollutants frequently occur in mixtures with other natural as well as synthetic organic compounds (Keith and Telliard 1979; Pfeffer 1979). Because PNP biodegradation can be greatly affected by the presence of other organic compounds, especially toxic aromatic compounds (Beltrame et al. 1984; Schmidt et al. 1987; Zaidi and Mehta 1995), the study on the effect of alternate toxic compounds on the biodegradation of PNP is important from a practical aspect.

In our laboratory, we have studied the effect of toxic aromatic compounds on the degradation of PNP. For the study, phenol was selected as an alternate toxic aromatic compound from among various toxic compounds, because of its frequent occurrence in the environment. We have shown two different metabolic pathways of PNP and phenol degradation in *Nocardioides* sp. NSP41 (Cho et al. 1998). The aim of the present study was to investigate the PNP degradation in the presence of phenol by freely suspended and immobilized *Nocardioides* sp. NSP41.

Materials and methods

Microorganism

Nocardioides sp. NSP41 was isolated from industrial wastewater by selective enrichment as previously described (Lee et al. 1991). This strain was described as a new species of the genus *Nocardioides*, *Nocardioides nitrophenolicus* (Yoon et al. 1999). Pure cultures of strain NSP41 were used throughout the experiments.

Media

Three different media were used in this study. The medium composition (Medium I) for the inoculum preparation used for both freely suspended and immobilized cells contained (l^{-1}): K_2HPO_4, 5.0 g; $NaH_2PO_4{\cdot}2H_2O$, 0.4 g; KCl, 0.25 g; $MgSO_4{\cdot}7H_2O$, 0.25 g; and trace element solution (Lee et al. 1991), 1 ml, and pH was adjusted to 8.0. Acetate (0.2%) and NH_4Cl (0.1%) were supplied as carbon and nitrogen sources, respectively. The medium composition for freely suspended cells (Medium II) was basically the same as for the inoculum preparation except for the carbon sources. PNP and phenol were used as carbon sources. In order to improve the strength of the calcium alginate beads, the salt composition and concentration in the medium for immobilized cells were modified (Ferschl et al. 1991). The medium composition for immobilized cells (Medium III) contained in l liter of Tris-HCl buffer (50 mM, pH 8.0): K_2HPO_4, 0.02 g; KCl, 0.1 g; $MgSO_4{\cdot}7H_2O$, 0.1 g; $CaCl_2{\cdot}7H_2O$, 1.5 g; and trace element solution, 1 ml. PNP and phenol were used as carbon sources. For the degradation of phenol only, NH_4Cl (1.0 g l^{-1}) was used as the sole nitrogen source. As previously described, PNP was used as the sole nitrogen source for the simultaneous degradation of PNP and phenol (Cho et al. 1998).

Immobilization

The cells were immobilized in gel beads of calcium alginate as follows: (1) Exponentially growing cells were harvested into a paste by centrifugation at 5,000 rpm for 10 min. After discarding the supernatant, the cell paste was washed with sterilized distilled water twice and then resuspended in 1.5% sodium alginate. The final cell concentration was approximately 50 g wet weight l^{-1} of 1.5% sodium alginate. (2) A mixture of cells and sodium alginate was dropped into a 0.1 M $CaCl_2$ solution through the inner tube of a manufactured atomizer using a peristaltic pump (Kwack and Rhee 1992). Gel beads of about 1.0–1.2 mm in diameter were obtained by blowing air through the outer tube of the atomizer. One liter of 0.1 M $CaCl_2$ solution was used for 100 ml mixture of cell and sodium alginate. After immobilization, to harden the calcium alginate beads, one liter of fresh $CaCl_2$ solution was added and resuspended with gentle agitation for 2 h. Finally, these beads were transferred to Erlenmyer flasks.

PNP and phenol degradation experiments

For the freely suspended cell culture, exponentially growing cells were inoculated into 250-ml Erlenmyer flasks containing 50 ml of medium II at an initial cell concentration of 1.5 g dry cell weight (DCW) l^{-1}. The cultures were incubated in a shaker at 150 rpm and 30 °C. For the immobilized cell culture, 10 g wet weight of calcium alginate beads was inoculated into 250-ml Erlenmyer flasks containing 40 ml of medium III. The initial cell concentration was 2.5 g DCW l^{-1} medium. Like the freely suspended cell culture, the immobilized cell culture was incubated in a shaker at 150 rpm and 30 °C. The culture was carried out in a repeated batch mode. To exchange the medium, the

beads were first allowed to settle, the supernatant was then removed, and fresh medium was added to the Erlenmyer flasks.

Analytical methods

Samples were withdrawn periodically for analysis. Cell growth was monitored by measuring the optical density of the culture broth at 600 nm, and was expressed as DCW based on the standard curve. In order to estimate the cell growth of the immobilized cells, the immobilized cells were first freed from the calcium alginate beads by treatment with potassium phosphate buffer (0.2 M, pH 7.0) and the cell concentration was determined by measuring the optical density (Keweloh et al. 1989). For the analysis of PNP and phenol, the culture broth sample was centrifuged and the supernatant directly analyzed. The concentrations of PNP, phenol, and 4-nitrocatechol were determined by isocratic reverse-phase high performance liquid chromatography (HPLC) equipped with a Nova-pak C_{18} column (Millipore Waters, Milford, MA., USA) and a Waters model 44l UV detector. For the quantification of compounds, the external standard method was used at 280 nm (Cho et al. 1998).

Determination of specific degradation rate, volumetric degradation rate, and biomass yield coefficient

The kinetics of growth and substrate degradation in batch culture are described by simple differential equations. Cell growth is described by

$$\frac{dX}{dt} = \mu X$$

where X is the cell concentration (dry cell weight), t is the cultivation time, and μ is the specific growth rate. The specific degradation rate of the substrate, q, was determined as follows:

$$-\frac{dS}{dt} = qX$$

where S and q are the concentration of substrate and specific degradation rate, respectively. Integrating the above equations, one obtains

$$X = X_0 \cdot e^{\mu t}$$

$$[S]_0 - [S] = q \cdot \frac{1}{\mu}(X - X_0)$$

where X_0 and $[S]_0$ are the initial cell and substrate concentrations, respectively. The parameters μ and q can be found by linear regression techniques. Hence a plot of $([S]_0 - [S])$ versus $(X - X_0)/\mu$ should give a straight line with a slope of q. Only the data confined to the exponential growth phase was used (Ozturk and Palsson 1990).

The volumetric degradation rate, r, is defined as follows:

$$r = \frac{[S]_0 - [S]_{t_c}}{t_c}$$

where t_c denotes the total cell culture time of each batch cycle, and $[S]_{t_c}$ denotes the final substrate concentration during the exponential growth phase of each batch cycle (Rhee et al. 1996).

The biomass yield coefficient, $Y_{x/s}$, was obtained from the linear relationship between final cell density and initial substrate concentration (Stanlake and Finn 1982).

Results

Freely suspended cell culture at varying initial cell concentrations and phenol concentrations

In order to determine the effect of cell concentration on the phenol degrading activity of cells at varying phenol concentrations, freely suspended cell cultures with varying initial cell concentrations were carried out.

Figure 1 shows the significant interdependence among the times needed for phenol degradation, phenol concentration, and initial cell concentration. When phenol concentrations were lower than 1000 mg l^{-1}, phenol was completely degraded regardless of the initial cell concentrations used. However, when the phenol concentration was increased to approx. 1400 mg l^{-1}, phenol could be completely degraded only at an initial cell concentration higher than approx. 1.5 g DCW l^{-1}. At a cell concentration of 1.5 g DCW l^{-1}, strain NSP41 showed the highest volumetric phenol degradation rate (r_{Phenol} = 21.0 mg l^{-1} h^{-1}) at a phenol concentration of 400 mg l^{-1} as the sole carbon source. In the range of phenol concentrations used in this experiment, a lag time for the cell growth was observed. When the phenol concentration was increased, the lag time increased concomitantly. When the phenol concentration was higher than 1000 mg l^{-1}, the cell concentration was decreased significantly during lag time because of the toxicity of phenol

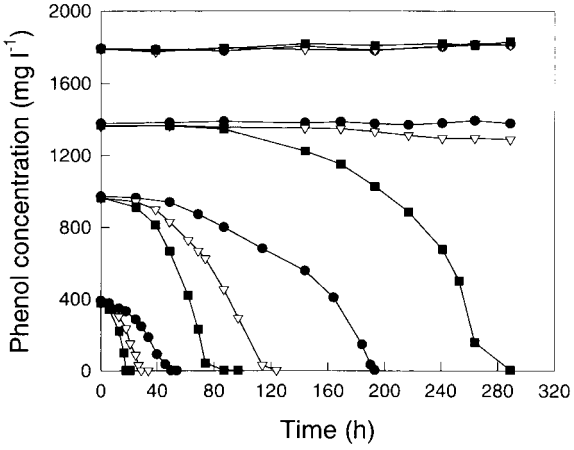

Figure 1. Effect of initial phenol and cell concentration on phenol degradation by freely suspended cells. Initial cell concentration used (g l^{-1}): ● 0.3; ▽ 0.9; ■ 1.5.

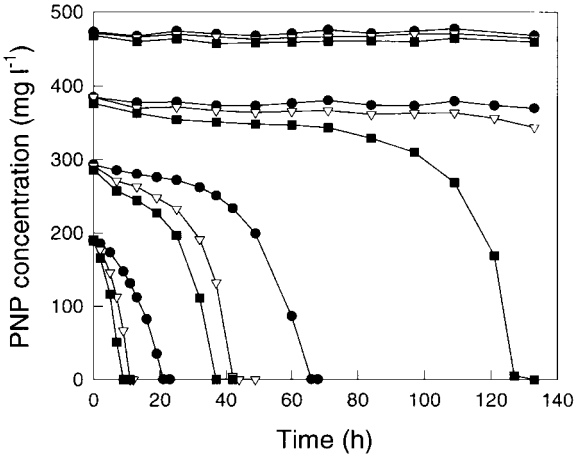

Figure 2. Effect of initial cell concentration on PNP degradation by freely suspended cells at varying PNP concentrations. Initial cell concentration used (g l^{-1}): ● 0.3; ▽ 0.9; ■ 1.5.

to the cells. For example, at the phenol concentration of 1400 mg l^{-1}, the initial cell concentration of 1.5 g DCW l^{-1} was decreased to approximately 1.15 g DCW l^{-1} during lag phase.

Freely suspended cell culture at varying initial cell concentrations and PNP concentrations

Figure 2 shows the effect of initial cell concentrations on the PNP degradation by freely suspended cells, when PNP was used as the sole carbon, nitrogen, and energy source. Because PNP was more toxic to cells than phenol, PNP severely inhibited PNP degradation and cell growth of strain NSP41. When the PNP concentration was lower than 300 mg l^{-1}, PNP was completely degraded regardless of the initial cell concentrations used. However, when the PNP concentration was increased to approx. 400 mg l^{-1}, PNP could be completely degraded only at an initial cell concentration higher than approx. 1.5 g DCW l^{-1} (Table 1). Furthermore, during the PNP degradation, we could not observe any significant cell growth because of the toxicity of PNP and the low biomass yield ($Y_{X/\mathrm{PNP}}$ = 0.28 g g^{-1}) as compared to the biomass yield ($Y_{X/\mathrm{Phenol}}$ = 0.41 g g^{-1}) with phenol as the sole source of carbon.

As shown in Figure 1 and Figure 2, the maximal degradable concentrations of phenol and PNP could be increased at higher initial cell concentrations. In addition, cultures with high cell concentrations were able to degrade substrates faster than those with low cell concentrations regardless of substrate concentrations.

Effect of varying phenol concentrations on the degradation of PNP by freely suspended cells

In order to test the effect of an alternate toxic compound on the degradation of PNP, phenol was used as a supplementary toxic compound. Figure 3 shows the effect of different initial phenol concentrations when the initial PNP concentration was fixed. Initial cell concentration was 1.5 g DCW l^{-1}. PNP and phenol could be degraded simultaneously by strain NSP41. When the concentration of phenol was increased from 100 mg l^{-1} to 400 mg l^{-1}, the specific PNP degradation rate (q_{PNP}) at the concentration of 200 mg l^{-1} was decreased from 0.028 to 0.021 h^{-1}, but the specific phenol degradation rate (q_{Phenol}) was increased from 0.010 to 0.042 h^{-1} (refer to the Table 2). However, when PNP concentration was increased more than 300 mg l^{-1} in the presence of phenol, PNP could not be degraded completely (data not shown).

As shown in Figure 3A, when the phenol concentration was lower than the PNP concentration, 4-nitrocatechol (4-NC) was transiently accumulated after the depletion of phenol. However, the accumulation of 4-NC had no significant effect on the volumetric PNP degradation rate.

Immobilized cell culture at varying PNP concentrations

In order to determine the effect of immobilization on PNP-degrading activity and the tolerance of cells for PNP toxicity, immobilized cell cultures were carried out at various PNP concentrations. To clearly show the

Table 1. Volumetric PNP degradation rate (r_{PNP}) and specific PNP degradation rate (q_{PNP}) of freely suspended and immobilized cells at varying initial PNP concentrations

Initial cell Concentration ($g\ l^{-1}$)		PNP concentration ($mg\ l^{-1}$)						
		100	200	300	400	500	600	700
Freely suspended cells								
1.5	r_{PNP} ($mg\ l^{-1}\ h^{-1}$)	31.2	21.0	7.7	2.8	0.1	n.d.[a]	n.d.
	q_{PNP} (h^{-1})	0.028	0.019	0.013	0.009	<0.001	n.d.	n.d.
Immobilized cells								
2.5	r_{PNP} ($mg\ l^{-1}\ h^{-1}$)	35.0	26.0	10.2	9.7	9.3	8.8	0.5
	q_{PNP} (h^{-1})	0.014	0.010	0.004	0.004	0.004	0.004	<0.001

[a]n.d. = not determined.

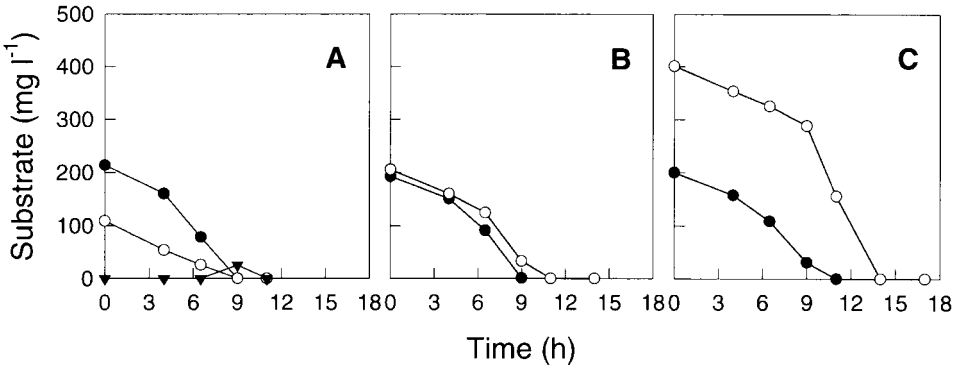

Figure 3. Effect of phenol on PNP degradation in freely suspended cell culture: ● PNP concentration, ○ phenol concentration, ▼ 4-nitrocatechol concentration. Initial cell concentration was 1.5 $g\ l^{-1}$. Initial PNP and phenol concentration used ($mg\ l^{-1}$): (*A*) 200, 100; (*B*) 200, 200; (*C*) 200, 400.

effect of immobilization, the specific PNP degradation rate (q_{PNP}) and the volumetric PNP degradation rate (r_{PNP}) are summarized in Table 1. When the initial cell concentration used was 2.5 g DCW l^{-1}, PNP at concentrations of up to 600 $mg\ l^{-1}$ could be degraded completely within 70 h. When the PNP concentration and initial cell concentration were 200 $mg\ l^{-1}$ and 1.5 g DCW l^{-1} respectively, the q_{PNP} of freely suspended cells was 0.019 h^{-1}, which is approximately 2 times higher than that of immobilized cells. Thus, the specific PNP degradation rate of the cells was decreased by immobilization. The reduction of q_{PNP} after immobilization might have been due to internal diffusional resistance in the alginate beads. However, a high r_{PNP} and increased tolerance for PNP toxicity could still be obtained due to the high cell concentration and immobilization (refer to Table 1). Furthermore, immobilized cells are more suited for a continuous operation than freely suspended cells because of the minimal washout of immobilized cells.

Influence of phenol on the degradation of PNP by semi-continuous culture of immobilized cells

To test the influence of phenol on PNP degradation by immobilized cells and the feasibility and reusability of immobilized cells for continuous cultures, calcium-alginate immobilized *Nocardioides* sp. was cultivated in a medium with 200 $mg\ l^{-1}$ PNP and varying phenol concentrations.

Figure 4A shows the cell concentration during the immobilized cell culture. No significant change in the immobilized cell concentration was observed during the culture. This might be due to the accumulation of dead cells in the alginate beads (Lee et al. 1994; Rhee et al. 1996). The concentration of freely suspended cells in the medium during immobilized cell culture was less than 5% of the total cell concentration, which was neglected in the estimation of q_{PNP}. Figure 4B shows the semi-continuous degradation of PNP by immobilized cells in the presence of phenol. Like the freely suspended cells, the immobilized cells

Table 2. Volumetric degradation rate (r) and specific degradation rate (q) of PNP and phenol by freely suspended and immobilized cells at varying substrate concentrations

Initial cell concentration ($g\ l^{-1}$)		Substrate concentration ($mg\ l^{-1}$)					
		PNP (200) + Phenol (100)		PNP (200) + Phenol (200)		PNP (200) + Phenol (400)	
		PNP	Phenol	PNP	Phenol	PNP	Phenol
Freely suspended cells							
1.5	r ($mg\ l^{-1}\ h^{-1}$)	23.8	12.1	21.4	18.7	18.2	28.6
	q (h^{-1})	0.028	0.010	0.026	0.022	0.021	0.042
Immobilized cells							
2.5	r ($mg\ l^{-1}\ h^{-1}$)	30.9	20.5	24.8	24.8	22.2	34.8
	q (h^{-1})	0.013	0.009	0.010	0.010	0.009	0.014

could degrade PNP simultaneously with phenol. At low phenol concentration (100 mg l^{-1}), 4-NC was accumulated transiently and further mineralized. The increase in the concentration of supplemented phenol slightly decreased the PNP degradation rate of the immobilized cells as shown in Table 2. Figure 4C shows the change in the specific degradation rate (r_{PNP} and r_{Phenol}) and the volumetric degradation rate (q_{PNP} and q_{Phenol}) during the semi-continuous culture of the immobilized cells. When the phenol concentration was increased from 100 to 400 mg l^{-1}, the r_{PNP} and q_{PNP} were decreased by 28% and 31%, respectively. In addition, when the PNP concentration was increased to 400 mg l^{-1} in the presence of phenol, PNP could not be completely degraded (data not shown).

In order to clearly show the effects of phenol on the degradation of PNP by immobilized cells, the q_{PNP} and r_{PNP} of the freely suspended cells and immobilized cells were summarized in Table 2. As previously described, the q_{PNP} of the cells was decreased by immobilization. However, when immobilized cells were used, the maximum concentration of PNP degraded in the presence of phenol increased from 200 to 400 mg l^{-1}. Thus, the high r_{PNP} and the increased tolerance for PNP toxicity in the presence of phenol could still be obtained due to the high cell concentration and immobilization. The immobilized cells could be reused 12 times without losing their degradation activity during the culture period tested. The average r_{PNP} was 26.0 $\pm$ 6.0 mg l^{-1} h^{-1} during the semi-continuous culture of the immobilized cells.

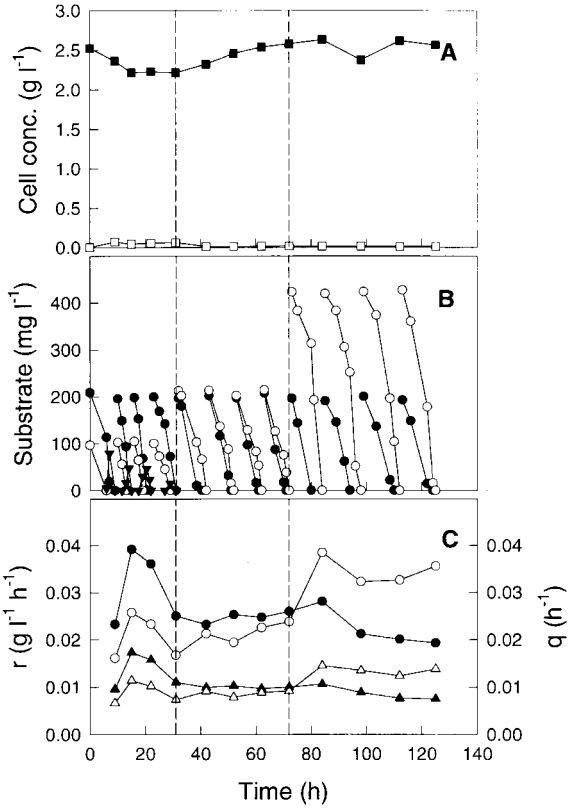

Figure 4. Effect of phenol on degradation of PNP in immobilized cell culture: (*A*) ■ immobilized cell concentration, □ free-suspension cell concentration in the medium; (*B*) ● PNP concentration, ○ phenol concentration, ▼ 4-nitrocatechol concentration; (*C*) ● r_{PNP}, ○ r_{Phenol}, ▲ q_{PNP}, △ q_{Phenol}.

Discussion

Since soil and water polluted with a toxic organic compound can sometimes contain lots of other toxic compounds, the biodegradation kinetics of some contaminants may be significantly changed by the presence of other contaminants (Beltrame et al. 1984; Schmidt et al. 1987; Zaidi and Mehta 1995). Thus, one desirable characteristic of the microorganisms used for biode-

gradation is their ability to degrade a toxic organic compound in the presence of another metabolizable toxic organic compound.

Here, the effect of phenol on PNP-degrading activity and cell growth of freely suspended and immobilized *Nocardioides* sp. NSP41 was investigated at high concentrations of PNP and phenol. There are a few reports on the biodegradation kinetics of phenol and PNP in lake waters (Jones and Alexander 1986; Wiggins and Alexander 1988). However, these determination were made at low concentrations of toxic organic compounds in natural environment.

In the freely suspended cell culture, cultures with a high cell concentration degraded phenol and PNP faster than those with a low cell concentration. In addition, the maximal degradable concentration of phenol or PNP increased as the initial cell concentration was increased. It has been shown that high initial cell concentration results in a high degradation rate of toxic compounds (Balfanz and Rehm 1991; Menke and Rehm 1992; Lee et al. 1994). These data obtained from freely suspended cell cultures suggest that a high cell concentration is desirable for the biodegradation of PNP and phenol.

When the phenol concentration was increased in the simultaneous degradation experiment, the specific PNP degradation rate and the volumetric PNP degradation rate decreased. Furthermore, the maximal degradable concentration of PNP was decreased by the supplementation of phenol. Kinetically, the effect of PNP addition on phenol degradation was also inhibitory (Cho et al. 1998). This probably was the result of increasing the overall concentrations of toxic organic compounds (Menke and Rehm 1992). As reported previously, in the presence of phenol, PNP degradation kinetics could be significantly changed (Schmidt et al. 1987; Wiggins and Alexander 1988; Zaidi and Mehta 1995). However, because of the difference in the concentration of aromatic compounds and cells, direct comparison of our kinetic data with others is difficult only via mathematical models. Furthermore, whether the metabolic pathway of PNP and phenol are cometabolic or not has not been described. This result means that the kinetics of PNP degradation can be changed significantly depending on the concentrations of other toxic compounds and kinds of microorganisms.

Immobilized cells, like freely suspended cells, can also simultaneously degrade PNP and phenol. In immobilized cell cultures, the r_{PNP} and the tolerance of cells for PNP toxicity were improved through the use of high cell concentrations, as previously reported by Heitkamp et al. (1990). Regardless of the presence of PNP, the q_{Phenol} increased in proportion to the concentration of phenol in the range of 100–400 mg l^{-1}. However, the effect of phenol on PNP degradation in immobilized cell cultures did not significantly change as compared with that in freely suspended cell cultures.

At low phenol concentration, phenol was completely mineralized before PNP was completely degraded as shown in Figure 3A and Figure 4B. After the depletion of phenol, 4-nitrocatechol (4-NC) was transiently accumulated and then mineralized. In a previous report (Cho et al. 1998), we showed that PNP was degraded via a hydroquinone pathway and that phenol degraded through a catechol pathway in strain NSP41. A proportion of PNP remaining after the depletion of phenol might have been transformed to 4-NC via a catechol (phenol degradation) pathway. This result shows that two different enzyme systems in *Nocardioides* sp. were induced for the simultaneous degradation of PNP and phenol. Because the accumulated 4-NC disappeared immediately, the 4-NC accumulation can not be a significant problem in the biodegradation.

Owing to a high cell concentration, the r_{PNP} at 200 mg l^{-1} PNP in the immobilized cell culture was higher than that in the freely suspended cell culture. Furthermore, the immobilized cells could be reused 12 times without losing their simultaneous degradation of PNP and phenol during the culture period tested, demonstrating their potential for continuous degradation of PNP and phenol in industrial wastewaters.

In conclusion, the use of immobilized *Nocardioides* sp. NSP41 for the degradation of PNP and phenol in industrial wastewaters is feasible because of the simultaneous degradation of PNP and phenol, the high volumetric degradation rate, and the reusability of the immobilized cells.

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