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Simultaneous degradation of *p*-nitrophenol and phenol by a newly isolated *Nocardioides* sp.

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A *p*-nitrophenol (PNP)- and phenol-mineralizing bacterium (strain NSP41) was isolated from an industrial wastewater and identified as a member of the genus *Nocardioides*. PNP was degraded via a hydroquinone pathway, and phenol was degraded through a catechol pathway in strain NSP41. Both enzyme systems for the degradation of PNP and phenol were induced simultaneously in the presence of both compounds. Although both enzyme systems were induced at the same time, PNP and phenol were degraded by the hydroquinone and catechol pathway, respectively. However, during the simultaneous degradation in the low phenol concentration, after the exhaustion of phenol, some PNP was transformed by the catechol pathway and 4-nitrocatechol was transiently accumulated. Kinetically, the addition of phenol greatly enhanced the apparent PNP degradation rate, which may be due to the increased cell mass by the assimilation of phenol.

Key Words——4-nitrocatechol; hydroquinone; *Nocardioides* sp. NSP41; *p*-nitrophenol; phenol; simultaneous degradation

Nitroaromatic compounds have been used as dyes, pesticides, and explosives. Among these compounds, nitrophenols widely occur as contaminants in the natural environment. Because of the well-known toxicity of nitrophenols for man and animals, their biodegradation has been extensively studied. Especially, the biodegradations of *p*-nitrophenol (PNP), a hydrolytic product of parathion or methyl parathion (Nelson, 1982; Rani and Lalithakumari, 1994), have been described in many researches (Bruhn et al., 1987; Hanne et al., 1993; Jain et al., 1994; Spain and Gibson, 1991).

In natural ecosystems, organic pollutants frequently occur in mixtures with other natural and synthetic organic compounds (Bond and Straub, 1974). Because biodegradation of one aromatic compound could be affected greatly by the presence of other aromatic compounds (Beltrame et al., 1984; Schmidt et al., 1987; Zaidi and Mehta, 1995), the study on the simultaneous degradation of toxic aromatic compounds is important in practical aspects. Recently, it has been reported that methyl- and chloroaromatic compounds are simultaneously degraded by a single organism (Pettigrew et al., 1991; Rojo et al., 1987; Taeger et al., 1988). These compounds were simultaneously degraded by a converged pathway that structurally similar intermediates were transiently produced and then degraded by the same pathway.

Concerning *p*-nitrophenol, it has been reported that there are two kinds of biodegradation pathways: hydroquinone in a *Moraxella* sp. (Spain and Gibson, 1991), and 4-nitrocatechol in an *Arthrobacter* sp. (Jain et al., 1994). On the other hand, phenol is known to be degraded via a catechol pathway (Bayly and Wigmore, 1973; Gaal and Neujahr, 1979). Hanne et al. (1993) reported that a *Nocardia* sp. has two alternative pathways, that is, the hydroquinone pathway induced by PNP and the catechol pathway induced by phenol. However, there have been no previous reports on the simultaneous degradation of PNP and phenol.

In this paper we identified different metabolic pathways of PNP and phenol degradation by a newly isolated bacterial strain and studied the induction of the pathways and the simultaneous degradation of PNP and phenol.

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Materials and Methods

Chemicals. All chemicals used were of a reagent grade. Phenol, *p*-nitrophenol, catechol, hydroquinone, and 4-nitrocatechol were purchased from Aldrich Chemical Company (Milwaukee, WI, USA).

Isolation and characterization of a PNP-degrading bacterium. Some PNP-degrading bacterial strains were isolated from industrial wastewaters by a selective enrichment procedure described by Lee et al. (1991). The enrichment culture was performed in 50 ml nitrogen-free mineral salt medium (see media and culture conditions) supplemented with 100 mg/L PNP as a sole source of carbon, nitrogen, and energy. Strain NSP41, which exhibited the highest PNP degrading activity, was chosen for further study.

The isolate was identified on the basis of morphological, physiological, and chemotaxonomic characterizations and phylogenetic inference based on 16S rDNA sequence. Gram staining, morphology, oxidase, catalase, oxidation-fermentation, and physiological properties were tested according to the procedures outlined in "Manual of Methods for General Bacteriology" (Smibert and Krieg, 1981). The API 20 NE system (Bio Merieux, SA, France) and Biolog GP MicroPlates were used to test physiological characteristics. The G+C content was determined by the method of Tamaoka and Komagata (1984). Fatty acids were extracted and analyzed according to the instructions of the Microbial Identification System (MIDI: Microbial ID, Inc., Newark, DE, USA). The diamino acid of the peptidoglycan was determined by the method described previously (Komagata and Suzuki, 1987). Menaquinone was analyzed as described previously (Komagata and Suzuki, 1987) by using reversed-phase HPLC. The 16S rDNA sequencing and phylogenetic analysis of strain NSP41 were performed as described previously (Yoon et al., 1997).

Media and culture conditions. The medium composition for the isolation of PNP-degrading bacteria contained (per liter) K_2HPO_4, 1 g; $NaH_2PO_4 \cdot 2H_2O$, 0.5 g; KCl, 0.25 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; and trace elements solution (Lee et al., 1991), 1 ml, and pH was 7.2. PNP was supplied as a sole carbon and nitrogen source. The medium composition for main cultures was modified by increasing the concentration of buffer (15 mM, pH 8.0) in the isolation medium. For the degradation of phenol, NH_4Cl (1.0 g/L) was used as a sole nitrogen source.

The cells grown overnight in the minimal salt medium with 0.2% sodium acetate (Hanne et al., 1993), was inoculated to a 250 ml Erlenmeyer flask containing 50 ml of minimal medium and incubated at 30°C in a shaking incubator at 150 rpm. The optimum culture conditions were determined as pH 8.0 and 30°C.

Utilization of different aromatic hydrocarbons. To define the range of aromatic hydrocarbons that this strain can utilize, 50 mg/L of various aromatic compounds were added to the 10 ml minimal salt medium in a 50 ml cap tube. NH_4Cl was used as a sole nitrogen source. The disappearance of substrates and the increase of cell mass were determined by a spectrophotometer at maximum absorbance of each compound and 600 nm, respectively.

Induction and degradation experiments. Resting cells were used to elucidate the first-step degradation metabolite of PNP and phenol. Resting cells were obtained by growth in the mineral salt medium with 0.5 mM PNP and 0.5 mM phenol, respectively. The cells were harvested, washed twice with sterilized distilled water, resuspended in 50 ml of phosphate buffer pH 8.0 (optical density at 600 nm=1.0), and incubated with 0.5 mM substrates in a rotary shaker (Lenke et al., 1992). The disappearance of PNP and phenol and the formation of intermediate metabolites were determined by HPLC and gas chromatography/mass spectrometry (GC/MS).

Analytical methods. Cell growth was monitored by measuring the optical density of the culture broth at 600 nm and was expressed as dry cell weight (D.C.W.) based on the standard curve. For the determination of the nitrite ion concentration in the culture fluid, the photometric method was used (Hanson and Phillips, 1981).

For the analysis of PNP and phenol, the culture broth sample was centrifuged and the supernatant directly analyzed. The concentrations of PNP and phenol 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 441 UV detector. For the quantification of compounds, the external standard method was used at 280 nm. The mobile phase was composed of methanol–water–acetic acid (500 : 500 : 2, v/v/v). The injection volume of samples was 10 µl. The amounts of PNP and phenol were also measured spectrophotometrically by reading the absorption maximum of the respective compound. The extinction coefficients of PNP and phenol were 17,700 $M^{-1}\,cm^{-1}$ at above pH 9 and 400 nm, and 1,500 $M^{-1}\,cm^{-1}$ at 268 nm, respectively.

For the analysis of intermediate metabolites, the metabolites were extracted by using ethylacetate (pH 2.0) and analyzed with gas chromatography/mass spectrometry (Fisons, Altrincham, England) as described previously (Bae et al., 1996a).

Nucleotide sequence accession number. The 16S rDNA sequence of strain NSP41 has been deposited in the GenBank database under accession number

AF005024.

Results and Discussion

Isolation and characterization of a PNP-degrading bacterium

A PNP-degrading bacterium (strain NSP41) was isolated from industrial wastewater serially enriched with PNP as a sole source of carbon, nitrogen, and energy. Some phenotypic characteristics of strain NSP41 are presented in Table 1. This strain contained the LL-type diaminopimelic acid in the cell wall and MK-8(H_4) as a major menaquinone. Strain NSP41 had a complex cellular fatty acid profile containing saturated, unsaturated, *iso*-, *anteiso*-, and 10-methyl-branched fatty acids, and the main fatty acid (found in strain NSP41) was 14-methylpentadecanoic acid (*iso*-$C_{16:0}$). The genomic DNA G+C content of strain NSP41 is 71.4 mol%. Figure 1 shows the position of strain NSP41 at the phylogenetic tree based on the 16S rDNA sequence.

From the result of taxonomic properties that were determined mainly by chemotaxonomic characteristics, and of phylogenetic inference that was based on 16S rDNA sequence, strain NSP41 was identified as a member of the genus *Nocardioides*. The isolate (*Nocardioides* sp. NSP41) has been deposited in the Korean Collection for Type Cultures (KCTC) as KCTC 9800.

Mineralization of PNP by strain NSP41

To see the mineralization of PNP as a single substrate by the strain NSP41, the fermenter culture was carried out. In the 5 L bench-top fermenter culture (working volume, 3.0 L; impeller speed, 150 rpm; aera-

Table 1. Some phenotypic characteristics of a PNP-degrading bacterium, strain NSP41.

Gram stain	+	Assimilation of	
Morphology	coccus/rod[a]	Glucose	+
Size	0.5–1.0 μm	Arabinose	−
Flagellum	single polar	Mannose	−
Spore formation	−	Mannitol	−
Oxidase	+	N-Acetyl-glucosamine	−
Catalase	+		
O/F test[b]	inert	Maltose	−
H_2S production	−	Gluconate	+
Indole production	−	Caprate	−
Voges-Proskauer	+	Adipate	−
Arginine dehydrogenase	−	Malate	+
		Citrate	−
Lysine decarboxylase	−	Phenyl-acetate	−
Tryptophan deaminase	−	Acetate	+
Urease	+	Lactose	+
β-Glucosidase	−	Acid from	
Protease	−	Glucose	−
β-Galactosidase	−	Rhamnose	−
NO_3^- to NO_2^-	−	Sucrose	−
NO_3^- to N_2	−	Inositol	−

[a] Coccus/rod: this strain has displayed a rod shape in the early log phase and a coccus shape in the stationary phase.
[b] O/F, oxidation-fermentation test.

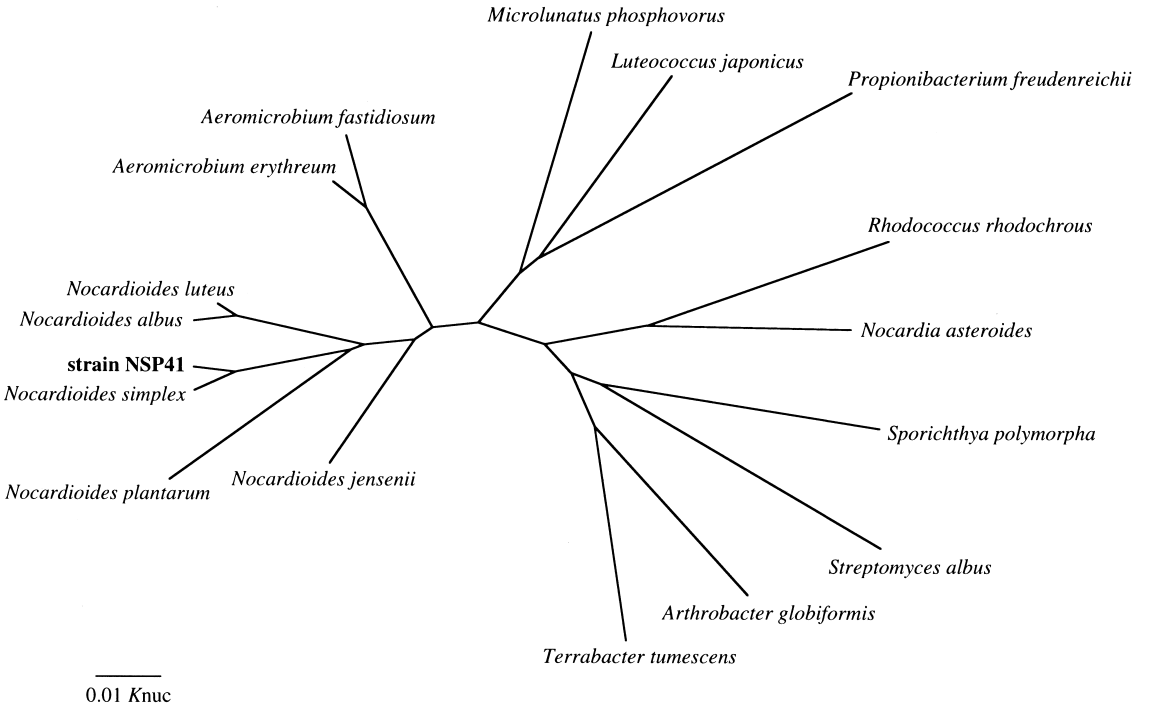

Fig. 1. Phylogenetic tree showing the position of strain NSP41, *Nocardioides* species, and some other actinomycete taxa. The scale bar represents 1 nucleotide substitution per 100 nucleotides.

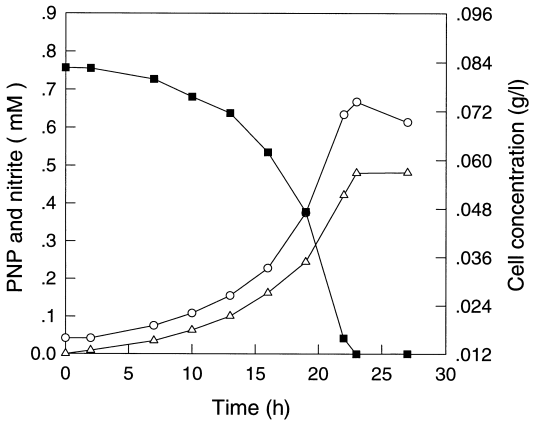

Fig. 2. Time course of a batch culture of *Nocardioides* sp. NSP41 in a 5 L fermenter with PNP (■) as a sole substrate.

Cell concentration (○) was detected by a spectrophotometer at 600 nm and calculated by an optical density vs. a dry cell weight standard curve. Nitrite (△) release was quantified photometrically.

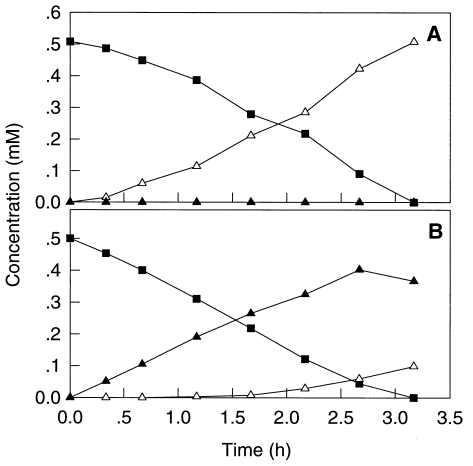

Fig. 3. Conversion of PNP by resting cells of *Nocardioides* sp. NSP41.

Resting cells were obtained by the growth in mineral medium with 0.5 mM PNP (A) and 0.5 mM phenol (B), respectively. The concentrations of PNP (■) and 4-NC (▲) were determined by HPLC. Nitrite (△) was estimated photometrically.

tion rate, 1.0 v/v/m), strain NSP41 was capable of mineralizing 0.75 mM of PNP completely (Fig. 2).

A strain of *Pseudomonas putida* released nitrogen from *m*-nitrophenol as ammonium ion through a separate, reductive enzymatic reaction (Zeyer and Kearney, 1984). However, as Jain et al. (1994) have reported nitrite is released from the PNP degradation by *Arthrobacter* sp., this strain NSP41 also released nitrite during the mineralization of PNP. The difference between the amount of PNP mineralized and that of nitrite accumulated in the culture medium might be caused by the assimilation of nitrite as a nitrogen source, because no nitrogen-containing metabolites were accumulated during the PNP degradation. This result showed that strain NSP41 could utilize PNP as a sole source of carbon, nitrogen, and energy.

Strain NSP41 could degrade PNP up to the initial concentration of 1.5 mM. At higher concentrations, the degradation of PNP was completely inhibited (data not shown).

Metabolic pathway of PNP and phenol degradation

When the nitrophenol isomers and other aromatic compounds as a sole carbon source were examined, strain NSP41 showed the substrate specificity for the *para*-site substituted phenols, such as *p*-nitrophenol, *p*-chlorophenol, *p*-bromophenol, *p*-iodophenol, and *p*-fluorophenol. To determine the metabolic pathway of PNP and the phenol degradation, we carried out the resting cell experiments, and the results are shown in Fig. 3. The resting cells (optical density at 600 nm= 1.0) pregrown with 0.5 mM PNP transformed PNP completely without the accumulation of any intermediate metabolites. PNP was directly degraded with the stoichiometric release of nitrite (Fig. 3A). In the presence of 2,2′-dipyridyl as an inhibitor, hydroquinone was detected as a common metabolic intermediate during the degradation of these *para*-site substituted phenols. This suggests that the first-step enzymes of strain NSP41 for the aromatic ring fission of PNP are involved in the *para*-site specific hydroxylation.

PNP is known to be degraded by two kinds of pathways, hydroquinone and catechol, as reported by Spain and Gibson (1991), Hanne et al. (1993), and Jain et al. (1994). Our results showed that the strain NSP41 has the hydroquinone pathway for PNP degradation. Bae et al. (1996 a) reported that cells with the hydroquinone pathway could have a higher degradation rate and growth rate compared with that of the catechol pathway.

Induction of metabolic pathways for the degradation of PNP and phenol

To determine whether the enzyme systems involved in the simultaneous degradation of PNP and phenol are identical, the induced cell and resting cell experiments were conducted. Cells pregrown with PNP or phenol were inoculated for the PNP or phenol degradation. PNP-induced cells (optical density at 600 nm= 0.05) degraded PNP faster than noninduced cells did without a lag time, but they could degrade phenol only after about 10 h of lag time. Moreover, phenol-induced cells could degrade phenol without a lag time, but they degraded PNP with a long lag time.

Resting cells (optical density at 600 nm=1.0) pregrown with 0.5 mM phenol transformed PNP to 4-nitrocatechol (4-NC). It disappeared slowly during further incubation. A slow decrease of 4-NC was due to the enzymatic decomposition of 4-NC, which was accompanied by the liberation of stoichiometric amounts of

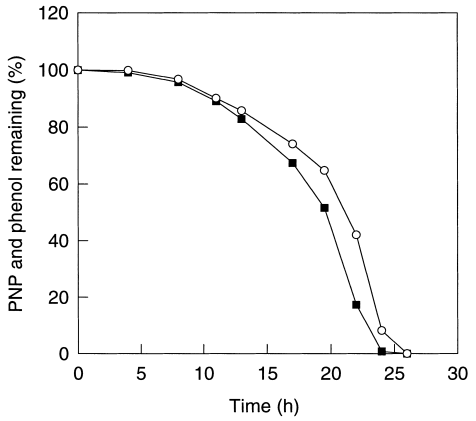

Fig. 4. Simultaneous degradation of PNP and phenol by *Nocardioides* sp. NSP41.

PNP and phenol were used as both substrates with 0.5 mM. The concentration of PNP (■) and phenol (○) were determined by HPLC.

nitrite (Fig. 3B). This clearly showed that the enzymes for the degradation of PNP and phenol were inducible.

Hanne et al. (1993) reported that *Nocardia* sp. strain TW2 has two alternative pathways whose expression depends on the inducer used. The results of the induced resting cell experiments showed that the enzyme system involved in the degradation of PNP or phenol was induced according to the presence of the respective substrate.

Simultaneous degradation of phenol and PNP

The kinetics of the biodegradation of target organic compounds could be altered by the presence of other compounds that the organism can mineralize (Beltrame et al., 1984; Schmidt et al., 1987; Zaidi and Mehta, 1995). To study the effect of other toxic aromatic compounds for PNP degradation, phenol was added to the culture medium. Figure 4 shows that PNP (0.5 mM) and phenol (0.5 mM) were degraded simultaneously. During the simultaneous degradation of PNP and phenol, no metabolites were detected by HPLC.

The simultaneous degradation of PNP and phenol by the respective PNP- and phenol-induced cells was carried out at a constant PNP concentration (1.0 mM) and varying phenol concentrations (0.5, 1.0, and 1.5 mM). Although the increased phenol concentration reduced the apparent degradation rate of PNP, the degradation rates of PNP and phenol were not significantly different in each instance. In the low phenol concentration (0.5 mM), phenol was completely mineralized before PNP was degraded completely. After the exhaustion of phenol, the enzyme induced for the phenol degradation transformed a part of the remaining PNP to 4-NC. Then PNP and 4-NC were directly mineralized (Fig. 5A). Concerning the same PNP and

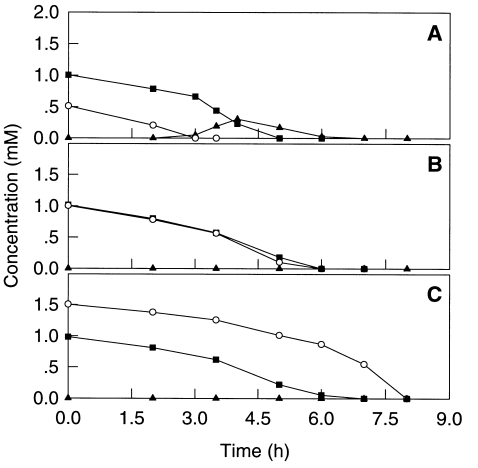

Fig. 5. Degradation of PNP and phenol by *Nocardioides* sp. NSP41 in different phenol concentrations.

The initial cell concentration was 1.5 g dry cells/L. The initial PNP (■) concentration was 1.0 mM. The initial phenol (○) concentrations were A: 0.5 mM, B: 1.0 mM, and C: 1.5 mM. The concentrations of PNP, phenol, and 4-NC (▲) were determined by HPLC.

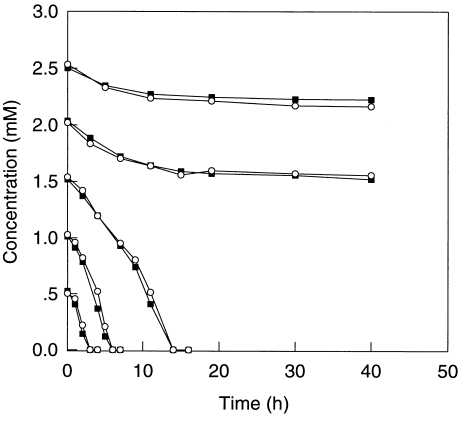

Fig. 6. Effect of initial concentration of PNP and phenol on the simultaneous degradation by *Nocardioides* sp. NSP41.

The initial cell concentration was 1.5 g dry cells/L. PNP (■) and phenol (○) were completely mineralized up to 1.5 mM simultaneously.

phenol concentration (1.0 mM), both substrates were degraded simultaneously at the same rate (Fig. 5B). In the same concentrations of PNP and phenol, both compounds were degraded simultaneously without the production of any other intermediates. Concerning the high phenol concentration (1.5 mM), PNP was degraded faster and was completely exhausted before phenol was degraded (Fig. 5C). No degradation products were determined by the HPLC detection after the complete mineralization of PNP.

The effects of initial concentrations on the simultaneous degradation of PNP and phenol at varying equimolar concentrations were investigated. Strain NSP41 was able to degrade simultaneously up to 1.5 mM of the PNP and phenol concentrations. When

Table 2. Effects of phenol addition on the kinetics of PNP degradation by *Nocardioides* sp. NSP41.

Substrate	Concentration (mM)	Specific growth rate (h^{-1})	Specific degradation rate (h^{-1})
PNP only	0.5	0.0458±0.003	0.0751±0.008
Phenol only	0.5	0.1762±0.038	0.2056±0.013
PNP	0.5	0.1054±0.025	0.0977±0.016
+Phenol	+0.5		0.0797±0.006

the PNP and phenol concentrations were increased to 2.0 mM, neither compound could be degraded completely (Fig. 6). This seems to be due to the toxicity by the increase in the total concentration of PNP and phenol. In the simultaneous degradation of PNP and phenol, no diauxic growth patterns occurred. This indicated that different enzyme systems were induced simultaneously in the PNP and phenol degradation. However, chloro- and methylaromatic compounds were simultaneously degraded via the same initial degradation pathway with the conversion to the corresponding substituted catechols (Pettigrew et al., 1991; Rojo et al., 1987; Taeger et al., 1988). Bae et al. (1996b) also reported that the mixture of 4-chlorophenol and phenol was degraded via the *meta*-cleavage pathways of 4-chlorocatechol and catechol, respectively.

Kinetically, the addition of phenol enhanced the apparent PNP degradation rate. Table 2 shows the effect of the phenol addition on the kinetics of PNP degradation. The rapid degradation of PNP in the presence of phenol seemed to be due to the increased cell mass rather than to the enhancement of the specific PNP degrading activity (Bae et al., 1996b). On the other hand, Schmidt et al. (1987) reported that phenol had an inhibitory effect on the kinetics of PNP mineralization by *Pseudomonas* sp.

In conclusion, our results indicate that the first-step intermediate of the PNP degradative pathway is hydroquinone, whereas that of the phenol degradative pathway is catechol in a newly isolated strain, *Nocardioides* sp. NSP41. The enzyme systems involved in the PNP and phenol degradation were inducible by two compounds. PNP and phenol could simultaneously induce two different enzyme systems and could be degraded simultaneously.

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