J. Microbiol. Biotechnol. (2006), **16**(11), 1740–1746

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Isolation of a *Pseudomonas* sp. Capable of Utilizing 4-Nonylphenol in the Presence of Phenol

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Received: May 5, 2006
Accepted: June 23, 2006

Abstract Enrichment techniques led to the isolation of a *Pseudomonas* sp. strain P2 from municipal waste-contaminated soil sample, which could utilize different isomers of a commercial mixture of 4-nonylphenol when grown in the presence of phenol. The isolate was identified as *Pseudomonas* sp., based on the morphological, nutritional, and biochemical characteristics and 16S rDNA sequence analysis. The β-ketoadipate pathway was found to be involved in the degradation of phenol by *Pseudomonas* sp. strain P2. Gas chromatography-mass spectrometric analysis of the culture media indicated degradation of various major isomers of 4-nonylphenol in the range of 29–50%. However, the selected ion monitoring mode of analysis of biodegraded products of 4-nonylphenol indicated the absence of any aromatic compounds other than those of the isomers of 4-nonylphenol. Moreover, *Pseudomonas* sp. strain P2 was incapable of utilizing various alkanes individually as sole carbon source, whereas the degradation of 4-nonylphenol was observed only when the test organism was induced with phenol, suggesting that the degradation of 4-nonylphenol was possibly initiated from the phenolic moiety of the molecule, but not from the alkyl side-chain.

Key words: Biodegradation, endocrine disruptor, nonylphenol, phenol, *Pseudomonas* sp.

Potential human exposure to a wide variety of chemicals occurs through commonly used products, many of which are harmful to human beings. Alkylphenols are among those chemicals, which are the starting material for the production of a number of non-ionic surfactants, called "alkylphenol ethoxylates" (APEs). APEs are widely used as emulsifying agents in latex paints, and in various industrial and domestic consumer products such as plastic additives and detergents, as well as in the form of pesticide adjuvants [40, 51]. Among the APEs, the most widely used are the nonylphenol ethoxylates (NPEs). During sewage treatment, the NPEs are rapidly but incompletely degraded, leaving behind the more recalcitrant metabolites 4-nonylphenols (4-NPs), short-chain NPEs, and their carboxylic derivatives [13, 43]. Leaching of nonylphenol (NP) has also been detected from plastic materials [37] and polyvinyl chloride (PVC) food packaging films [19]. NPs have largely been detected in water samples as well as in sediments [1, 4].

NP has been found to be acutely toxic to aquatic life, even in minute concentrations [18, 32, 51], and has been shown to have estrogenic properties both *in vivo* and *in vitro* [24, 37, 49]. Picomolar to nanomolar concentrations of NP could mimic estradiol function [5, 6]. NP was observed to induce proliferation of tumor cells and also trigger mitotic activity in rat endometrium [37]. Moreover, NP inhibits ATPase activity [27], and also enhances the generation of reactive oxygen species in human blood neutrophils [30]. It has been found to stimulate ligand-dependent gene transcription in both yeast and mammalian cells [49] and induce apoptosis by regulating Fas/FasL gene expression in rat thymus [50]. NP is thus considered as a chemical of major environmental concern around the world.

To date, there have been a number of reports describing the degradation of NP by some strains belonging to the genera *Sphingomonas*, *Sphingobium*, and *Pseudomonas* [9, 10, 12, 15–17, 20, 35, 36, 38, 44]. Although it has been reported that degradation of NP is facilitated by linearity in the alkyl chain [7, 45, 47], the situation, however is different for branched isomers of NP, where the quaternary α-carbon atom of the branched alkyl chain leads to poor degradability. On the other hand, microbial degradation of higher alkylphenols studied in pure culture showed that the degradation is initiated at the phenolic moiety rather than at the alkyl chain [2, 15, 20, 35, 38, 39]. Conversion of α-secondary 4-alkylphenols to their corresponding α-keto

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derivatives and then incorporation of an oxygen atom between the carbon atom and the acyl moiety by a Baeyer-Villiger mechanism, followed by cleavage of the resulting esters by a hydrolytic mechanism, have been observed in several bacteria [11, 22]. Degradation of the branched isomer of NP by a *Sphingomonas* sp. strain TTNP3 was reported; the mechanism involves a type-II *ipso*-substitution to form hydroquinone and nonanol [8, 9, 38]. Degradation of numbers of α-quaternary NP isomers by another bacterium, *Sphingomonas xenophaga* Bayram, was found to initiate with the hydroxylation at the *ipso*-position, yielding 4-alkoxyphenols, followed by subsequent detachment of the alkyl moiety as alcohols [17]. Cometabolic degradation of linear 4-NP by a *Pseudomonas* strain INA06 has also been reported [2]. In the present study, we describe the isolation and characterization of a *Pseudomonas sp.* strain P2 that can utilize a commercial mixture of 4-nonylphenol when grown in the presence of a catalytic amount of phenol. A quantitative estimation of the biodegradation of major isomers of 4-nonylphenol is reported.

MATERIALS AND METHODS

Chemicals

All the specialty chemicals used in this study were obtained from either Merck (Germany), Aldrich Chemical Co. (Milwaukee, WI, U.S.A.), Sigma (St. Louis, MO, U.S.A.), or Hi-Media (India). All other chemicals and reagents used were of analytical grade and used without any purification.

Enrichment and Isolation of Organism

Enrichment of cultures was started by inoculating the consortia obtained from municipal waste-contaminated soil samples in liquid mineral salt medium (MSM, pH 7.5) containing (per liter) 3.34 g of K_2HPO_4, 0.87 g of NaH_2PO_4, 2.0 g of NH_4Cl, 123 mg of nitrilotriacetic acid, 200 mg of $MgSO_4 \cdot 7H_2O$, 12 mg of $FeSO_4 \cdot 7H_2O$, 3 mg of $MnSO_4 \cdot H_2O$, 3 mg of $ZnSO_4 \cdot 7H_2O$, and 1 mg of $CoCl_2 \cdot 6H_2O$, individually with NP (1.0 g/l) and NP (1.0 g/l) supplemented with phenol (0.25 g/l) or *n*-hexadecane (0.25 g/l) as the sole carbon and energy sources at 28°C. When growth was observed, the enrichment process was repeated, and after several transfers under the same conditions, the enriched cultures were subsequently purified by plating on nutrient agar medium. Each type of colony was then isolated and subcultured individually in MSM along with the preferred carbon source(s), as mentioned above.

Characterization of Bacteria

Morphological features were studied using a phase contrast microscope (Olympus CX40, Olympus, Japan). Conventional biochemical tests were performed using standard methods [33]. The 16S rDNA gene was amplified using universal bacterial specific primers f27 and r1492 [21], and was sequenced according to the manufacturer's specifications for *Taq* DNA polymerase-initiated cycle sequencing reactions using fluorescently labeled dideoxynucleotide terminators with an ABI PRISM 377 automated sequencer (Perkin-Elmer Applied Biosystems, Inc.). Sequence homologies were analyzed using BLAST version 2.2.12 of the National Center for Biotechnology Information [3]. Multiple sequence analyses were done using ClustalW version 1.83 [42], and a consensus neighbor-joining tree [31] was constructed following the majority rule out of 100 phylogenetic trees derived using the program NEIGHBOR of the PHYLIP package [14]. The genetic distances were computed by the Kimura 2-parameter method. The resulting tree was plotted using TREECON [46].

Culture Conditions for Phenol and Nonylphenol Degradation

Cells were grown in 100-ml Erlenmeyer flasks containing 25 ml of MSM supplemented with phenol (0.5 g/l) as the sole carbon source, by incubating them at 28°C on a rotary shaker (180 rpm) for different periods of time. Solid media contained 2% agar (Hi-Media, India). For 4-NP degradation, MSM containing 4-NP (0.3 to 1.8 g/l) as the carbon source was inoculated with cells grown in the presence of phenol (0.2 to 0.4 g/l) and incubated at the same conditions as above. Enzyme induction and protein synthesis on phenol degradation were examined by resting cell transformation of induced and non-induced cells with phenol. Phenol and succinate-grown starter cultures were used for the growth of experimental cultures. Cells at the late log phase were harvested by centrifugation (8,000 ×*g*, 10 min), washed twice by equal volume of potassium phosphate buffer (50 mM, pH 7.5), and finally resuspended in the same buffer to give an optical density (OD_{660}) of 1.0. Phenol (0.25 g/l) was added with or without chloramphenicol (25 μg/ml) in phenol and succinate-grown cell suspensions, and incubated at 28°C for different periods of time to monitor the extent of phenol degradation. For the quantitative estimation of biodegraded 4-NP isomers, the strain P2 was grown in MSM containing 4-NP (0.3 g/l) and a minute amount of phenol (75 mg/l), and they were incubated for 5 days under shake culture condition, as mentioned above. Unless stated otherwise, each experimental set was cultivated in triplicate.

Isolation of Metabolites

After incubation, the spent cultures were acidified to pH 1.5 to 2.0 by concentrated hydrochloric acid and extracted thrice with an equal volume of ethyl acetate. The combined extracts were then dried by evaporation under reduced pressure, and the residues were dissolved in ethyl acetate for further analysis.

Preparation of Cell-Free Extracts and Enzyme Assay

Cells grown in MSM in the presence of phenol (0.25 g/l) were harvested at the mid-exponential phase by centrifugation at 8,000 ×*g* for 10 min at 4°C. The pellet was washed twice with 10 volumes of 50 mM potassium phosphate buffer (pH 7.5) and resuspended in 1 to 2 volumes of the same buffer. The cell suspensions were ultrasonicated for 2 min at 4°C in seven pulses. The ultrasonicated cell suspensions were centrifuged at 20,000 ×*g* for 20 min at 4°C. The supernatants were used as cell-free enzymes for further studies. One unit of enzyme activity was defined as the amount to degrade 1 μmol of substrate per minute under the assay conditions, and the specific activity was defined as units per mg of protein. Protein was measured by the method of Lowry *et al.* [25] with bovine serum albumin as the standard.

Metabolism of catechol by cell-free extracts of phenol and succinate-grown culture were recorded with UV-Visible spectra on a Cary 50 Bio UV-Visible spectrophotometer (Varian Australia Pty. Ltd.) using 1-cm path-length quartz cuvettes. Data were analyzed by the Varian Cary Win UV Scan application.

Analytical Methods

Metabolites were resolved by a high-performance liquid chromatography (HPLC) system (Waters, U.S.A.) on an analytical Novapak C_{18} reverse-phase column attached to a Waters 515 solvent delivery system. The biodegraded products were eluted with a programmed 45-minutes linear methanol-water gradient (from 50:50, v/v to 95:5, v/v) as solvent system at a flow rate of 1.0 ml/min and detected by a Waters 486 UV detector at 254 nm.

An analysis of the biodegraded products of phenol and quantitative estimation of residual 4-NP isomers following biodegradation were performed by gas chromatography-mass spectrometry (GC-MS) using a Varian 3800 GC column with a Saturn-2000 mass spectrometer (Varian Inc., Palo Alto, CA, U.S.A.) equipped with a 30 m× 0.25 mm (0.25-μm film thickness) HP-5MS capillary column. The temperature program gave a 1.5 min hold at 70°C, an increase to 200°C at 10°C/min, further increase to 280°C at 5°C/min, and a 10 min hold at 280°C. The injection volume was 1 μl, and the carrier gas was helium (1.5 ml/min). The mass spectrometer was operated at an electron ionization energy of 70 eV. Degradation was monitored by measuring residual 4-NP concentrations using phenanthrene as an external standard. For this, the dried ethyl acetate extracts of spent cultures were dissolved in equal volumes of ethyl acetate (40 μl) and were spiked with 0.1 mg of phenanthrene. Parallel controls, MSM containing NP only, were also processed as above. Quantitative estimation of the 4-NP isomers in the biodegraded samples was made relative to the peak area of the external standard. All the samples were dissolved in 1 ml of ethyl acetate prior to GC-MS analysis. Quantitative estimation of phenol was done by the Folin-Ciocalteu method [34].

The 16S rDNA sequence accession number (DDBJ/EMBL/GenBank) of strain P2 is DQ404013.

RESULTS AND DISCUSSION

Isolation of Bacteria

The consortia obtained from municipal waste-contaminated soil samples failed to grow in MSM containing NP as the sole carbon and energy source. It also did not grow in MSM supplemented with NP and *n*-hexadecane. However, growth was observed when MSM was supplemented with NP and phenol. Several transfers under the same conditions allowed the enrichment of cultures, which were subsequently purified by plating on nutrient agar medium. Each type of colony was then isolated and subcultured individually in MSM along with NP and phenol. This screening ultimately led to the isolation of single strain P2, which could grow on NP in MSM in the presence of a catalytic amount of phenol.

Taxonomic Identification of Strain P2

Based on its morphological, nutritional, and biochemical characteristics, the strain P2 was identified as the genus *Pseudomonas*. The strain produced a non-diffusible fluorescent pigment on King's B medium [23]. Biochemical tests showed motility, with oxidase, catalase, nitrate reduction, and arginine dihydrolase reaction positive gelatin hydrolysis and acid production from glycerol were negative; and there was growth in the presence of 2% NaCl, but no growth in the presence of 5% NaCl or above. The partial 16S rDNA gene sequence (1,405 bp) of the strain P2 was determined. Analysis of this sequence using BLAST search revealed 98.4 and 98.3% sequence identities to *Pseudomonas plecoglossicida* strain FPC 951T and *Pseudomonas putida* strain KL33, respectively. However, in the phylogenetic tree (Fig. 1), the strain P2 clearly belonged to the *Pseudomonas* lineage, as evidenced by the high bootstrap values. Although the combined analyses indicated a strong correlation in genus level, these data were not sufficient to identify the strain up to the species level. Thus, the strain P2 has been identified as *Pseudomonas* species only at this point.

Induction of Phenol Degradation

The relationship between enzyme induction and phenol degradation was studied with *Pseudomonas* sp. strain P2 cultures grown in the presence and absence of phenol and chloramphenicol. The cultures grown with constant exposure to phenol were highly induced for phenol degradation. Under resting cell transformation, these cultures degraded 39% of phenol within 24 h and 99% within 72 h at 28°C. Cells grown for 4 days in the presence of succinate

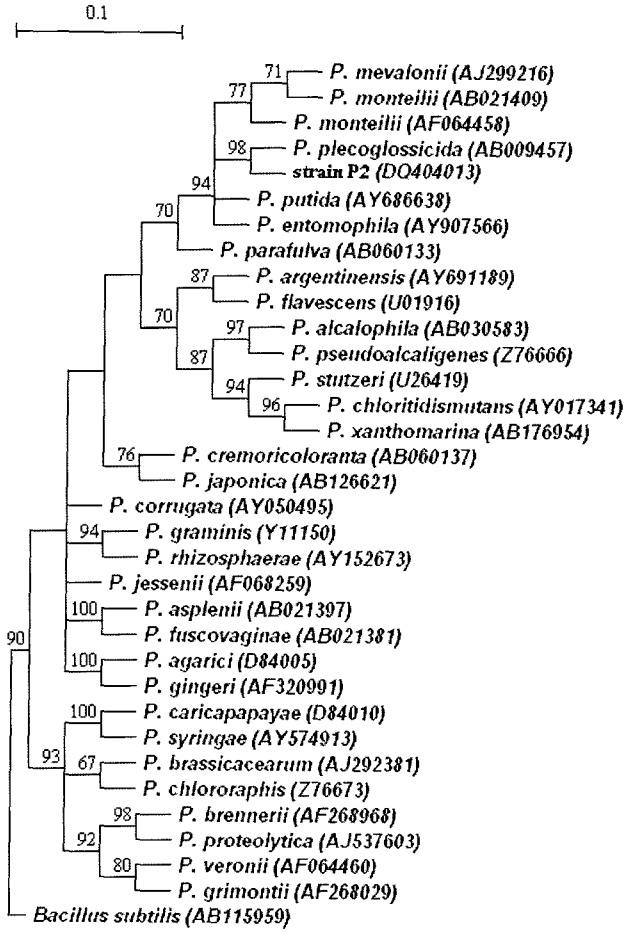

Fig. 1. Phylogenetic tree based on the 16S rDNA gene, showing the relation between strain P2 and representative strains of the genera *Pseudomonas*.

The accession numbers of the sequences used are given in parentheses. Numbers at nodes indicate levels of bootstrap support based on neighbor-joining analysis of 100 resampled datasets. Bootstrap values below 60% are not shown. Bar represents 0.1 substitutions per nucleotide position.

showed a longer lag phase and could degrade up to 27% of phenol within 72 h. The bacterial protein synthesis inhibitor, chloramphenicol, was added initially to cultures pregrown with or without phenol, to determine whether the phenol-degrading enzymes were constitutive or inducible. Induced cultures that were dosed with chloramphenicol at 0 h could degrade 28% of phenol during 24 h of exposure. However, no substantial degradation of phenol by this culture was observed beyond 24 h. On the other hand, chloramphenicol dosed at 0 h to succinate-grown culture inhibited phenol degradation to less than 4% in 72 h. In all experiments, abiotic loss of phenol was detected in the range of 2–3% after 72 h. Apart from that, the specific activity for catechol-1,2-dioxygenase in phenol-grown cells was shown to be 19.26 μmol/min/mg of protein, whereas no such ring-cleavage activity was observed in succinate-grown cells. Therefore, it may be concluded from the above that both phenol hydroxylase and catechol-1,2-dioxygenase involved in phenol degradation are inducible with phenol.

Biodegradation Potential of Strain P2

The test organism could grow on phenol as the sole source of carbon and energy and could completely degrade phenol within 72 h under shake culture conditions. HPLC analysis of metabolites of the ethyl acetate extract of the spent culture of the strain P2 grown on phenol showed barely any accumulation of metabolites. However, the resting cell suspension of phenol-grown cells on phenol furnished one detectable metabolite eluted at 10.8 min, and was identified as catechol, based on the retention time of authentic catechol (data not shown). The presence of catechol in the ethyl acetate extract of the resting cell culture on phenol by phenol-grown cells was further confirmed by GC-MS analysis, where the mass spectrum of the metabolite was detected at 8.73 min with the significant ions at m/z 110 (M^+, 100), 92 (10), 81 (11), 64 (33), 63 (13). Furthermore, catechol was found to be metabolized by the phenol-grown cells of the strain P2. The cell-free extract-mediated metabolism of catechol was demonstrated by the spectrophotometric analysis (Fig. 2). A sharp increase of A_{260} is indicative of the *ortho*-cleavage of catechol by catechol-1,2-dioxygenase with the formation of *cis,cis*-muconic acid [28]. Thus, it is concluded that phenol is metabolized via catechol and *cis,cis*-muconic acid, leading to the tricarboxylic acid cycle. Detection of catechol as an intermediate and the presence of catechol-1,2-dioxygenase activity suggest that phenol hydroxylase (a monooxygenase) is the first committed step in the biodegradation of phenol by the strain P2.

Pseudomonas sp. strain P2 could utilize a commercial mixture of 4-NPs as co-substrate in the presence of phenol.

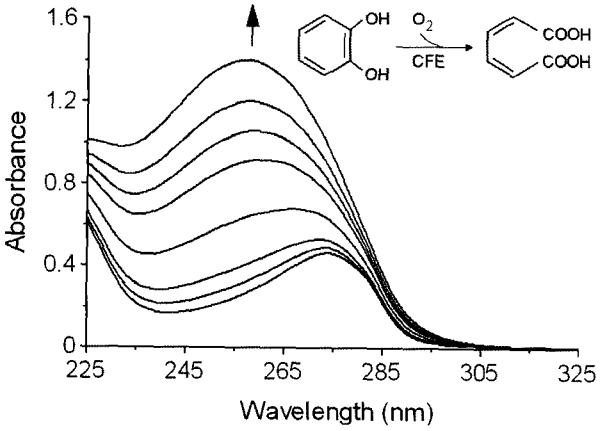

Fig. 2. Spectral changes during metabolism of catechol by cell-free extract of phenol-grown cells of *Pseudomonas* sp. strain P2.

The sample and reference cuvettes contained 50 mM potassium phosphate buffer (pH 7.5) in 1-ml volume. The sample cuvette also contained 90 nmol of catechol. Spectra were recorded at 0, 5, 9, 13, 15, 17, and 20 min after the addition of 115 μg of crude protein to both cuvettes. The arrow indicates increase in absorbance at 260 nm.

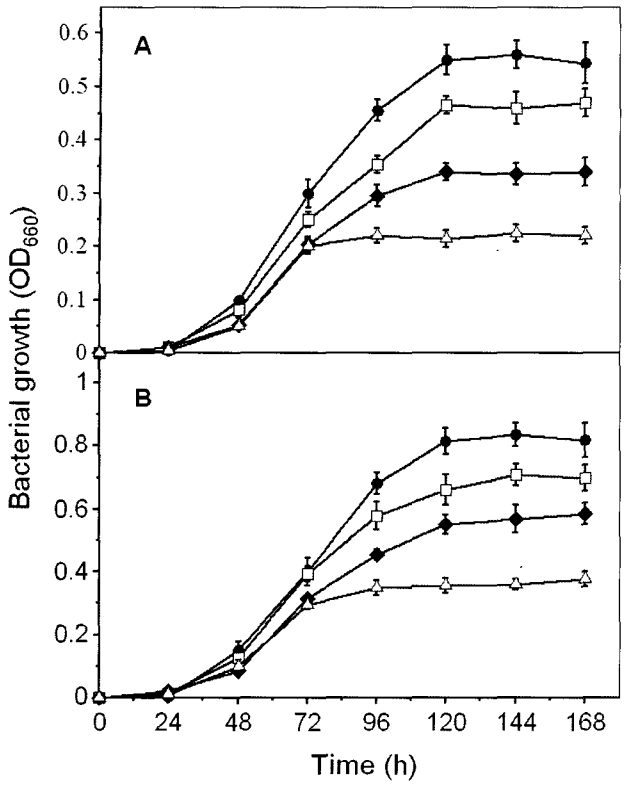

Fig. 3. Growth of *Pseudomonas* sp. strain P2 in the presence of phenol and phenol plus 4-NP.
Phenol concentration was kept at 0.2 g/l (**A**) and 0.4 g/l (**B**); 4-NP concentration was gradually increased from 0 g/l (△), to 0.6 g/l (◆), 1.2 g/l (□), and 1.8 g/l (●). Vertical bars represent mean±standard deviations from triplicate measurements.

The growth profiles of the strain P2 on phenol in the presence or absence of 4-NP are shown in Fig. 3. It has been observed that growth in the presence of phenol only became saturated after 72 h of incubation. However, differential growth on addition of 4-NP was observed beyond 24 h, where linearity in growth profile was observed with increasing concentrations of 4-NP, which was saturated at around 120 h of incubation. This suggests that, although 4-NP was not used as the sole carbon source by the strain P2, 4-NP was also utilized for its growth, once induced with phenol.

Technical nonylphenol is a mixture of more than 22 4-NP isomers [41, 48], which differ in the structure of nonyl moiety attached to the phenol ring. Although separation of about 40 alkylphenol isomers from technical 4-NP has recently been achieved by using comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry [26], GC-MS analysis of the commercial 4-NP used in this study revealed 11 well-resolved major isomers. The biodegradation profile of the commercial 4-NP isomers by the strain P2 in the presence of a catalytic amount of phenol during 5 days of incubation is presented in Fig. 4. Although the degree of degradation of individual

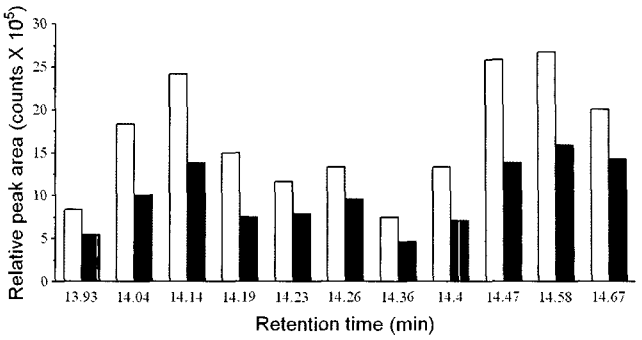

Fig. 4. Degradation profile of different isomers of 4-NP by *Pseudomonas* sp. strain P2.
Data represent the GC-MS analysis of the peak area of the individual isomers of the biodegraded sample incubated for 5 days (■) in comparison with their corresponding abiotic controls (□). Individual isomers are represented by their corresponding GC retention time. Standard deviations were in the range of ±2–7% from triplicate measurements.

isomers varied in the range of 29 to 50%, none of the major isomers so detected was found to escape from the degradation process. The extent of degradation of the NP isomers seems to be dependent on the structural complexity of the alkyl side chain. The strain P2 could not utilize long- or medium-chain alkanes as carbon sources, since no growth was detected when *n*-hexane, *n*-heptane, and *n*-hexadecane were used individually as substrates. Moreover, the m/z 107 selected ion monitoring (SIM) mode of analysis of the total ion chromatogram showed the absence of any alkyl-substituted phenolic compounds in the 4-NP biodegraded samples apart from the unutilized 4-NP isomers. Similar SIM mode of analysis was also reported in the degradation of NP by strain TTNP3, where the metabolic pathway was predicted to initiate with the fission of the phenol ring [38]. But a type-II *ipso*-substitution mechanism has recently been proposed in the degradation of NP by the strain TTNP3 [8, 9]. Similarly, *ipso*-substitution was also observed during degradation of branched NP by the bacteria *Sphingomonas xenophaga* Bayram, which could utilize NP isomers with α-quaternary carbon atoms only [16, 17]. In this study, however, the enzymes involved in NP degradation were induced by phenol, where the degradation of phenol itself proceeds via the β-ketoadipate pathway. Moreover, during growth on 4-NP in the presence of phenol, the culture did not turn yellow, thus ruling out the possibility of the *meta*-cleavage of the aromatic ring of 4-NP, since *meta*-cleavage of catechol yields 2-hydroxymuconic semialdehyde, a yellow product that absorbs at 377 nm [29]. Although this work did not address the mechanism of NP degradation by the strain P2, it can be suggested that the enzymes involved in phenol degradation may also be involved in initiating the degradation of NP isomers via hydroxylation of the phenolic moiety followed by the *ortho*-cleavage pathway. The complex structural nature of the alkyl side-chain may be accounted for by the differential extent of degradation of various

isomers of commercial 4-NP towards phenol-induced enzyme(s) in the strain P2. Nevertheless, at this stage, the involvement of *ipso*-substitution could not be ruled out. Unfortunately, identification of pathway intermediates of 4-NP degradation by GC-MS analysis remains unsuccessful because of the complex assemblage of the commercial NP mixture. *Pseudomonas sp.* strain P2 can, therefore, be considered as a candidate for further investigations on the mechanism of 4-NP degradation, using different NP isomers to understand the role of enzymes involved in the degradation process.

Acknowledgment

Financial support for this work was provided by Bose Institute, Kolkata, India.

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