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Isolation and characterization of *Arthrobacter* sp. HY2 capable of degrading a high concentration of *p*-nitrophenol

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ABSTRACT

A soil bacterium strain, capable of using *p*-nitrophenol (PNP) as its sole source of carbon and energy, was isolated by enrichment on minimal salt medium (MSM). On the basis of a phylogenetic analysis of 16S rRNA gene sequences the bacterium is a species of *Arthrobacter*, closely related to *Arthrobacter ureafaciens* DSM 20126. This strain has an unusually high substrate tolerance for PNP degradation in MSM. Greatest degradation of PNP was observed at 30 °C and under slightly alkaline pH (pH 7–9) conditions. Effective degradation rates slowed as the concentration of PNP was increased. Addition of glucose from 0.1% to 0.5% generally enhanced the degradation of PNP at high concentration (400 mg/l) although acidification as a result of glucose metabolism had a negative effect on PNP depletion. Biodegradation of PNP at high concentration was greatly accelerated by β-cyclodextrin at a concentration of 0.5%, indicating that β-cyclodextrin could be a promising addictive for effective PNP bioremediation.

1. Introduction

The wide use of nitroaromatics as synthetic intermediates in the manufacture of pharmaceuticals, pigments, dyes, plastics, pesticides and fungicidal agents, explosives, and industrial solvents (Spain, 1995) leads to the accumulation of nitrophenols (Schackmann and Muller, 1991). *p*-Nitrophenol (PNP) is probably the most important among the mono-nitrophenols in terms of the quantities used and potential environmental contamination (Karin and Gupta, 2002). In the United States, the industrial releases of PNP reported to the Environmental Protection Agency totalled 772 lb in 2004 (Perry and Zylstra, 2007). PNP has often been detected in wastewater, rivers, soils, and ground water (Wan et al., 2007). Because it is toxic to many living organisms and may accumulate in the food chain (Donlon et al., 1996), PNP has been rated a priority pollutant by the United States Environmental Protection Agency (EPA, 1980), which recommends restricting PNP concentrations in natural waters to <10 ng/l (Gemini et al., 2005).

To minimize the adverse effect of PNP, an effort has been made to develop microbial systems for the treatment of contaminated soils and water. Screening potential microorganisms is a critical step in the construction of an effective remediation system. Several bacterial strains able to utilize PNP as their sole source of carbon and energy have been documented. These include species of *Arthrobacter* (Chauhan et al., 2000; Jain et al., 1994; Li et al., 2008), *Bacillus* (Kadiyala and Spain, 1998), *Burkholderia* (Bhushan et al., 2000; Chauhan et al., 2000), *Ochrobactrum* (Qiu et al., 2006, 2007), *Pseudomonas* (Kulkarni and Chaudhari, 2006; Liu et al., 2005; Prakash et al., 1996; Shinozake et al., 2002; Zaidi and Mehta, 1996), *Rhodobacter* (Roldan et al., 1998), *Rhodococcus* (Shinozake et al., 2002), and *Strenotrophomonas* (Liu et al., 2007). Because the toxicity of high concentrations of PNP inhibits its degradation by most microorganisms (Hanne et al., 1993), the screening of microbes capable of degrading high concentrations of PNP is becoming a priority research objective in the field of PNP biodegradation. To date, very few bacteria have been documented as exhibiting high PNP tolerance. Examples include *Pseudomonas putida* (Kulkarni and Chaudhari, 2006) and *Stenotrophomonas* sp. (Liu et al., 2007).

Optimizing process parameters based on an understanding of the effects of abiotic factors on the capacity of microorganisms is important when designing bioremediation strategies. Although PNP degradation has attracted considerable interest, most studies have been conducted at relatively low PNP concentrations (Hanne et al., 1993; Qiu et al., 2007). There are very few investigations focused on improving the degradation rate of PNP at toxic concentrations. One example is a study on the effects of glucose and nitrogen on PNP degradation by *P. putida* at 300 mg/l (Kulkarni and Chaudhari, 2006). Although the toxicity of aromatics is reduced when they form complexes with cylcodextrins (Schwartz and Bar, 1995; Del Valle, 2004), the effect of CDs on the degradation of high concentrations of PNP has not yet been investigated.

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Given this background, we first screened and characterized a soil bacterium capable of utilizing PNP at high concentrations. We then investigated the effect of certain abiotic factors and additives on the degradation of PNP in liquid cultures to provide practical information for the design of an effective strategy suitable for remediation of PNP at high concentrations.

2. Methods

2.1. Chemicals

PNP of chromatographic grade was purchased from Fluka (Buchs, Switzerland); glucose and yeast extract used were manufactured by Difco (Detroit, USA). β-Cyclodextrin was purchased from Calbiochem (Merck Beijing, China). All other chemicals used were of the highest purity commercially available.

2.2. Culture medium

The composition of the soil enrichment medium was peptone 10 g, glucose 1 g, KH_2PO_4 1 g, NaCl 1 g in 1 l sterile water (pH 7.2). Minimal salts medium (MSM) contained (mg/l) $MgSO_4{\cdot}7H_2O$ 120, $ZnSO_4{\cdot}7H_2O$ 5.0, $Na_2MoO_4{\cdot}2H_2O$ 2.5, KH_2PO_4 400, $Na_2HPO_4{\cdot}7H_2O$ 700, $CaCl_2$ 14, $FeSO_4$ 0.13, $(NH_4)_2SO_4$ 500 according to Pesce and Wunderlin (2004) with minor modifications.

2.3. Isolation and identification of bacteria

Enrichment was performed by successive sub-culturing of samples. One gram of soil sample, obtained from the premises of a pesticide factory (located in Anyang, Henan Province, China) was used as the inoculum for 50 ml soil enrichment medium supplemented with PNP (5 mg/l final concentration). These cultures were incubated at 30 °C with agitation (150 rpm) for a week and were then transferred into MSM supplemented with 5 mg/l PNP as the sole carbon source. After sub-culturing (5%) in MSM with increasing concentrations of PNP at up to 50 mg/l for six generations, pure cultures were obtained by performing appropriate serial dilutions of the enrichment culture in MSM and plating them onto MSM plates containing 50 mg/l PNP. The isolates which grew fastest and rapidly turned the culture from yellow to colorless were selected for further investigation.

An isolate, designated HY2, capable of degrading PNP quickly was further characterized morphologically and biochemically by the China General Microbiological Culture Collection Center (Beijing, China). Universal primers for bacteria were used for polymerase chain reaction (PCR) amplification: forward primer (8f: 5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse primer (1492r: 5′-TAC CTT GTT ACG ACT T-3′) (Weisburg et al., 1991). Identification was made by comparing the sequence data obtained in the manner described above with 16S rRNA sequence data available online in public databases. The partial 16S rRNA gene sequence has been deposited in the GenBank Database under Accession No. FJ477386.

2.4. Biodegradation test

The inocula for PNP degradation experiments were prepared by growing bacteria in 50 ml of Luria–Bertani (LB) medium (Sambrook and Russell, 2001) supplemented with 50 mg/l PNP and incubating for 36 h at 30 °C on a shaker at 150 rpm. The culture was harvested aseptically. The cells were washed thoroughly with MSM (4000*g*, 10 min, 4 °C) and suspended in sterile MSM as the inoculum. Appropriate modifications were made to the incubation systems in order to analyze the effects of various factors on PNP degradation. Filter-sterilized glucose or β-cyclodextrin solution was added into MSM to different initial concentrations. At appropriate intervals, aliquots of samples were removed to determine periodically the amount of PNP and/or nitrite in the media, and also to evaluate microbial growth. All the experiments were performed in triplicate. Un-inoculated controls were maintained in all experiments. Results are reported as the average of three replicates.

2.5. Analytical method

PNP was quantified by HPLC (Agilent 1100, Agilent Technologies, Palo Alto, CA, USA) conducted at room temperature using Agilent Zorbax 300SB-C18 column and acetonitrile:water = 15:85 (water contains acetic acid, 650:1, v:v; pH 3.0) as the mobile phase at a flow rate of 0.70 ml/min. The analysis was performed at 290 nm, column pressure of 71 bar, column temperature at 40 °C. Sample volume was 5 μl.

Nitrite ion was quantified on the basis of the standard curves prepared using sodium nitrite (DU-800 spectrophotometer, Beckman Coutler Inc., Fullerton, CA, USA) according to the method of Montgomery and Dymock (1961).

3. Results

3.1. Isolation and identification of a PNP-degrading bacterium

A soil bacterial strain capable of utilizing PNP as its sole carbon and energy source was isolated by enrichment technique. This isolate was identified by the China General Microbiological Culture Collection Center (Beijing, China) as a Gram-positive and aerobic bacterium. Analysis of 16s rRNA sequences, using the Seqmatch tool at the Rimobomal Database Project release 10 online server, placed HY2 within the genus *Arthrobacter* with 100% confidence. HY2 showed the greatest similarity (99.3%) with the type sequence of *Arthrobacter ureafaciens* DSM 20126 (GenBank No. X80744). The closest-match non-type sequence (99.9% identity) is that of *Arthrobacter* sp. AD37 (GenBank No. EU672425).

HY2 growth was observed after incubation in MSM containing 50 mg/l of PNP within 6 h (Fig. 1). The growth of HY2 cells was concomitant with the decrease of PNP, indicating that HY2 could use PNP as its sole source of carbon and energy. Nitrite was detectable after 2 h of incubation, and was simultaneously released in stoichiometric quantities with PNP depletion (Fig. 1). No PNP degradation, nitrite release, or cell growth was detected in un-inoculated controls (data not shown).

PNP depletion and the release of nitrite was in a stoichiometric manner in our experimental system (Fig. 1), thus PNP degradation could be determined by recording the release of nitrite. In the interest of simplicity, we examined the effects of various factors on PNP degradation by determining the release of nitrite instead of PNP depletion.

3.2. Effect of temperature and pH on PNP degradation

The impacts of temperature and pH on PNP degradation were shown in Figs. 2 and 3. Although measurements were made every 2 h up to 12 h, only results obtained at 6 h are presented for clarity.

PNP degradation occurred at temperatures from 15 to 35 °C (Fig. 2). The greatest release of nitrite was observed at 30 °C. Temperatures over 35 °C or below 20 °C were unfavorable for PNP degradation (Fig. 2).

PNP degradation was observed over a wide range of pH from 5 to 10 (Fig. 3). The optimal pH for PNP degradation was found to be alkaline (pH 7–9). A pH lower than 6 or higher than 10 was shown to be less suitable for PNP degradation (Fig. 3).

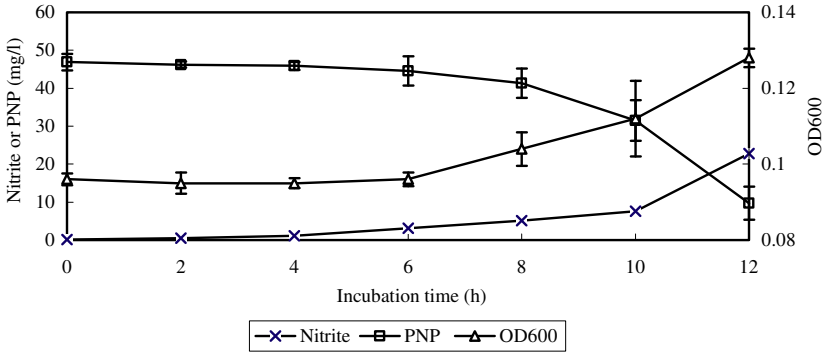

Fig. 1. Degradation of PNP, nitrite release (on the left *Y* axis), and cell growth (on the right *Y* axis) of *Arthrobacter* sp. HY2 in 50 ml of minimal salt medium containing PNP (50 mg/l) at pH 7.0 and 30 °C.

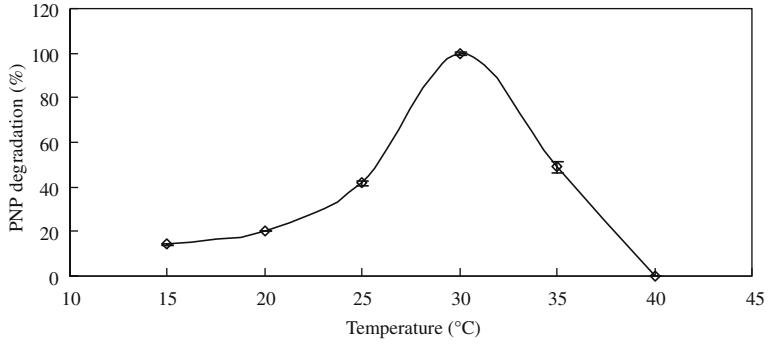

Fig. 2. Effect of temperature on PNP degradation. Data are mean percentage PNP degradation by *Arthrobacter* sp. HY2 cultivated in minimal salt medium containing 50 mg/l of PNP with an initial OD600 of 0.15 for 6 h at pH 8.0 and 30 °C.

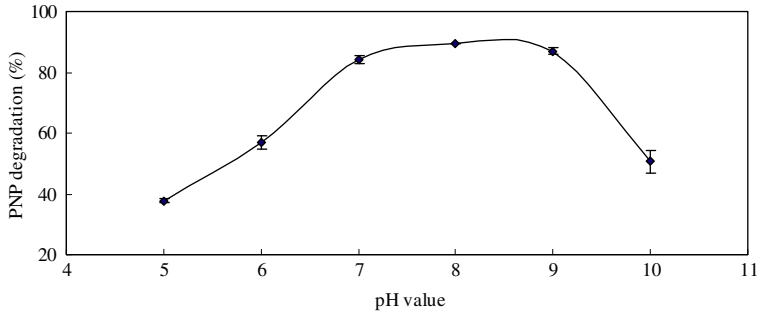

Fig. 3. Effect of pH on PNP degradation by *Arthrobacter* sp. HY2. Data are mean percentage PNP degradation by *Arthrobacter* sp. HY2 cultivated in minimal salt medium containing 50 mg/l of PNP with an initial OD600 of 0.15 for 6 h at 30 °C and different pH.

3.3. Effect of PNP concentration

Rates of PNP degradation by HY2, ranging from 200 to 500 mg/l, were tested in MSM at pH 8.0 and 30 °C. Our data show that effective degradation rates were hampered as initial PNP concentration increased (Fig. 4). For example, more than 90% PNP was depleted within 24 h if the initial concentration of PNP was below 250 mg/l, while 350 mg/l and 400 mg/l of PNP were totally depleted in 48 h and 168 h, respectively. Minor degradation of PNP was detected at 450 mg/l after incubation for over 48 h. No PNP degradation was observed at a concentration of 500 mg/l of PNP for a period of 7 days.

3.4. Effect of extra carbon source on PNP degradation at 400 mg/l

MSM enriched by the addition of glucose (0.1–0.5%) greatly improved the degradation of PNP at 400 mg/l (Fig. 5, top). Greater degradation occurred at 0.1% glucose than that at 0.3% or 0.5%. Dynamic alteration of pH in the medium containing glucose was observed (Fig. 5, bottom). In the presence of 0.1% glucose, the pH value in the medium decreased gradually from initial 7.8 to final 6.8 within 96 h, a pH condition favorable for PNP utilization. In contrast, with the addition of 0.3% and 0.5% glucose, the pH dropped gradually from an initial pH of 7.7 to pH 6.9 within 36 h (Fig. 5, bottom), and then decreased sharply and remained at around pH 5.2, respectively after 48 h, a pH value which has proved limiting for PNP degradation. No obvious acidification and only a marginal depletion of a high concentration of PNP were observed in the medium without glucose (Fig. 5).

3.5. Effects of β-cyclodextrin on PNP degradation at 400 mg/l

The effect of β-cyclodextrin on PNP degradation is shown in Fig. 6. Higher concentrations of β-cyclodextrin had a significantly

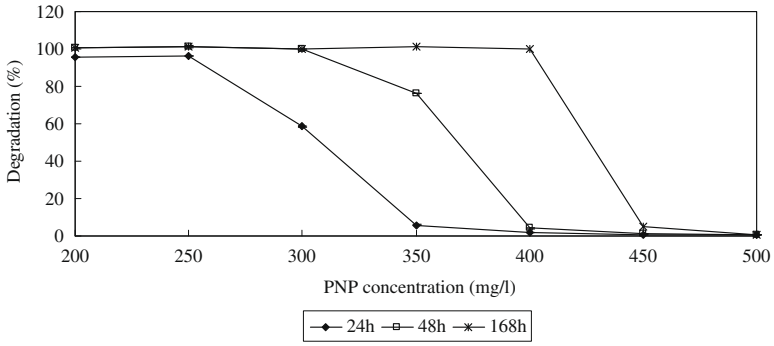

Fig. 4. Effect of PNP concentration on PNP degradation by *Arthrobacter* sp. HY2 under 30 °C and pH 8.0 conditions with an initial OD600 of 0.15.

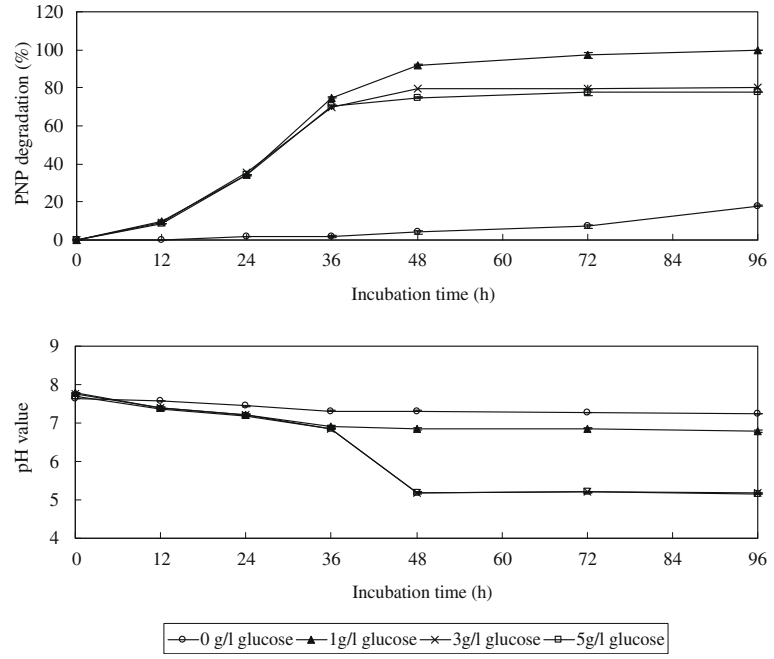

Fig. 5. PNP degradation and pH change in the minimal salt medium containing 400 mg/l of PNP, with or without the addition of glucose, inoculated with *Arthrobacter* sp. HY2. The initial OD600 was 0.15 and incubation temperature was 30 °C.

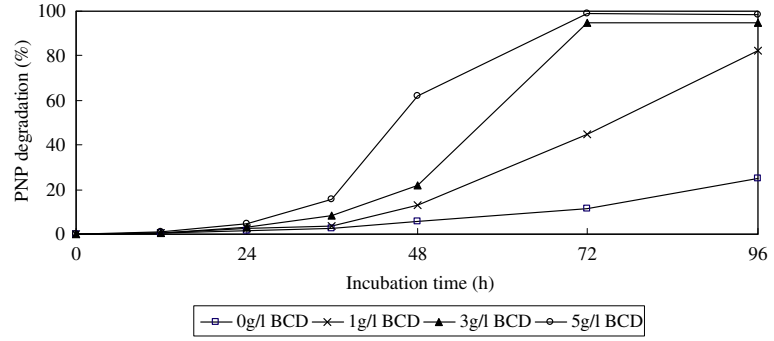

Fig. 6. PNP degradation in the minimal salt medium containing initial 400 mg/l of PNP with or without addition of β-cyclodextrin (BCD), inoculated with *Arthrobacter* sp. HY2. The initial OD600 was 0.15 and incubation condition was temperature of 30 °C, pH of 8.0.

more powerful enhancing effect on PNP degradation. With addition of β-cyclodextrin in the media at 5 g/l, almost all PNP (99%) was depleted within 72 h, while only 11.5% PNP was transformed in the absence of β-cyclodextrin (Fig. 6).

4. Discussion

The efficiency of microbial degradation is often seriously impeded by the inaccessibility of lipophilic compounds to the

microorganism carrying out the degradation, and the toxicity which substrates may have for the microorganisms (Schwartz and Bar, 1995). PNP is water-soluble, so the main obstacle to PNP degradation is its toxicity. It has been well known that PNP is highly toxic to most microorganisms (Roldan et al., 1998; Labana et al., 2005) and that the toxicity of PNP at high concentrations limits its degradation (Hanne et al., 1993). Microbes with high tolerance to PNP have undoubted advantages for the clearance of high concentrations of PNP. However, to date, few bacteria have been documented to be PNP-tolerant (Kulkarni and Chaudhari, 2006; Li et al., 2008; Liu et al., 2007). HY2's tolerance of PNP up to 400 mg/l (Fig. 4), a concentration proven to be toxic to most microorganisms (Kulkarni and Chaudhari, 2006), and HY2's ability to degrade PNP effectively over a relatively wide pH range (Fig. 3), make this isolate a realistic candidate for bioremediation.

It has been suggested that the toxicity of PNP increases with a decrease in pH (Zeyer et al., 1986). The initial pH value and the potential range of pH in the processing system are therefore important parameters and must be considered when constructing a PNP remediation system (Qiu et al., 2007). Our results of PNP degradation at varying pH values suggest that a slightly alkaline pH is favorable for PNP degradation by HY2 (Fig. 3). Other studies on different bacteria (Kulkarni and Chaudhari, 2006; Labana et al., 2005; Qiu et al., 2007; Unell et al., 2008) have also reported faster degradation of PNP at high pH, probably due to the increased bioavailability and decreased toxicity of PNP, and optimal metabolic activity of the bacterial cells (Wan et al., 2007).

Glucose is often used as a carbon source for bacteria in order to enhance their growth and metabolism. We assessed the effect of different glucose concentrations on PNP degradation. Our results show that the addition of glucose at 0.1–0.5% enhances the PNP degradation. The enhancement effect is not positively related to the glucose concentration, better degradation occurs at 0.1% glucose than that at 0.3% or 0.5% (Fig. 5). In contrast, Kulkarni and Chaudhari (2006) examined the impact of 0.4 g/l glucose on PNP degradation at 300 mg/l by *P. putida* and concluded that the higher concentration of glucose in the minimal medium did not favor PNP degradation. A possible explanation for this discrepancy between the two systems may lie in the degree of acidification during glucose metabolism. In the presence of glucose, the pH of the medium dropped from pH 7.0 to pH 3.0 within 6 h, resulting in the inhibition of PNP degradation by *P. putida* (Kulkarni and Chaudhari, 2006). In our biodegradation system, the pH remained at a value greater than pH 6.8 for 36 h, which was favorable for PNP degradation. Although additional carbon source such as glucose usually accelerate PNP biodegradation activity, it is worth noting that the metabolism of additives may acidify the environment, depending on the amount added, and this may contribute to a decrease of PNP degradative efficiency (Qiu et al., 2007; Li et al., 2008).

Given that toxicity is the main obstacle to PNP degradation, the addition of compounds which alleviate this may improve PNP degradation. Cyclodextrins (CDs) are natural, non-toxic cyclic oligosaccharides. CD molecules have an external hydrophilic shell and a hydrophobic internal cavity. This structure gives CDs the ability to accommodate guest molecules in the cavity via the formation of inclusion compounds (Schwartz and Bar, 1995). Our results showed that β-cyclodextrin, the cheapest cyclodextrin, significantly increased the biodegradation of high concentrations of PNP (Fig. 6). One explanation for the enhancement of biodegradation is that the soluble β-CD–PNP complex reduces the effective concentration of PNP, so that HY2 is exposed to less toxic concentrations. This kind of toxicity alleviation by the CD complex has also been documented in other studies of the degradation of aromatics (Schwartz and Bar, 1995; Boyle, 2006). Cyclodexitrins (CDs) have been used to enhance biodegradation of toxic compounds (Bardi et al., 2000; Boyle, 2006; Cai et al., 2006; Garon et al., 2004; Wang et al., 1998; Zhou et al., 2007). β-Cyclodextrin may be a promising additive for the treatment of PNP contamination, not only for its considerable acceleration of PNP degradation, but also for its non-toxicity, relatively low cost, biocompatibility, and biodegradability (Bardi et al., 2000; Del Valle, 2004). Further work is needed to explore the optimal ratio between cyclodextrin and PNP for the greatest enhancement of PNP degradation at a lower cost.

5. Conclusion

A soil bacterium, *Arthrobacter* sp. HY2, which utilizes PNP as a carbon and energy source and which tolerates PNP up to 400 mg/l was isolated successfully. The concentration of PNP and the pH proved to be two important factors affecting the rate of PNP degradation by HY2. The addition of glucose at 0.1 g/l and β-cyclodextrin at 3–5 g/l greatly improved PNP degradation by HY2. The findings concerning *Arthrobacter* sp. HY2 may have direct relevance to bioremediation of PNP and related pollutants in contaminated water.

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