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Biodegradation of *p*-nitrophenol by *Rhodococcus* sp. CN6 with high cell surface hydrophobicity

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ABSTRACT

Rhodococcus sp. CN6, isolated from a pesticide industry's effluent-sediment, was able to completely degrade and utilize 100 mg/L *p*-nitrophenol (PNP) as the sole carbon, nitrogen and energy sources for growth in the minimal salt media (MSM) within 12 h. To study the applicability of the strain for bioremediation of PNP, its degradation potential was examined in the presence of different supplemented carbon and nitrogen sources in MSM with 100 mg/L PNP. Dextrin was experienced as the best supplemented carbon source used by the strain CN6 during degrading PNP. Addition of ammonium nitrate could also increase the PNP degradation rate. Preliminary studies on the surface characters of *Rhodococcus* sp. CN6 were undertaken for the sake of exploring its high efficiency on the degradation of PNP. Microbial adherence to hydrocarbons (MATH) assays illuminated that the strain CN6 was of higher hydrophobicity while grown on higher concentration of PNP. The results suggested that the strain CN6 could be used as a potential and efficient PNP degrader for the bioremediation of contaminated sites.

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1. Introduction

p-Nitrophenol (PNP), i.e. 4-nitrophenol is a widely distributed xenobiotic nitroaromatic compound in the environment throughout the world, especially with the manufacture of drugs, dyes, pigments, plastics explosives, fungicide, industrial solvents and phosphororganic insecticides [1–4]. PNP may have the potential to leach through soil and enter groundwater; besides it is hardly degradable and persistently toxic to the environment [5]. PNP is considered as a priority pollutant by the Environmental Protection Agency (EPA) of USA, and its concentration in natural waters is restricted to less than 10 mg/L [6,7]. Because of its extensive contamination to soil and water, in particular, the groundwater, it had arose public concerning and its fate in the environment has been studied extensively [3,8]. Among few strategies employed to remove the pollutant, microbial degradation is a safe, economical and efficient technology [9–12]. It is believed that the microbes enriched and screened from execrable environmental conditions generally comprise the specific regulation mechanisms for the degradation pathways, besides the desired catalyzing enzyme clusters [13,14]. Thus, several PNP-degrading microorganisms have been successfully isolated from the contaminated sites during the recent few decades, of which few bacteria, including *Flavobacterium*, *Moraxella*, *Nocardia*, *Pseudomonas* and *Arthrobacter* can metabolize PNP through removing their nitro-groups as nitrites, and simultaneously utilize PNP as a source of carbon or/and nitrogen for growth [15–17].

Most of the existing researches have focused on the PNP metabolic pathways by various microbes [7]. However, the estimates of the cell surface hydrophobicity (CSH) still have not been reported for any PNP-degrading bacteria. CSH has been believed to be an important response to various environmental factors, and also suggesting a definite relation with the degradation of the hydrophobic organic pollutant in the water environment [18–20]. The bacteria with hydrophobic cell surface have distinct advantages over that with hydrophilic surface in terms of tolerance to toxicant and cell adherence. During bioremediation of toxic chemicals, the ability of adherence is one of the great importance for microorganisms. It is accepted that adherence to surfaces is the first step in the process of removing pollutants for microbes. Rosenberg reported that hydrophobicity assays are positively correlated to adhesion tests in many instances [21]. The degradation rate of contamination by the high hydrophobic bacteria was quicker than that by the bacteria of low hydrophobicity. The ecological significance of the CSH of the bacteria was explored to supply new theoretical basis for the bioremediation of organic pollution such as PNP in the environment [22].

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In this study, a naturally isolated strain was identified and examined for its ability to grow on and degrade PNP. The isolate CN6, identified as a strain of *Rhodococcus* sp., was able to metabolize and utilize PNP as sole carbon, nitrogen and energy sources for growth. Furthermore, its high CSH was also first investigated. Biodegradation assays indicated that it might be a very potential and efficient biocatalyst for biotreatment of industrial wastewaters and bioremediation of contaminated soils.

2. Materials and methods

2.1. Bacterial strain

An indigenous bacterial strain was isolated from effluent-sediment collected from a local pesticide industry in Shandong Province, China, which had produced pyrethroids, carbamates and organophosphorus pesticides for many years. The strain CN6, used as PNP degrader, was able to grow utilizing PNP as the sole carbon, nitrogen and energy sources on the minimal medium. For short-term storage, the strain was maintained on mineral salt medium containing 200 mg/L PNP at 4 °C.

2.2. Chemicals

PNP (>98% purity) and *p*-nitrocatechol (>97% purity) was purchased from Aldrich–Sigma Chemicals Co. Glucose, sucrose, maltose, D-galactose, dextrin, soluble amylum, sodium nitrate, ammonium nitrate and ammonium sulphate were of analytical reagent grade and procured from Tianjin Chemical Reagent Co. Ltd., China. Methanol was of HPLC purity grade. All other solvents and chemicals used were of highest purity grade and commercially available.

2.3. Media and conditions for the growth of culture

The constituents of mineral salt medium (MSM) were (per liter) $K_2HPO_4{\cdot}3H_2O$, 0.75 g; KH_2PO_4, 0.2 g; $MgSO_4{\cdot}7H_2O$, 0.09 g; $FeSO_4{\cdot}7H_2O$, 0.06 g at pH 7.0. The PNP stored solution was prepared aseptically by rationing into sterile media to get wanted concentrations. Various carbon (namely; glucose, D-galactose, maltose, sucrose, dextrin, soluble amylum) or nitrogen (viz., sodium nitrate, ammonium nitrate and ammonium sulphate) supplements were added to MSM to find their effect on PNP degradation by *Rhodococcus* sp. CN6. Before every experiment, the strain was transferred to nutrient broth media (0.3% beef-extract, 0.5% NaCl and 1.0% tryptone) in 250-mL Erlenmeyer flasks and incubated in the orbital shaker (200 rpm, 30 °C) for 18 h. The culture was centrifuged at 10,000 rpm for 10 min at 4 °C. The cell pellet was resuspended in fresh MSM to around 0.6 OD units and then the aliquots of these cell suspensions were separately used as inocula. For the biodegradation assays, 1% (v/v) of the bacterial cells suspension prepared as inocula was inoculated to the MSM supplemented PNP up to desired concentrations in 250-ml Erlenmeyer flasks and incubated in the orbital shaker at 200 rpm and 30 °C.

2.4. Amplification of the 16S rRNA gene from the PNP-degrading bacteria

The genomic DNA was extracted as described previously [23]. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) with primers: forward prime (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse prime (5′-GGCTACCTTGTTAC GACT-3′). PCR products were cloned into a pMD18-T vector (TaKaRa). Positive clones comprising the amplified 16S rRNA gene fragment were screened out and sent to BGI Life Tech Co. Ltd., China for sequencing using an ABI 3730 genetic analyzer. The determined 16S rDNA sequence was aligned with those available in the public database BLAST search of the GenBank/EMBL/DDBJ [24].

2.5. Analytical methods

Bacterial growth was monitored by the absorbency at 600 nm throughout the studies. Aliquots (5 mL) of the culture grown on PNP or *p*-nitrocatechol as the sole carbon, nitrogen and energy sources were harvested at regular time intervals and centrifuged at 10,000 rpm, 4 °C for 10 min. The supernatant fluid of each sample was passed through a 0.45-μm filter and then submitted to analyze the amount of remaining PNP or *p*-nitrocatechol spectrophotometrically; while a simple control media, missing PNP or *p*-nitrocatechol, was used as blank. The spectrophotometric determinations of PNP and *p*-nitrocatechol was monitored by a spectrophotometer (SCINCO S-3100, Korea) at 397 and 254 nm, respectively. The samples were also analyzed by high performance liquid chromatography (HPLC, Thermo Finnigan, U.S.A.) using a Zorbax SB-C 18 column (250 mm × 4.6 mm, 5 μm) with PDA detector at the wavelength range from 200 to 600 nm. The mobile phase was a mixture of methanol and water (70:30, v/v) at a flow rate of 1.0 mL/min. The injection volumes were 10 μL. The compounds were identified by comparison of HPLC retention times and UV–vis spectra to those of authentic standards.

2.6. Measurement of the relative CSH

The relative CSH of the isolate during growth on PNP was determined using the microbial adherence to hydrocarbon (MATH) method of Rosenberg et al. with some modifications [18]. The hydrocarbon *p*-xylene was employed for CSH determination of *Rhodococcus* sp. strain CN6, while *n*-hexadecane and *n*-octane were excluded from the test because they were found to produce unreliable hydrophobicity results. Cells were harvested by centrifugation (10,000 rpm, 10 min, 4 °C) and washed twice with 50 mM phosphate buffer (pH 7.0, 22.2 g $K_2HPO_4{\cdot}3H_2O$; 7.26 g KH_2PO_4 per liter of deionized water) to remove any interfering solutes. The cells were resuspended with the same buffer to give a final concentration of cells of approximately OD_{600} = 0.6. Aliquots of the suspension was added to glass tubes and overlaid with 1 mL of *p*-xylene. The resultant aqueous/organic mixtures were mixed by vortexing for 2 min. After equilibration for 40 min, the lower aqueous phase was then carefully removed and its absorbance at 600 nm was measured. The CSH value was calculated as the percentage of the decrease in absorbance of the aqueous phase before and after mixing with *p*-xylene, using the following equation: CSH (%) = $100 \times (A_i - A_f)/A_i$; A_i, initial optical densities at 600 nm of the aqueous phase; A_f, final optical densities at 600 nm of the aqueous phase.

3. Results and discussion

3.1. Characterization of PNP-degrading bacteria

An indigenous bacterial strain CN6, capable of utilizing PNP as sole carbon, nitrogen and energy sources, was selected and identified. The bacterium was Gram-positive, oxidase-positive, catalase-positive, aerobic and non-motile. Based on the partial 16S rDNA sequence analysis, the isolate showed very high identity with *Rhodococcus* sp. (Fig. 1) and was given the distinguishing code CN6. The determined 16S rDNA sequence was deposited in the genbank nucleotide sequence databases under accession No. EU266492.

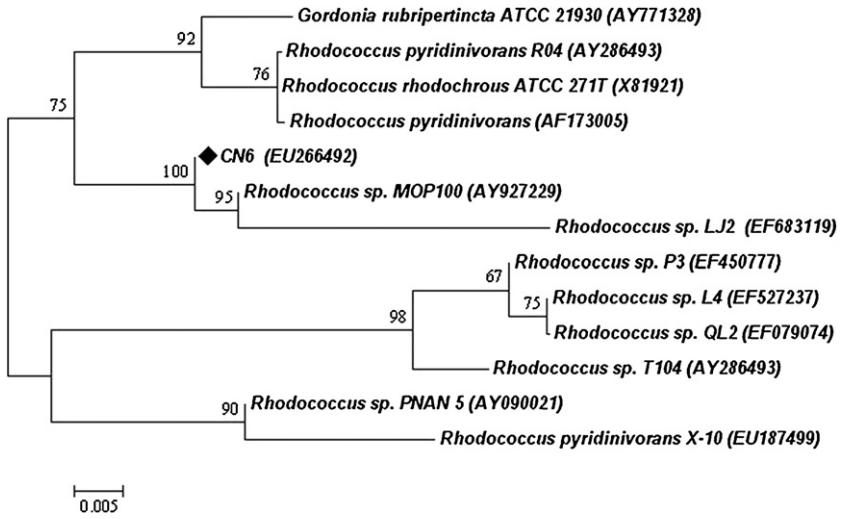

Fig. 1. The phylogenetic tree of strain CN6 based on 16S rRNA gene sequence analysis. Bootstrap values expressed as percentages of 1000 replications are shown at the branch points. GenBank accession numbers are shown in parentheses.

3.2. Bacterial growth and degrading ability of Rhodococcus sp. strain CN6

The time-course study of degrading PNP by *Rhodococcus* sp. strain CN6 and its biomass change grown on MSM containing PNP is shown in Fig. 2. Generally, 100 mg/L PNP was proved to be toxic to most microorganisms [25,26]. *Rhodococcus* sp. CN6 could nearly degraded 100 mg/L PNP completely within 12 h, with concomitant increase in biomass from initial 0.051 OD units to final 0.348 OD units. When initial PNP concentration was high, the growth of *Rhodococcus* sp. CN6 was inhibited with a lag phase of about 4 h. Parallel to entering the exponential growth phase, the bacteria rapidly consume PNP with a faster kinetics. Once the microbes had adapted to the toxic substrate, the bacterial growth and its PNP degrading ability increased rapidly. In controls without incubation or without added PNP to media, abiotic degradation and bacterial growth were negligible throughout the study. Nishino and Spain have also reported that the PNP degradation ability of *Pseudomonas putida* JS444 was coupled to the biomass present in the culture [27]. The bacterial growth's profile exhibited that *Rhodococcus* sp. CN6 can metabolize and utilize PNP as sole carbon, nitrogen and energy sources for growth.

3.3. Degradation of p-nitrocatechol by Rhodococcus sp. strain CN6

No *p*-nitrocatechol was detected during the degradation of PNP by *Rhodococcus* sp. CN6, although *p*-nitrocatechol is a known intermediate produced during the PNP degradation by Gram-positive bacteria [13,17,28,29]. This was probably because *p*-nitrocatechol from PNP biodegradation was transient presence before further metabolized. Therefore, it was interesting to study the ability of *Rhodococcus* sp. CN6 to degrade *p*-nitrocatechol. *Rhodococcus* sp. strain CN6 was shown to be capable of degrading 50 and 100 mg/L *p*-nitrocatechol within 4 and 7 h, respectively (Fig. 3). In controls without incubation, abiotic degradation was negligible throughout this study. The increase in cell density was also observed, simultaneously, during *p*-nitrocatechol metabolized. After degradation, the biomass increased to 0.183 and 0.247 OD units from initial 0.05 OD units, respectively. However, the growth of *Rhodococcus* sp. strain CN6 was not remarkable when it was incubated in MSM without *p*-nitrocatechol as added nutrition. This suggested that *Rhodococcus* sp. strain CN6 was able to utilize *p*-nitrocatechol as sole nitrogen source more than sole carbon and energy sources because no other nitrogen source abetted its growth in the sterile medium.

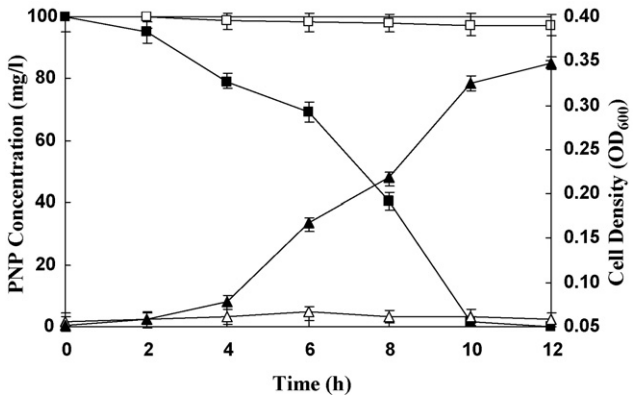

Fig. 2. Degradation of PNP by *Rhodococcus* sp. strain CN6 and bacterial growth monitored by measuring optical densities at 600 nm. □, PNP control without inoculated; ■, PNP inoculated; △, OD_{600} control without PNP in media; ▲, OD_{600} with PNP in media. The standard errors were within 5% of the mean. All the values are averages of three replicates from three independent experiments.

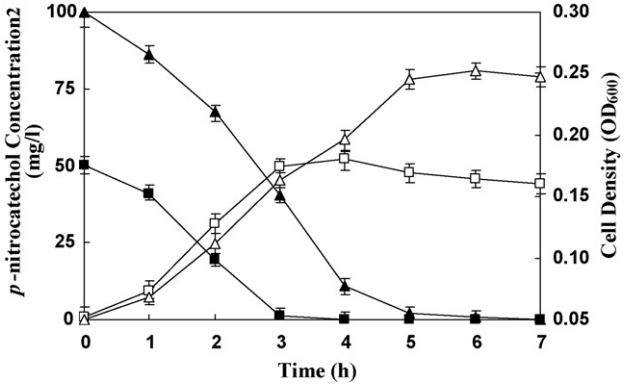

Fig. 3. Degradation of *p*-nitrocatechol by *Rhodococcus* sp. strain CN6 and bacterial growth monitored by measuring optical densities at 600 nm. ■, 50 mg/L *p*-nitrocatechol inoculated; □, OD_{600} with 50 mg/L *p*-nitrocatechol; ▲, 100 mg/L *p*-nitrocatechol inoculated; △, OD_{600} with 100 mg/L *p*-nitrocatechol. The standard errors were within 5% of the mean. All the values are averages of three replicates from three independent experiments.

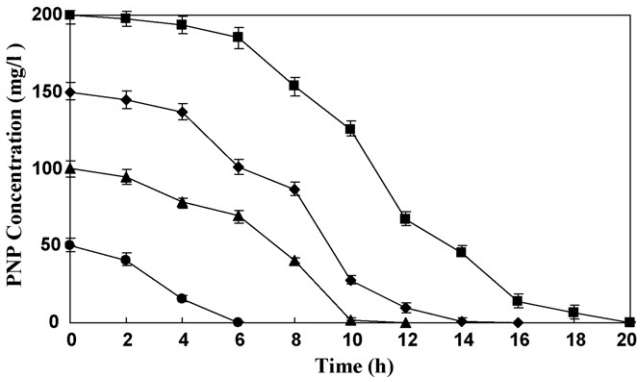

Fig. 4. Degradation of different PNP concentrations by *Rhodococcus* sp. CN6. ●, 50 mg/L PNP; ▲, 100 mg/L PNP; ♦, 150 mg/L PNP; ■, 200 mg/L PNP. The standard errors were within 5% of the mean. All the values are averages of three replicates from three independent experiments.

3.4. Effect of PNP concentration on degradation by Rhodococcus sp. strain CN6

In general, PNP degradation was concentration dependent, which is reasoned from the degradation mode of *Rhodococcus* sp. CN6 on different PNP concentrations (Fig. 4). As a result of toxic to the microbes, PNP severely inhibited its depletion with the PNP content increase in MSM without other nutritious supply. *Rhodococcus* sp. CN6 utilized nearly complete 50 mg/L PNP in 6 h of incubation, followed by 100 mg/L in 12 h, 150 mg/L in 16 h and 200 mg/L in 20 h. The strain CN6 even degraded up to 300 mg/L PNP in more than 62 h of incubation under the same conditions. It was observed that PNP were easily degraded at lower concentrations, whereas PNP were not depleted effectively at high concentrations. This was mostly due to the toxic inhibitory effect of PNP at a high content on the microbes. These results illuminated that the strain CN6 was potential to remove various concentrations of PNP with a high degradation rate and extent.

3.5. Effect of carbon on PNP degradation

In order to design the effective and efficient conditions to accelerate PNP degradation, it is necessary to study the effects of different supplemented carbon sources. For this purpose, various carbon sources (at 0.5 and 1.0 g/L, respectively) were added to MSM in the presence of 100 mg/L PNP, including monosaccharide (glucose and D-galactose) disaccharide (maltose and sucrose) and polysaccharide (dextrin and soluble amylum).

Table 1 depicts the growth and biodegradation efficiency of *Rhodococcus* sp. CN6 in the presence of different carbon sources. All added carbon sources except 1.0 g/L amylum supported the growth of *Rhodococcus* sp. CN6 to different extent. Compared to control, 1.0 g/L glucose best supported increase in biomass to 0.645 OD units at the cost of delayed PNP degradation. Minimum growth had been observed in the presence of 1.0 g/L soluble amylum. Maximum rate of PNP degradation occurred at 1.0 g/L dextrin, while obvious repression of PNP biodegradation was observed, in the presence of 1 g/L glucose. According to the study described by Meenal Kulkarni and Ambalal Chaudhari, *P. putida* rapidly degraded 20 and 50 mg/L PNP but retarded bioconversion of higher concentration, at the similar content of glucose (0.4 g/L) [7]. Acidic environment resulted from rapid glucose depletion inhibited the degradation of PNP at high concentration. Addition of 0.5 g/L glucose and 1.0 g/L D-galactose did not exhibit significant effect on PNP degradation though they hastened remarkable increase in biomass. Dextrin of different concentrations shortened the time required for complete PNP degradation and hence accelerated metabolism rate. Presumably, the toxicity of 100 mg/L PNP overstepped the accepted tolerance of microorganisms and intensively repressed the growth of strains. However, the toxicity of PNP to bacteria was reduced while addition of dextrin was saturated with PNP. Sequentially, the lag growth time of microbes was reduced and then the rate of PNP degradation was promoted. The results above indicated that dextrin was the best supplemented carbon source for the PNP-degrading *Rhodococcus* sp. strain CN6. The higher concentration of each carbon source was more effective for the formation of biomass, but the effect was reverse to the rate of PNP degradation. All the results indicated that the strain CN6 could metabolize and utilize PNP as the sole carbon source for growing without any added carbon source.

3.6. Effect of nitrogen on PNP degradation

Supplemental substrates such as nitrogen sources have shown positive effect on nitroaromatics biodegradation in most of the previous studies [30,31]. The degradation kinetics of PNP could also be optimized through the addition of glucose and a suitable nitrogen source [5]. While the growth repression resulted from them has been rarely reported. The effect of various nitrogen sources at different concentrations was studied since supplemental nitrogen source played an important role in bacterial growth and biodegradation (Table 2). However, no significant change in the growth of *Rhodococcus* sp. CN6 after addition of different nitrogen sources, except 0.05

Table 1
Effect of various carbon sources on degradation of PNP at 100 mg/L by bacteria

Carbon source	Concentration (g/L)	OD_{600} [a]	Time required for complete PNP degradation (h)	Rate of PNP degradation (mg/(L h))
Control	–	0.338 ± 0.0025	12.1 ± 0.21	8.26
Glucose	0.5	0.547 ± 0.0036	12.2 ± 0.12	8.20
	1.0	0.645 ± 0.0107	14.3 ± 0.26	6.99
D-Galactose	0.5	0.515 ± 0.0026	11.8 ± 0.15	8.47
	1.0	0.521 ± 0.0055	12.2 ± 0.25	8.20
Maltose	0.5	0.411 ± 0.0036	11.2 ± 0.26	8.93
	1.0	0.565 ± 0.0065	12.4 ± 0.23	8.06
Sucrose	0.5	0.583 ± 0.0092	13.7 ± 0.35	7.30
	1.0	0.642 ± 0.0081	14.2 ± 0.40	7.04
Dextrin	0.5	0.408 ± 0.0045	10.8 ± 0.36	9.26
	1.0	0.479 ± 0.0031	10.7 ± 0.32	9.35
Amylum	0.5	0.369 ± 0.0035	13.8 ± 0.29	7.25
	1.0	0.322 ± 0.0042	14.1 ± 0.45	7.09

±Standard deviation; minimal salt medium (MSM) with initial cell density of 0.05 OD_{600} units at 30 °C was used. Rate of PNP degradation (mg/(L h)) = 100/time required for complete PNP degradation. Control = PNP 100 mg/L without added carbon source. All the values are averages of three replicates from three independent experiments.

[a] OD_{600} was recorded at the time of complete exhaustion of PNP.

Table 2
Effect of various nitrogen sources on degradation of PNP at 100 mg/L by bacteria

Nitrogen source	Concentration (g/L)	OD_{600} [a]	Time required for complete PNP degradation (h)	Rate of PNP degradation (mg/(L h))
Control	-	0.338 ± 0.0025	12.1 ± 0.21	8.26
$NaNO_3$	0.05	0.337 ± 0.0015	12.3 ± 0.15	8.13
	0.5	0.350 ± 0.0039	11.9 ± 0.12	8.40
	1.0	0.341 ± 0.0055	12.5 ± 0.17	8.00
NH_4NO_3	0.05	0.365 ± 0.0031	12.2 ± 0.20	8.19
	0.5	0.392 ± 0.0047	11.8 ± 0.23	8.47
	1.0	0.323 ± 0.0040	13.6 ± 0.26	7.35
$(NH_4)_2SO_4$	0.05	0.345 ± 0.0042	12.1 ± 0.15	8.26
	0.5	0.368 ± 0.0035	12.0 ± 0.06	8.33
	1.0	0.334 ± 0.0045	13.3 ± 0.25	7.52

±Standard deviation; minimal salt medium (MSM) with initial cell density of 0.05 OD_{600} units at 30 °C was used. Rate of PNP degradation (mg/(L h)) = 100/time required for complete PNP degradation. Control = PNP 100 mg/L without added nitrogen source. All the values are averages of three replicates from three independent experiments.
[a] OD_{600} was recorded at the time of complete exhaustion of PNP.

and 0.5 g/L ammonium nitrate and 0.5 g/L ammonium sulphate. The maximum bacterial growth as well as the maximum degradation rate was observed in the presence of 0.5 g/L ammonium nitrate. Though the concentration of 0.5 g/L was represented the positive effect to biomass formation and PNP degradation for each nitrogen source, added nitrogen sources did not evidently influence the rate of PNP biodegradation. No further increase in biomass was observed in the absence and presence of added nitrogen source.

3.7. Hydrophobicity of *Rhodococcus* sp. CN6 during growth on PNP

The effect of different PNP concentration on CSH during degradation was also investigated. Fig. 5 shows that the time-course of CSH change in 50, 100 and 150 mg/L PNP, respectively. When *Rhodococcus* sp. CN6 was grown on 50 mg/L PNP, the MATH assays gave a maximum hydrophobicity of only less than 30%. However, with concomitant increase in PNP concentration from 50 to 100 mg/L, the maximum hydrophobicity rose to 66.1%, while a further rise in PNP concentration to 150 mg/L increased maximum hydrophobicity to 69.6%. The results in this study suggested that the hydrophobicity of *Rhodococcus* sp. CN6 increased with rise in PNP concentration. That is to say, bacterial hydrophobicity was linked to substrate toxicity. Farrell and Quilty also reported that owing to toxic effects of mono-chlorophenol, *P. putida* CP1 cells were more hydrophobic when they were incubated on higher concentrations of mono-chlorophenol [22]. Gram-positive bacteria were generally considered as more hydrophobic than Gram-negative microbes due to markedly hydrophobic constituents of the cell outer membrane [32]. The high hydrophobicity of *Rhodococcus* sp. CN6 can enhance the ability of adherence to pollutant surfaces and hence accelerate its biodegradation. So, *Rhodococcus* sp. CN6 was a potential and efficient strain for bioremediation of PNP contaminative sites. However, the hydrophobicity of a given strain may largely depend on characters of itself and environmental factors. For the application of *Rhodococcus* sp. CN6 during the practical PNP pollution treatment, further research works are required. In the present instance, a research for the constituents of the bacterial cell outer membrane and the optimum culture conditions is currently in progress, with a view towards the relationship between high hydrophobicity and degradation ability.

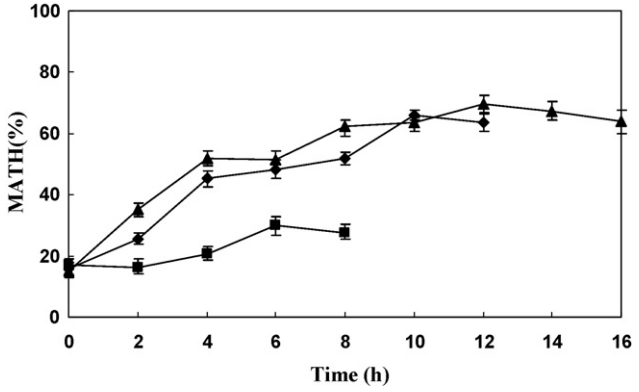

Fig. 5. Hydrophobicity (as MATH %) of *Rhodococcus* sp. CN6 during degrading different PNP concentrations. ■, 50 mg/L PNP; ♦, 100 mg/L PNP; ▲, 150 mg/L PNP. The standard errors were within 5% of the mean. All the values are averages of three replicates from three independent experiments.

4. Conclusions

An efficient PNP degrader, strain CN6, was isolated from a local pesticide industry's effluent-sediment and was identified as a member of *Rhodococcus* sp. of *Actinobacteria*. The organism was able to withstand and degrade PNP, as well as *p*-nitrocatechol at unusually high concentration within the lesser time, which constituted the basic requirement in the wastewater treatment strategies.

The experiments performed with *Rhodococcus* sp. CN6 showed that the degradation of PNP was enhanced through the suitable addition of carbon source like dextrin and nitrogen source such as ammonium nitrate. The MATH assays illuminated that the organism was highly hydrophobic, therefore, capable of efficiently adhere to the surface of pollutant to be degraded. The obtained results also proved that the concentration of the toxic pollutant to be degraded was of prime importance while designing an effective strategy for bioremediation of contaminated sites.

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