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Biodegradation of *p*-nitrophenol by methyl parathion-degrading *Ochrobactrum* sp. B2

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Abstract

Ochrobactrum sp. B2, a methyl parathion-degrading bacterium, was proved to be capable of using *p*-nitrophenol (PNP) as carbon and energy source. The effect of factors, such as temperature, pH value, and nutrition, on the growth of *Ochrobactrum* sp. B2 and its ability to degrade *p*-nitrophenol (PNP) at a higher concentration ($100\,mg\,l^{-1}$) was investigated in this study.

The greatest growth of B2 was observed at a temperature of 30 °C and alkaline pH (pH 9–10). pH condition was proved to be a crucial factor affecting PNP degradation. Enhanced growth of B2 or PNP degradation was consistent with the increase of pH in the minimal medium, and acidic pH (6.0) did not support PNP degradation. Addition of glucose (0.05%, 0.1%) decreased the rate of PNP degradation even if increased cell growth occurred. Addition of supplemental inorganic nitrogen (ammonium chloride or ammonium sulphate) inhibited PNP degradation, whereas organic nitrogen (peptone, yeast extract, urea) accelerated degradation.

Keywords: *p*-nitrophenol; Biodegradation; *Ochrobactrum*

1. Introduction

The wide use of nitroaromatics, as synthetic intermediate in the manufacture of pharmaceuticals, pigments, dyes, plastics, pesticides and fungicidal agents, explosives and industrial solvents (Spain, 1995), leads to accumulation of nitrophenols (Schackmann and Muller, 1991). *p*-Nitrophenol (PNP) is probably the most important among the mononitrophenols in terms of the quantities used and potential environmental contamination (Karin and Gupta, 2002). PNP has been classified as a priority pollutant by the United States Environmental Protection Agency (EPA, 1980), which recommends restricting PNP concentrations in natural waters to $<10\,ng\,l^{-1}$ (Genini et al., 2005). Therefore, PNP poses significant health and environmental risks, because it is toxic to many living organisms and it may accumulate in the food chain (Donlon et al., 1996).

As a result of growing awareness over pollution caused by PNP release, efforts are being made to minimize its adverse effect. Microbial systems and bioremediation programs are becoming the method of choice over other traditional methods such as land-filling, incineration, excavation, etc. for the treatment of contaminated soils and waters (Labana et al., 2005). Although the nitrogroup of PNP enhances the resistance of the aromatic ring to biodegradation, several bacterial strains able to utilize PNP as sole carbon and energy source have been described. These include species of *Arthrobacter* (Jain et al., 1994; Chauhan et al., 2000), *Bacillus* (Kadiyala and Spain, 1998), *Burkholderia* (Bhushan et al., 2000; Chauhan et al., 2000), *Pseudomonas* (Prakash et al., 1996; Zaidi and Mehta, 1996; Liu et al., 2005; Kulkarni and Chaudhari, 2006), and *Rhodobacter* (Roldan et al., 1998).

We have previously reported the isolation and characterization of the bacterium *Ochrobactrum* sp. B2, which is capable of hydrolyzing methyl parathion and using PNP as sole source of carbon and energy (Qiu et al., 2006). Although the screening of potential microbes is a critical

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step in construction of a system for remediation, an understanding of the interplay between the biotic and abiotic factors is also important. In this study, we attempted to examine the effects of various factors on the degradation of PNP and growth of B2 in liquid cultures, in order to provide practical information for the design of an effective remediation strategy.

2. Materials and methods

2.1. Chemicals

PNP of chromatographic grade was purchased from Fluka (Buchs, Switzerland); glucose and yeast extract were the products of Difco (Detroit, USA). All other chemicals used were of the highest purity available.

2.2. Bacterial strain

The wide-type *Ochrobactrum* sp. B2 is a rod- shaped, Gram-negative soil bacterium, which has been described by Qiu et al (2006).

2.3. Biodegradation test

The inocula for the experiments of PNP degradation study were prepared by growing bacteria in 50 ml of minimal salt medium (MSM) (Sutherland et al., 2000) supplemented with 0.1% glucose and 50 mg l^{-1} PNP, and incubated for 36 h at 30 °C on a shaker at 150 rpm. The culture was asceptically harvested and the cells were washed thoroughly with MSM (8000 × *g*, 10 min, 4 °C). The cells were suspended in sterile MSM and added as the inoculum. Appropriate modifications were made to the incubation systems in order to analyze the effects of abiotic factors on the growth of B2 and its ability in depleting PNP. After appropriate intervals, aliquots of samples were removed in order to determine both the amount of remaining PNP and/or the amount of nitrite released, and also to evaluate microbial growth. All the experiments were performed in triplicate. Un-inoculated control tubes were maintained in all experiments. The results are reported as an average of three replicates.

2.4. 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^{-1}. 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 quantitatively determined based on the standard curves prepared using sodium nitrite (DU-800 spectrophotometer, Beckman Coutler Inc., Fullerton, California, USA) by the method of Montgomery and Dymock (1961).

3. Results

3.1. Effect of temperature

The experiments carried out to determine the optimal temperature for the PNP showed that degradation occurred over a wide range of temperatures (Fig. 1). The greatest growth of B2 and PNP depletion was observed at 30 °C. Temperature over 30, 35, and 40 °C were proved to be unfavorable for both growth of B2 and PNP degradation.

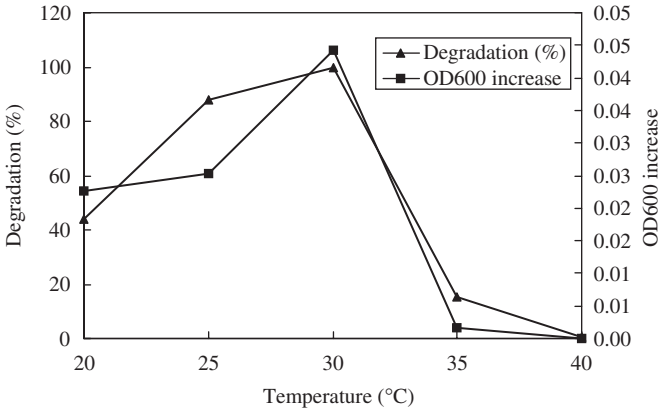

Fig. 1. The effect of temperature on B2 growth and PNP degradation. The initial PNP concentration was 100 mg l^{-1}, and initial OD600 was 0.02. The data were the results after incubation for 48 h.

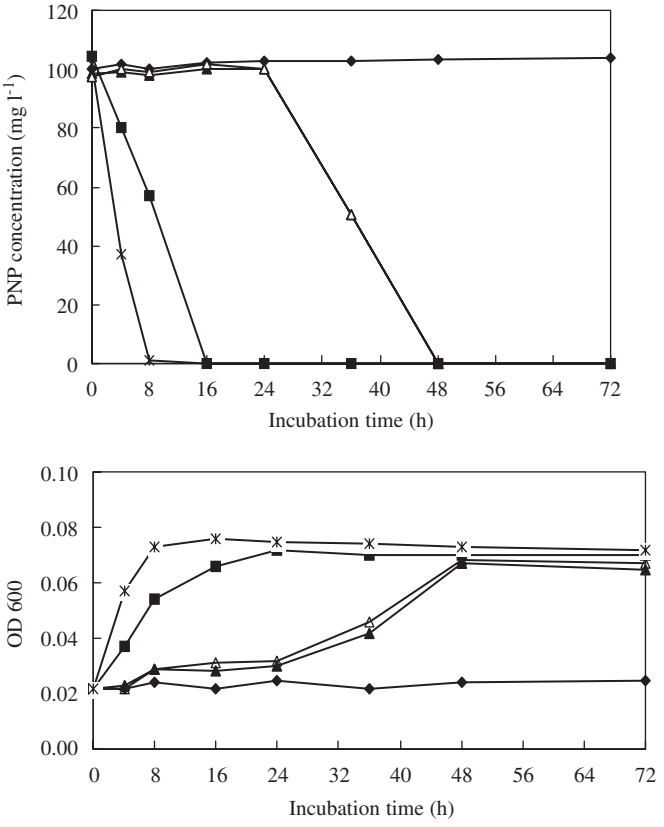

Fig. 2. Effect of initial pH values on PNP degradation by B2 (top), and the growth of B2 (bottom). ♦, pH = 6; ▲, pH = 7, △, pH = 8, ■, pH = 9, *, pH = 10.

3.2. Effect of pH

To explore the effect of pH, MSM was adjusted separately to pH 6.0, 7.0, 8.0, 9.0, and 10.0. The optimal of pH for PNP degradation was found to be alkaline (Fig. 2). If the initial pH was 10,98% of PNP was depleted after incubation for 8 h . PNP disappeared completely at

pH 9.0 after incubation for 16 h. At acidic condition (pH = 6.0), no degradation of the added 100 mg l^{-1} PNP was detected, probably due to growth inhibition of B2. The increase of OD600 of the cultures accompanied with the degradation of PNP, indicated that growth of B2 relied on PNP as its energy and carbon source. The greatest and fastest growth occurred at pH 10. No PNP degradation was observed in the incubations without the inoculum at any tested pH conditions.

3.3. Effect of PNP concentration

To study the effect of PNP concentration on the rate of degradation by B2, different concentrations, ranging between 15 and 300 mg l^{-1}, were tested. Effective degradation rates appeared hampered as the PNP concentration increased. Fig. 3 showed that the periods of acclimation and PNP depletion increased if increasing initial PNP was added in the medium. PNP was completely removed by B2

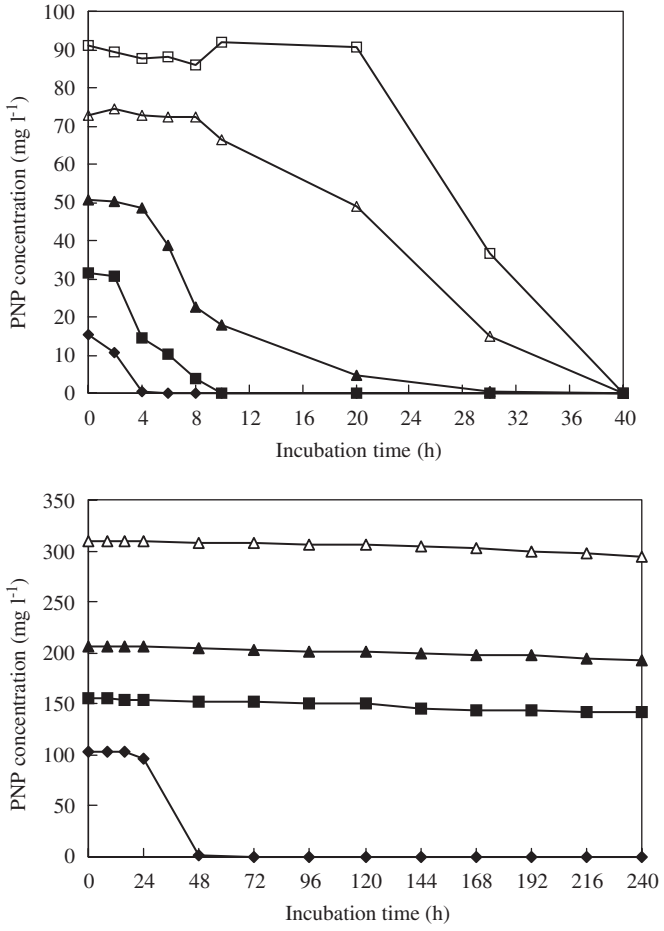

Fig. 3. The degradation of PNP by B2 at different initial PNP concentrations (the value at time zero). The initial OD 600 of inoculated B2 was 0.03.

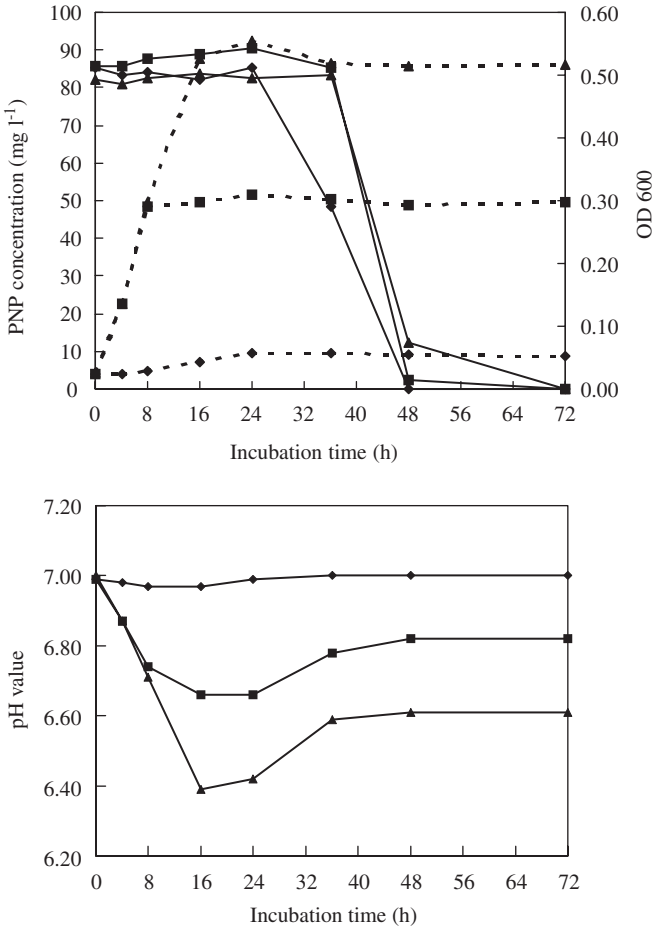

Fig. 4. PNP degradation, B2 growth (top) and pH changes (bottom) in the MSM media with or without addition of glucose. ♦, without glucose; ■, with addition of 0.05% glucose; ▲, with addition of 0.1% glucose. Dashed lines represent OD 600.

Table 1
The PNP concentration in the media inoculated with different amounts of B2

Amount of inoculum (initial OD 600)	PNP concentration (mg l^{-1}) after varying incubation times (h)					
	0	24	48	72	120	168
No-inocula	135.1±5.5	138.5±6.3	140.3±5.9	140.3±6.9	144.3±7.2	151.4±12.8
0.014	136.8±8.3	137.9±7.4	139.8±7.1	140.9±6.2	143.6±8.0	149.1±9.2
0.026	135.1±4.1	138.2±3.8	139.0±3.4	141.6±3.6	143.9±4.1	148.9±4.5
0.058	132.9±8.6	134.8±6.9	138.1±8.4	138.3±9.0	143.4±10.6	146.9±9.6
0.174	134.9±6.9	132.9±5.7	138.4±5.5	139.4±6.5	144.3±7.3	154.0±16.4

Data are mean±SD of three replicates.

within 40 h if the initial concentration of PNP was under 100 mg l^{-1} (Fig. 3, top). However, when the PNP in the medium was over 100 mg l^{-1}, no significant degradation was observed over a period of 10 days (Fig. 3, bottom). The degradation of PNP at a relative high concentration (over 100 mg l^{-1}) was not enhanced by increasing inoculum size (Table 1).

3.4. Effect of additional carbon source

Glucose-enriched MSM at the experimental concentration did not enhance PNP degradation, but greatly increased the growth of B2 (Fig. 4, top). The OD600 in the medium with addition of 0.05% glucose was almost five times more than that without glucose, and greater growth occurred as the concentration of glucose increased. In MSM without glucose, *Ochrobactrum* degraded about 90 mg l^{-1} PNP within 48 h with a lag phase of about 24 h, whereas a lag of 36 h was observed in MSM with the addition of glucose. In the presence of 0.1% and 0.05% glucose, the pH in the incubation dropped gradually from initial 7.0 to 6.4 and 6.6 within 16 h, respectively (Fig. 4, bottom). pH increased afterwards and remained at 6.6 and 6.8, respectively after 36 h, when significant PNP degradation was observed. In contrast, no significant change of pH was monitored in the medium without glucose.

3.5. Effect of nitrogen source

Various nitrogen sources were used to investigate their effects on PNP degradation and on growth of B2 (Figs. 5a–d). B2 could grow in the absence of nitrogen, suggesting the nitrogen was not a necessary nutrient for its growth. Addition of 1 g l^{-1} inorganic nitrogen, such as ammonium chloride and ammonium sulfate, did not favor the growth of B2 or the degradation of PNP. In contrast, without added inorganic nitrogen in the medium, B2 showed a shorter lag in PNP degradation, paralleling with an increase of pH in the medium (Fig. 5d). No significant difference in the amount of nitrite produced was detected in the media with or without nitrogen addition, which indicated that B2 could not use the resultant nitrite as a nitrogen source (Fig. 5b).

4. Discussion

PNP has been reported to be highly toxic to some microorganisms (Roldan et al., 1998; Labana et al., 2005). The toxicity of higher concentrations of nitro-aromatics limits its degradation by microbes. Therefore, degradation of PNP is mostly studied at lower concentrations (Hanne et al., 1993). However, *P. putida* has been documented to be PNP-tolerant, and in about 72 h can completely degrade 500 ppm PNP, a concentration proven to be toxic to most of the microorganisms (Kulkarni and Chaudhari, 2005).

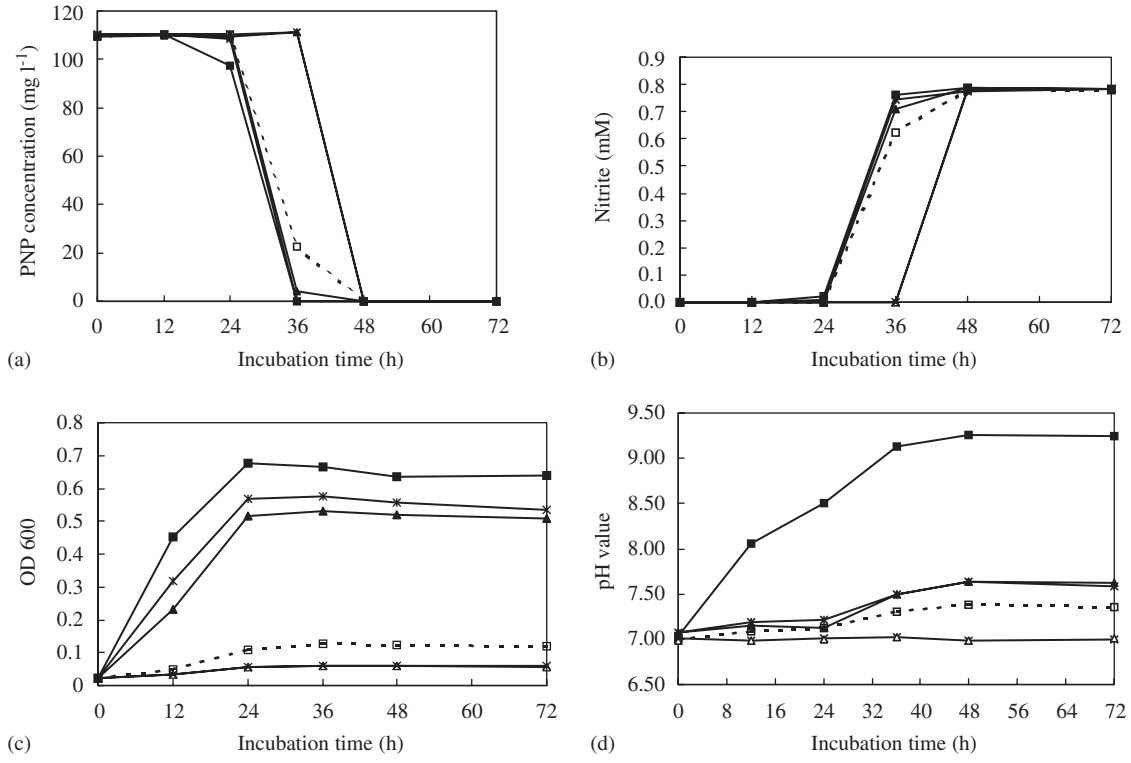

Fig. 5. Effect of nitrogen source on the PNP degradation and B2 growth. ▪□▪, without addition of nitrogen; ——, ammonium chloride; ——△——, ammonium sulfate; ■■■, urea; ——✳——, peptone; ——▲——, yeast extract.

Our results showed that the ability of B2 to degrade PNP was influenced by PNP concentration. No significant degradation was observed if the initial concentration of PNP was more than 100 mg l^{-1} (Fig. 3), suggesting that cell toxicity of PNP towards B2 existed. Improvement of the rate of PNP degradation by *Ochrobactrum* sp. B2 at higher concentrations by optimizing processing parameters is a subject for further investigation.

Our results of PNP degradation at varying pH conditions suggest that an alkaline pH condition is crucial to achieving PNP degradation by B2. It was suggested that toxicity of PNP increases with a decrease in pH (Zeyer et al., 1986). Hence, the acceleration of PNP degradation by B2 could perhaps be due to lower toxicity of PNP at alkaline pH. Other workers have reported better degradation of PNP (Kulkarni and Chaudhari, 2006; Labana et al., 2005) and other contaminants such as chlorpyrifos (Singh et al., 2003) at alkaline conditions.

Additional nutrients such as glucose and organic nitrogen greatly enhanced the growth of B2 (Figs. 4 and 5). Metabolism of glucose resulted in an acidic environment and this contributed to the decrease of PNP degradative activity of B2 (Fig. 4). The addition of urea largely accelerated the PNP degradation, and the acceleration of PNP degradation rate was concomitant with an increase of pH in the medium (Fig. 5). These results suggest that the pH change caused by additional nutrients is an important factor that needs to be considered when making a remediation strategy.

In conclusion, *Ochrobactrum* sp. B2 showed the capacity to degrade PNP for use as a carbon and energy source. Inorganic nitrogen was not necessary for B2 growth, and the nitrite was not aerobically transformed by B2. Addition of supplemental organic nutrients such as glucose, peptone, yeast extract and urea enhanced B2 growth. pH condition was proved to be a crucial factor affecting PNP degradation and B2 growth. The concentration of PNP and the pH condition would be of importance while developing an effective remediation strategy.

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