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Kinetics and mechanisms of *p*-nitrophenol biodegradation by *Pseudomonas aeruginosa* HS-D38

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Abstract

The kinetics and mechanisms of *p*-nitrophenol (PNP) biodegradation by *Pseudomonas aeruginosa* HS-D38 were investigated. PNP could be used by HS-D38 strain as the sole carbon, nitrogen and energy sources, and PNP was mineralized at the maximum concentration of 500 mg/L within 24 h in an mineral salt medium (MSM). The analytical results indicated that the biodegradation of PNP fit the first order kinetics model. The rate constant k_{PNP} is 2.039×10^{-2}/h in MSM medium, K_{PNP+N} is 3.603×10^{-2}/h with the addition of ammonium chloride and K_{PNP+C} is 9.74×10^{-3} /h with additional glucose. The addition of ammonium chloride increased the degradation of PNP. On the contrary, the addition of glucose inhibited and delayed the biodegradation of PNP. Chemical analysis results by thin-layer chromatography (TLC), UV-Vis spectroscopy and gas chromatography (GC) techniques suggested that PNP was converted to hydroquinone (HQ) and further degraded via 1,2,4-benzenetriol (1,2,4-BT) pathway.

Key words: *p*-nitrophenol biodegradation; *Pseudomonas aeruginosa*
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Introduction

Nitrophenols are widely used in the production of pesticides, herbicides, explosives, dyes and plasticizers (Kulkarni and Chaudhari, 2007). After being released into the environment, they become pollutants of wastewater, pesticide-treated soils, rivers and urban atmospheres. Hence, many of them are considered as priority pollutants (as HR-3 grade) and recommended restricting their concentrations in the natural water bodies to below 10 ng/mL by the US Environmental Protection Agency (Leung *et al*., 1997; USEPA, 1980).

p-Nitrophenol (PNP), one of the common and important mono-nitrophenols, is widely used for industrial production (Kitagawa *et al*., 2004), especially in the synthesis of the aspirin acetaminophen, the manufacture of pesticides such as parathion and methyl parathion. It is also the intermediate product of some organophosphorus pesticides and nitroaromatic herbicide degradation (Zhang *et al*., 2008; Liu *et al*., 2007; Spain and Gibson, 1991). As an environmental hazardous reagent, PNP has been extensively studied as a model of nitrophenols biodegradation because of its wide distribution in both terrestrial and aquatic environment, and difficulties in biodegradation by reason of its aromatic ring in PNP structure. In view of its toxic effects, efforts have been made to examine the removal of PNP from effluents. At present, the methods eliminating PNP include physico-chemical combination (Fares Al *et al*., 2008) and biodegradation (Genini *et al*., 2005; Tomei *et al*., 2006; Chen *et al*., 2002). Since bioremediation has the advantages of rapid, mild reaction conditions, cost-effective, friendly environmental and no secondary pollution, it offers a promising strategy for economical and safe detoxification of some wastes. Strains capable of degrading PNP, including species such as bacteria (Roldán *et al*., 1998; Liu *et al*., 2003; Labana *et al*., 2005) and microalgae (Sofia *et al*., 2003) have been isolated. Due to that the toxicity of high concentration of PNP limited its degradation by microbes (Kulkarni and Chaudhari, 2007), most of works mainly focused on PNP at low concentrations (50–150 mg/L) (Spain and Gibson, 1991; Qiu *et al*., 2007; Pakala *et al*., 2007). Biodegradation of PNP at high concentration (500 mg/L) with effective degradation rate was rarely reported. Naturally, an effective bioremediation strategy is constrained due to many factors, such as addition of carbon and nitrogen source. Biodegradation of PNP was generally tested in mineral salt medium (MSM), which avoided other carbon and nitrogen sources, and most studies indicated that supplemental substrate (glucose

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and ammonium chloride) had showed positively effect on nitrophenol biodegradation (Kulkarni and Chaudhari, 2006; Hess *et al*., 1990; Zhang *et al*., 2009a). On this background, characteristics of PNP degradation and the kinetics properties in the process with high concentration were investigated.

1 Materials and methods

1.1 Bacterial strain and culture medium

The wide-type bacterial strain HS-D38 capable of degradation methyl parathion as the sole source of carbon, nitrogen and energy, was originally isolated from cotton soils contaminated by organophosphorus pesticides. The strain was identified as *Pseudomonas aeruginosa* in our laboratory (Zheng *et al*., 2006).

HS-D38 strain was incubated in LB medium at 35°C for 15 h, then the cells were harvested, centrifuged (5000 r/min at 4°C, 10 min,), washed 3 times and suspended with sterile MSM (OD_{600} = 0.5) as prepared inoculums.

MSM was used for cultured medium of PNP degradation and the composition was as follows (g/L): KH_2PO_4 0.5, K_2HPO_4 1.5, NaCl 0.5, and $MgSO_4{\cdot}7H_2O$ 0.5, and 1.5 mL trace solution (Li *et al*., 2006). Glucose or ammonium chloride of 100 mg/L was added into MSM medium as supplemental substrate. All media were adjusted pH to 7.0 and autoclaved for 30 min.

1.2 Chemicals

Chemicals including *p*-nitrophenol, hydroquinone (HQ), 4-nitrocatechol (4-NC), 1,2,4-benzenetriol (1,2,4-BT) and quinone (QO) (⩾ 98.0% purity) were purchased from Sigma Chemical Co., USA. All other chemicals were of analytical grade from Shanghai Chemicals Co., China. Biochemical reagents were purchased from Takara Biotechnology Co., Ltd., China.

1.3 Metabolites extraction and preparation

On the basis of PNP mineralization time in MSM medium, the samples were collected at 8 h and 12 h for metabolite study. To recover enough metabolites, extractions were carried out from 1000 mL degradation samples by ethyl acetate, dehydrated with sodium sulfate, evaporated using a rotary evaporator, and then the sediment was redissolved into 1 mL methanol and filtered with a 0.22-μm membrane for thin-layer chromatography (TLC) and UV-Vis spectral analysis.

1.4 Analytical methods

In MSM medium, the yellow culture changed to colorless, indicating the PNP degradation and utilization by the HS-D38 strain. Bacteria growth was evaluated spectrophotometrically at 600 nm using a 722S spectrophotometer (Shanghai, China). The dynamic curves of PNP degradation were determined by sampling every hour and scanning with UV-Vis spectrophotometer (S-3100, Scinco Co., Ltd., Korea).

TLC was carried out as follows: the TLC plates were precoated with 0.25 mm, 20 cm × 20 cm silica gel 60 GF254 and developed with benzene-acetic acid (90:10 by volume). The bands, visualized by UV light at 254 nm, were scraped out and then extracted with methanol.

The PNP concentration was determined by gas chromatography (GC) using Agilent 6890N Series GC (Agilent Technologies Co., Ltd., USA), which was equipped with a fused silica capillary column (30 m × 0.25 mm × 0.25 μm HP-5MS), 7683 Series injector autosampler and a flame ionization detector (FID). FID was supplied with hydrogen (30 mL/min) and air (300 mL/min). Nitrogen was used as carrier gas. Headspace samples were injected into GC periodically. The injector temperature was 250°C, the detector temperature and the transfer line temperature were set at 280°C. The column temperature was programmed to rise at a rate of 10°C/min from 50 to 250°C, and then kept isothermal at 250°C for 5 min.

Chemical compounds of the metabolites sampled from TLC bands. The extractions were identified and quantified by comparing GC retention time (RT) and UV-Vis spectra with those of external standards.

2 Results and discussion

2.1 Kinetics of PNP degradation in MSM

In MSM, the yellow liquid gradually changed to colorless, whereas the contrast did not change after a specified period, which indicated the occurrence of PNP degradation. In order to determine the variation of the PNP concentration during the degradation course, dynamic scanning of samples at interval time by UV-Vis spectrophotometer was performed and the degradation amounts were measured. As shown in Fig. 1a, there was an absorption peak of PNP at 395 nm, and the peak declined slowly after an initial lag phase of 2 h. Subsequently, the peak decreased more rapidly in the next 8 h, indicating that the PNP degradation became active. By 10 h, the peak eventually disappeared and PNP concentration was undetectable, suggesting the completely degradation of 200 mg/L PNP (Fig. 1b). At the same time, an increase of OD_{600} values corresponding to the PNP decrease in the culture was also observed. The biomass increased from the initial 0.09 (OD_{600}) to 0.245 after 10 h, and the concentration of PNP from the initial 200 mg/L decreased gradually and finally no PNP could be detected (Fig. 2a). In the control experiment, no depletion of PNP concentration or increase of OD_{600} (bacteria not grew) was observed. These results indicated that HS-D38 strain could effectively degrade PNP at a concentration as high as 200 mg/L, and used it as the sole carbon, nitrogen and energy source.

Kinetics analysis was performed based on the principle that the whole cells were utilized as an enzyme system for PNP degradation (Dykaar and Kitanidis, 1996). The simulated kinetics curves meet first-order kinetic model. Our result is consisted with the previous report:

$$\ln(C_0/C) = kt \quad (1)$$

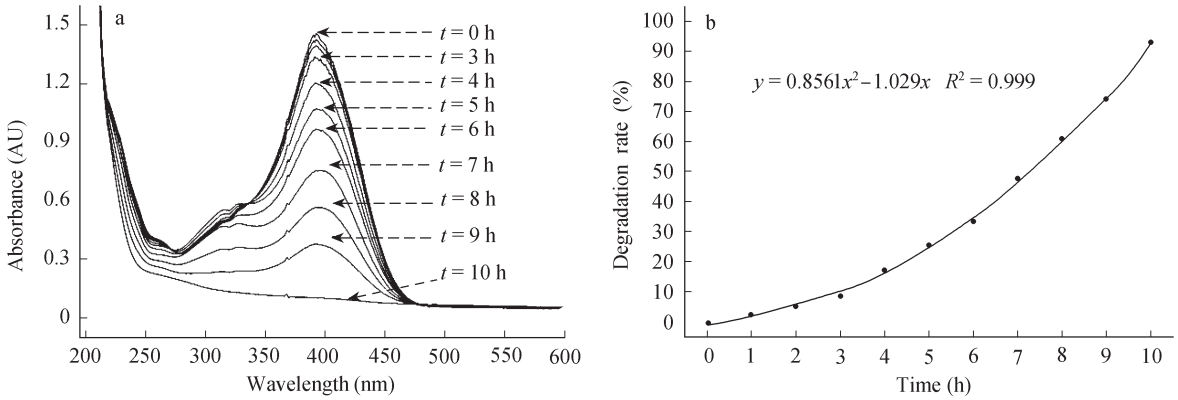

Fig. 1 Dynamic analysis of PNP degradation. (a) UV-Vis spectra of PNP degradation samples from 0 to 10 h; (b) PNP degradation rate.

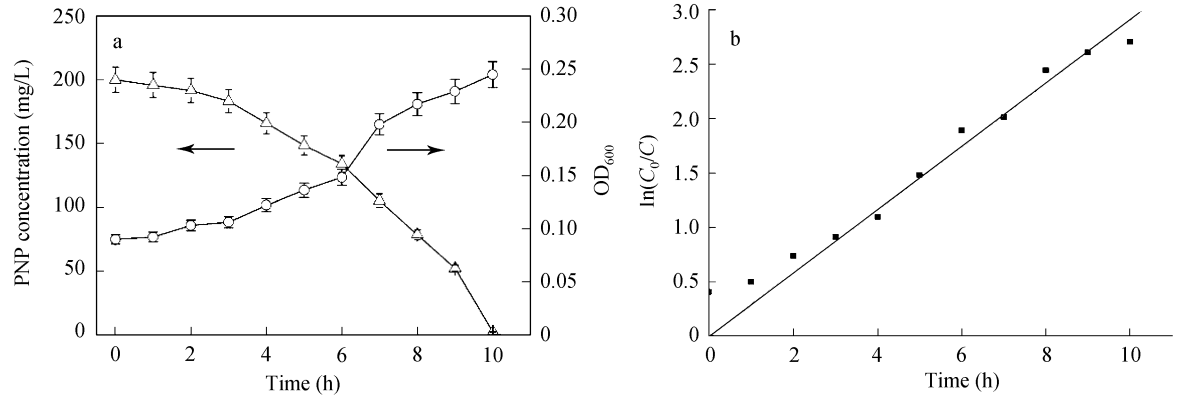

Fig. 2 Kinetics study of the PNP degradation in MSM. (a) the corresponding concentration and biomass vs. time; (b) the first order kinetics in PNP degradation (R^2 = 0.991).

where, C_0 is the initial concentration of PNP, C is the concentration of PNP at time t, and k is the reaction constant of first-order reaction. As shown in Fig. 2b, a linear relationship was obtained with k = 0.0291 h^{-1} and R^2 = 0.991.

2.2 Effects of supplemental carbon and nitrogen source

In MSM, the addition of ammonium chloride as nitrogen source further increased the PNP degradation amounts. However, glucose as an additional carbon source inhibited the degradation process (Fig. 3a).

From experimental data, the kinetics of PNP degradation process in MSM, MSM with additional ammonium chloride and MSM with additional glucose meet a first order model with a corresponding k value: k_{PNP}, K_{PNP+N} and K_{PNP+C}, respectively. PNP was degraded by HS-D38 more rapidly in MSM with additional ammonium chloride than in MSM with additional glucose (Fig. 3b). This was accord with the studies of Kulkarni and Chaudhari (2006). Supplemental substrates such as nitrogen source have shown positive effect on PNP biodegradation in most of the previous studies, whereas additional carbon source generally support the increase in biomass and inhibit the PNP degradation. Acidic environment resulted from rapid glucose depletion inhibited the degradation of PNP at high concentrations (Zhang *et al*., 2009b; Hess *et al*., 1990). Here reported that the effects of additional carbon and nitrogen sources on PNP bioremediation using HS-D38 strain are significant and should be noticed.

2.3 Effect of different initial concentrations for PNP degradation

As Table 1 shows, PNP was completely degraded within 6 h at 100 mg/L and within 9 h at 300 mg/L. The average degradation amount was up to 33.3 mg/(L·h) at 300 mg/L in MSM medium. HS-D38 strains grew well at the concentration range of 100–400 mg/L, while growing slowly at the concentration of 500 mg/L, at which PNP was degraded completely within 24 h. When the concentration exceeded 600 mg/L, PNP could not be degraded even after 144 h, indicating that a high concentration of PNP was toxic to cells and restrained HS-D38 growth (ATSDR, 1992).

Subsequent study indicated that *P. aeruginosa* HS-D38 could efficiently degrade PNP and use it as the sole carbon, nitrogen and energy source with high degradation amount and tolerable concentration. At the concentration as high

Table 1 Effects of degradation in different initial PNP concentrations

PNP concentration (mg/L)	Bacteria growth (OD_{600})	Time of complete degradation (h)	Degradation rate (mg/(L·h))
100	0.20 ± 0.03	6.0 ± 0.2	16.6 ± 3.31
200	0.78 ± 0.12	6.5 ± 0.2	30.7 ± 6.12
300	1.10 ± 0.09	9.0 ± 0.3	33.3 ± 2.52
400	0.27 ± 0.02	15.0 ± 0.6	26.6 ± 3.74
500	0.16 ± 0.01	24.0 ± 0.5	20.8 ± 2.81
600	0.05 ± 0.01	ND	ND

Data are expressed as means of three independent experiments ± SD.
ND: no degradation

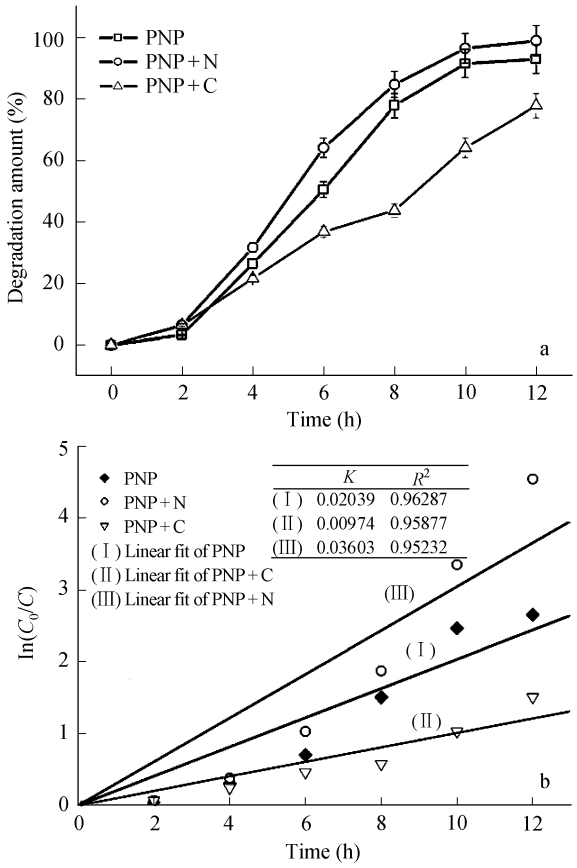

Fig. 3 Effects of supplemental carbon and nitrogen sources in PNP degradation in MSM (a) (initial volume 100 mL, C_{PNP} 200 mg/L, concentrations for both supplemental glucose and ammonium chloride 100 mg/L), and kinetics analysis (b).

as 500 mg/L, PNP could still be degraded within 24 h in MSM medium. Furthermore, HS-D38 strain could degrade other aromatic compounds, such as hydroquinone, aniline, naphthalene and phenol (Zheng *et al*., 2008). Compared with other bacteria capable of degrading PNP, *P. aeruginosa* HS-D38 strain was a highly efficient with a broad-spectrum degradative capability of the aromatic compound and a higher tolerable concentration, thus it will have a promising future in effective pollutants remediation.

Several PNP-degrading microorganisms have been successfully isolated from the contaminated sites during the recent few decades, including *Ochrobactrum*, *Moraxella*, *Nocardia*, *Arthrobacter*, *Ralstonia* and *Pseudomonas* (Gemini *et al*., 2005; Qiu *et al*., 2007; Wan *et al*., 2007; Liu *et al*., 2007; Spain and Gibson, 1991; Bhushan *et al*., 2000). They can metabolize PNP through removing their nitro-groups as nitrites and simultaneously utilize PNP as a source of carbon or/and nitrogen for growth at low concentrations (20–150 mg/L). The biodegradation of PNP at high concentrations (up to 500 mg/L) was rarely reported in MSM. Kulkarni and Chaudhari (2006) reported that *P. putida* strain could degrade PNP in minimal medium with 0.5 g/L yeast extract and acclimatized at respective PNP concentration for 18 h, and at OD_{600} = 0.5 inoculums, the strain metabolized 300 and 500 mg/L within 36 and 72 h, respectively, while HS-D38 could mineralized PNP at the maximum concentration of 500 mg/L within 24 h in

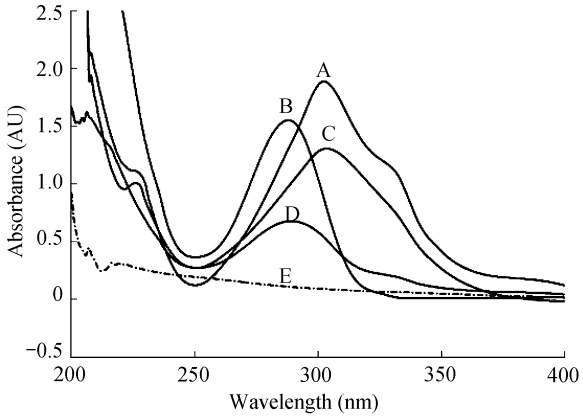

Fig. 4 Metabolites analysis with UV-Vis spectrophotometry. peak A: 302.5 nm, authentic standard PNP; peak B: 288.0 nm, authentic standard hydroquinone; peak C: band of R_f 0.37; peak D: band of R_f 0.79; peak E: extraction of degradation at 12 h.

MSM without acclimation phase. HS-D38 was the best in degrading with high efficiency at high PNP concentrations.

Keweloh and Heipieper (1996) reported that very high concentration of PNP has been attributed to changes in the membrane fluidity of *Pseudomonas*. Phenolic compounds, such as PNP, owing to their high aqueous solubility, have exerted toxic effects on membrane (Sikkema *et al*., 1995). *P. aeruginosa* has been reported to adapt to very high concentrations (2600 mg/L) of phenol and utilized it with 0.016 h^{-1} specific growth rate and 26.16 mg/(L·h) degradation rate (Afzal *et al*., 2007; Liu *et al*., 2004). Devleeschouwer *et al*. (1986) recorded notable difference of sensitivity of *P. aeruginosa* to chloroxylenol according to the growth medium with the difference components. Kulkarni and Chaudhari (2006) regarded it is rationalized that by cis-trans isomerization, *Pseudomonas* can quickly adapt to toxic organic substrates. The cis-to-trans conversion increases orderly nature of membrane and consequently decreases membrane fluidity. The most important adaptive response of bacteria against membrane-active substances is change(s) in fatty acid composition/protein:lipid ratio of membrane lipids. The cells of HS-D38 thus stabilize their membrane and allow them to stay in physiologically active condition in which they can adapt high PNP concentration.

2.4 Metabolites analysis by TLC and UV-Vis spectrophotometry

The intermediate metabolites were extracted and concentrated from the culture supernatant by solvent extraction under acidic conditions. TLC studies showed the presence of HQ (R_f 0.79), 1,2,4-BT(R_f 0.21) and PNP (R_f 0.37) in samples taken from 8 to 12 h intervals following the degradation of PNP by HS-D38 strain. The bands visualized at 254 nm were scraped out and then extracted with methanol. Extractions were scanned using UV-Vis spectrophotometry. Contrasting to the authentic standard compounds, the metabolites with the absorption maxima at 302.5 and 288.0 nm were identified as PNP and HQ, respectively (Fig. 4), indicating that HQ was the main metabolite in PNP degradation.

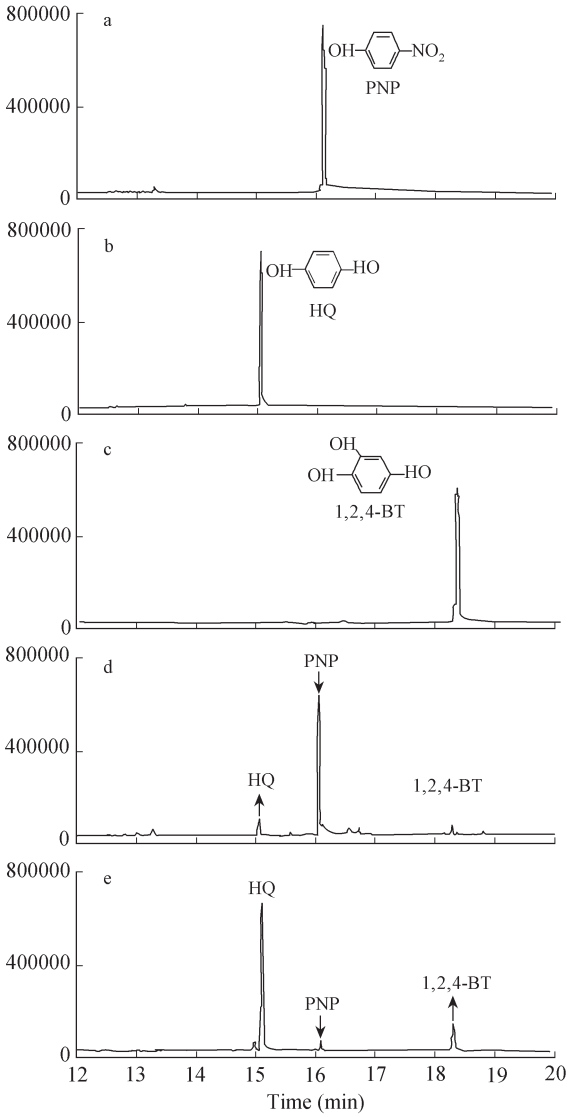

Fig. 5 GC analysis of metabolites in PNP degradation, the authentic standard of PNP (a), HQ (b), 1,2,4-BT (c) and the extraction of degradation at 8 h (d) and 12 h (e).

2.5 GC analysis of PNP degradation

The GC analysis result indicated that the retention time (RT) of the standard PNP, HQ and 1,2,4-BT was present at 16.1, 15.1 and 18.3 min, respectively (Figs. 5a, 5b, and 5c). After 8 h of biodegradation, the peaks of HQ and 1,2,4-BT were observed in addition to the peak of PNP, which corresponded well with their authentic standards, although the quantity of HQ and 1,2,4-BT were at a low level (Fig. 5d). The peak of PNP was sharply decreased and accompanied with the obvious enhancement of HQ peak and the 1,2,4-BT peak at 12 h, which indicates that enough metabolites were accumulated (Fig. 5e).

Two major initial degradation pathways of PNP by various microbes have been characterized. One is called HQ pathway in which PNP is converted to maleylacetate via hydroquinone (Zhang *et al*., 2009a), another is that PNP is converted via 4-nitrocathechol to 1,2,4-BT pathway (Chauhan *et al*., 2000). Our results indicated that HS-D38 degraded high concentration of PNP was also converted to hydroquinone via HQ pathway. According to the pathway of PNP degradation, 1,2,4-BT was probably converted from 4-nitrocathechol, whereas 4-nitrocatechol was not detected in all of the analysis techniques. The most probably reason was that hydroquinone was caused hydroxylation of the ring at the *ortho* position to 1,2,4-BT, which was reported in the study of *C. parapsilosis* CBS604 strain (Eppink *et al*., 2000) and remained unreported for *Pseudomonas*.

3 Conclusions

Organism was able to withstand and mineralized PNP at high concentration. Supplemental ammonium chloride as nitrogen source increased the PNP degradation, while glucose as additional carbon source had a reverse effect. Kinetics of PNP biodegradation fit the first order model very well, which constituted the basic requirement in an effective wastewater bioremediation strategy.

PNP was depredated and converted to HQ, then the HQ was converted to 1,2,4-BT, indicating that PNP was degraded via a HQ pathway. A further investigation of the enzyme mechanism of PNP degradation by *P. aeruginosa* HS-D38 strain is needed.

Acknowledgments

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