Biodegradation of *p*-nitrophenol by Immobilized *Rhodococcus* sp. Strain Y-1

Z. Leilei,* H. Mingxin, and Z. Suiyi
School of Urban and Environmental Sciences,
Northeast Normal University, 5268 Renmin Street,
Changchun, Jilin Province, People's Republic of China

Original scientific paper
Received: January 6, 2012
Accepted: June 6, 2012

A high efficiency *p*-nitrophenol (PNP) degrading bacterial strain Y-1 capable of utilizing PNP as sole carbon and energy source was screened and isolated from the activated sludge of Jilin Chemical Plant Oxidation Ditch in China. 16S rDNA sequencing identified strain Y-1 to be *Rhodococcus* sp. Polyvinyl alcohol (PVA) and sodium alginate were employed to immobilize the *Rhodococcus* sp. strain Y-1. Compared to the free cells, the immobilized cells possessed better PNP-degrading activity and stronger tolerance against the inhibitory effect of PNP. An orthogonal experimental design of L_9 (3^4) was used to investigate the effects of temperature, pH, and agitation rate on the biodegradation capabilities of the immobilized cells; the optimum conditions were as follows: 30 °C, pH at 7.0–7.5, shaken at 120 rpm. Moreover, immobilization supplemented with 1 g Fe_3O_4 significantly enhanced the PNP degradation capability (up to 500 mg L^{-1}). Our results indicated that immobilized *Rhodococcus* sp. strain Y-1 possessed good potential for application in the treatment of wastewater containing PNP.

Key words:
PNP, biodegradation, *Rhodococcus* sp. strain Y-1, immobilized bacterium, Fe_3O_4

Introduction

p-nitrophenol (PNP) is one of the most common and widely distributed water pollutants due to its commercial importance, primarily in the manufacturing processes of pesticides, herbicides, explosives, dyes, plasticizers, textiles, metal coatings, coal conversion and petroleum refining.[1] The compound is toxic by ingestion, contact, or inhalation even at low concentrations, thus posing a significant potential threat to human health and environmental conservation.[2] Traditionally, PNP and other aromatic compounds have been removed from industrial effluents by physical or chemical treatments, which, owing to their high operating cost or generation of secondary pollution, are often used as supplementary treatments. However, biodegradation techniques are preferable due to their economical advantage and the possibility of complete mineralization.[3] Bacteria, yeast, and fungi have been found to be capable of utilizing phenolic compounds as sole carbon and energy source. However, the microorganisms used in the biodegradation biotechniques were mostly domesticated flora from sludges, and the treatment effect is less than ideal due to a wide range of microbial flora within the complex. In recent years, biodegradation of phenolic compounds by bacteria has been extensively studied and several phenol-degrading bacterial strains such as *Pseudomonas* sp., *Arthrobacter* sp., *Rhodococcus* sp., *Burkholdria* sp., *Moraxella* sp. and *Bacillus* sp. have been isolated. It has been reported that the biodegradation rate of phenol can be improved by immobilizing the cells and entrapping them on a solid-based biomaterial such as alginate to obtain the maximum degradation capability.[7,12] Furthermore, high oxygen overvoltage anodes (often transition metal ions) such as iron oxides, which can generate •OH radicals from the oxidation of water, have been recently applied for the degradation or mineralization of organic contaminants. In this study, a bacterial strain that could effectively degrade phenol even at high concentration (500 mg L^{-1}), *Rhodococcus* sp. strain Y-1, was isolated and characterized from activated sludge. The strain was immobilized with polyvinyl alcohol (PVA) and sodium alginate to obtain the maximum phenol-degrading rate. The effects of temperature, pH, shaking speed, the amount of supplemented Fe_3O_4 and initial inoculum concentration of cells on biodegradation capabilities of immobilized strain Y-1 cells were investigated to explore the feasibility of the immobilized cells for treating wastewater containing PNP.

Materials and methods

Chemicals

Most of the chemicals used were purchased from Shanghai Dingjie Biological Technology Co. Ltd., Shanghai, China. Phenol crystals (99 % purity) and sodium alginate were purchased from

*Corresponding author: Zhang Leilei; Tel: +8610-52853660; Fax: +8610-82636378; E-mail: junwucao@hotmail.com

3B Pharmachem International Co., Ltd., Wuhan, China. Other chemicals obtained from commercial suppliers were analytical grade. PVA with an average degree of polymerization of 2400–2500 was purchased from Shanghai Solarbio Bioscience & Technology Co. Ltd. $CaCl_2$, saturated boracic acid solution and saline water (Shanghai Dingjie Biological Technology Co. Ltd.).[4]

Medium and culture conditions

For enrichment studies, the constituent of the medium (Luria-Bertani medium) included (per liter): beef extract 5.0 g, peptone 10.0 g, NaCl 5.0 g, agar 20.0 g, while pH was adjusted to 7.0–7.5.[5] The composition of basal salts medium included (per liter): K_2HPO_4 0.5 g, KH_2PO_4 0.5 g, NaCl 0.2 g, $MgSO_4$ 0.2 g, NH_4NO_3 1.0 g, and pH was adjusted to 7.0–7.3.[6,7] Solid media were prepared by the addition of 2 % agar to the medium. The media were autoclaved (120 °C/30 min) and cooled to room temperature prior to pH adjustment and addition of PNP (ethanol dissolved and aseptically filtered). To assess the biodegradation rate of the strain Y-1, PNP of different concentration was added to the sterilized basal salts medium as the sole carbon source.

Isolation of bacteria

1000 mL activated sludge (pH 7.6–8.0) was collected from Jilin Chemical Plant Oxidation Ditch, after 7 days of static precipitation, 2 mL supernatant of activated sludge sample was added to 2000 mL flask containing 200 mL enrichment medium at a shaking rate of 120 rpm for 30 minutes (30 °C), then centrifuged at 2000 rpm for 5 minutes; the supernatant of enriched culture was collected and 2 mL aliquots of supernatant were transferred to another enriching flask containing 200 mL enrichment medium, the transfer was repeated five times. 1 mL enriched culture was inoculated into 2000 mL flask containing 200 mL basal salts medium supplemented with stepwise increase of PNP (from the initial concentration of 60 mg L^{-1}, with a gradient concentration of 20 mg L^{-1}, to 500 mg L^{-1}) as sole carbon and energy source, dark cultured and domesticated at 120 rpm and 30 °C in a shaker for 48 h, three parallel groups were established in each batch. After five cycles of consecutive domestication had been carried out, the turbidity was measured as OD at 600 nm to screen the strain with highest growth rate, dilutions of culture were inoculated onto basal salts medium agar plates containing 1000 mg L^{-1} PNP as the sole carbon and energy source, the developed colonies were repeatedly streaked on agar plates for isolation of a pure culture, and the most efficient PNP degrading strain was isolated.[8]

Immobilization of bacterial cells

The most efficient PNP-degrading bacterial cells were collected by centrifugation at 120 rpm for 48 h, and then washed three times with distilled water. PVA solid powder and sodium alginate, supplemented with Fe_3O_4 were mixed and heated (90 °C) to dissolve. The concentrated cells were transferred to the cooled solution (the final bacterial concentration was OD_{600} = 0.5) and homogenized by stirring, the mixture was dropped into saturated boric acid solution with calcium chloride (1–3 %) using a syringe to form beads with 1–2 mm diameter. Finally, the beads were incubated in a 2000 mL flask containing 200 mL basal salts medium supplemented with 500 mg L^{-1} PNP at 30 °C and shaken at 120 rpm for 30 min,[9] and then washed thoroughly three times with saline.[10] The PVA and sodium alginate solution without the Fe_3O_4 was prepared as control.

Analytic methods

Bacterial growth

The isolated strain was grown at 30 °C shaken at 120 rpm for 48 h in enrichment medium. About 1 mL of the culture from the enrichment broth was inoculated in a 2 L flask containing 200 mL basal salts medium supplemented with 100 mg PNP as sole carbon and energy source. Bacterial growth was determined spectrophotometrically by measuring the OD_{600} at 6-hour intervals over 36 hours.[11]

Analysis of the concentration of PNP in medium

The concentration of PNP in the culture medium was determined according to the assay reported by Cheng *et al.* (2006).[12]

Scanning Electron Microscopy (SEM)

The beads bearing a network of immobilized cells grown in 500 mg L^{-1} PNP for 7 days were taken randomly from the bioreactor bed, washed three times with normal saline and washed twice with double distilled water, then fixed with 2.5 % (v/v) glutaraldehyde for 30 minutes. Then they were rinsed three times with phosphate buffer solution (PBS) and dehydrated in an ethanol gradient (50–70–80–90–95–100 % v/v) for 10 minutes at 4 °C, washed three times for 10 minutes with tert-butanol and then vacuum-cooled to sublimate the tert-butanol. The samples were coated with gold for 2 minutes and finally observed with a XL30 (FEI Ltd., USA) scanning electron microscope at an acceleration voltage of 20 kV.

Identification of strain Y-1 by 16s rDNA sequencing

16S rDNA was amplified with the primers 27F (5'-AGAGTT TGA TCC TGG CTC AG-3'); primers 2: 1492R (5'-TACCTTGTTACGACTT-3'), PCR reaction was carried out under the following conditions: initial denaturation at 94 °C for 5 minutes, 30 cycles of denaturation at 94 °C for 1 minute, annealing at 56 °C for 1 minute, and extension for 1.5 minutes, final extension at 72 °C for 10 minutes and storage at 4 °C. The PCR products were cloned to pMD-18T vector and sequenced by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. The final product of 1430bp was submitted to the GeneBank under accession number HM008918.1. The sequence was submitted and a BLAST search was performed to find the best hit in the NCBI's GeneBank database.

Results and discussion

Isolation and characterization of *Rhodococcus* sp. strain Y-1

By enrichment culture, the strains capable of growing in PNP as the sole carbon and energy source were isolated. The strain Y-1 was selected for further studies because of its high PNP-degrading rate (500 mg L^{-1} for 100 h). 16S rDNA gene sequencing identified the strain to be *Rhodococcus* sp. It showed 99 % identity to the *Rhodococcus* sp. WTZ-R2 (GeneBank Accession HM004214) with total score of 2582 (1433).

Immobilization of the strain

The PNP removal activity of the immobilized cells can be influenced by the operational process, especially the concentration of PVA, sodium alginate, $CaCl_2$, and the biomass.[13] To assess the biodegradability based on the four factors and enhance the stability of the immobilized cells,[14] a three-level orthogonal experiment (L_9,3^4) was employed to determine the optimum operational process.[15] Immobilized cells were inoculated into 100 mL of phenol-containing wastewater (500 mg L^{-1} phenol), and the PNP content was measured 48 hours later.[16] Based on four factors and three levels (Table 1), the experimental conditions for each test group were listed (Table 2).

According to the order of magnitude of R (maximum difference), the effect of all factors on PNP removal efficiency could be determined as D (ratio of Embedded reagent to bacterial culture) > A (concentration of PVA) > B (concentration of $CaCl_2$) > C (concentration of sodium alginate). The results indicated that the effect of biomass was more important in the immobilization process, the PNP degrading rate increased with the bacterial inoculum concentration. The PNP degradation rate of immobilized cells was also dependent on the concentration of PVA, H_3BO_3 and $CaCl_2$. However, no significant difference was observed in the different percentages of sodium alginate.[17–19] To obtain the highest PNP degrading rate, the optimal conditions should be 90 g L^{-1} PVA, 1.0 % sodium alginate,

Table 1 – *Orthogonal design of optimization of immobilization process*

	PVA (g L^{-1})	sodium alginate (%)	H_3BO_3+$CaCl_2$ (%)	Embedded reagent: bacterial culture (v:v)
1	100	2	4 + 1	30:1
2	100	1	4 + 2	25:1
3	100	0.5	4 + 3	20:1
4	90	2	4 + 2	20:1
5	90	1	4 + 3	30:1
6	90	0.5	4 + 1	25:1
7	80	2	4 + 3	25:1
8	80	1	4 + 1	20:1
9	80	0.5	4 + 2	30:1

Table 2 – *Orthogonal experiment design L_9 (3^4) in the optimization of immobilization process for PNP removal efficiency*

Run	A (PVA)	B (sodium alginate)	C ($CaCl_2$)	D (Embedded reagent: bacterial culture)	Removal Efficiency (%)
1	1	1	1	1	86.10
2	1	2	2	2	80.05
3	1	3	3	3	92.82
4	2	1	2	3	96.11
5	2	2	3	1	98.53
6	2	3	1	2	83.72
7	3	1	3	2	79.80
8	3	2	1	3	81.44
9	3	3	2	1	90.02
K_1	258.97	262.01	251.26	274.65	
K_2	278.36	260.22	266.18	243.57	
K_3	251.26	266.56	271.15	270.37	
R	27.1	6.34	19.89	31.08	

Note: K represents the total value of removal efficiency from the same factor; R is the maximum removal efficiency minus the minimum removal efficiency for the same factor.

3.0 % $CaCl_2$, with the ratio of the embedded reagent to bacterial inoculum concentration of 30:1 (v:v).

Operational ability of immobilized cells supplemented with Fe_3O_4

It has been shown that high oxygen overvoltage anodes (often transition metal ions), such as iron oxides, can generate •OH radicals from the oxidation of water and completely degrade or mineralize organic contaminants.[20] In this research, the effect of Fe_3O_4 was also investigated. Compared to the traditional immobilization methods, the addition of Fe_3O_4 enhanced the recovery efficiency of immobilized cells, with no negative effects on the degradation capability. The PNP degradation rate in 90 g L^{-1} PVA and 1.0 % sodium alginate mixture supplemented with 0.3, 0.5, 1.0, and 1.5 g Fe_3O_4 was respectively monitored. As expected, the percentage of the PNP degrading rate increased with the amount of Fe_3O_4 added (Fig. 1). However, when the amount of added Fe_3O_4 was increased above 1.0 g, the PNP degradation efficiency decreased dramatically. In addition, the surface of the immobilized cells supplemented with Fe_3O_4 was less smooth compared to the traditional ones (Fig. 2). Further increase in Fe_3O_4 amount may have reduced the space utilization for the bacteria, which led to the lower enzyme activity. Consequently, 1.0 g Fe_3O_4 was supplemented in the following studies.

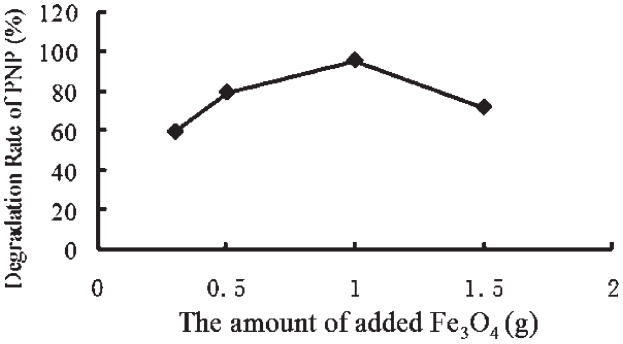

Fig. 1 – *Effect of Fe_3O_4 amount on PNP degradation of the immobilized bacterial cells*

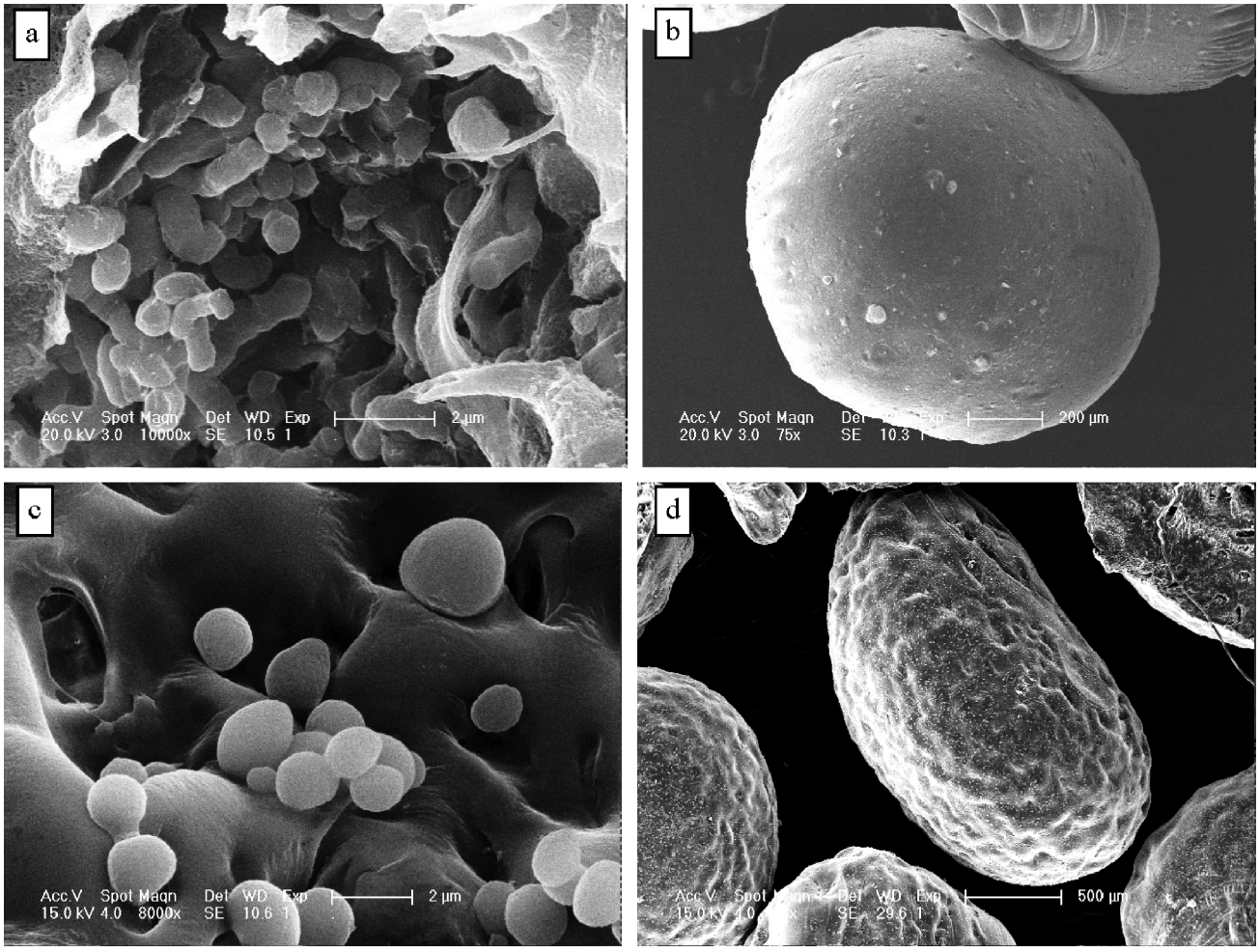

Fig. 2 – *Morphological features of the different immobilized cells under the scanning eletron microscope (XL30 scanning electron miscroscope, FEI); a) Immobilized bacterial cells obtained by traditional method, bar = 2 µm; b) Immobilized bacterial cells obtained by traditional method, bar = 200 µm; c) Immobilized bacterial cells with addition of Fe_3O_4, bar = 2 µm; d) Immobilized bacterial cells with addition Fe_3O_4, bar = 500 µm*

PNP degradation comparison between the free and immobilized cells

The same number of free and immobilized cells were inoculated into the wastewater containing 500 mg L^{-1} PNP, incubated at 30 °C and shaken at 120 rpm for 24 hours. Within the time period examined, the immobilized cells showed higher PNP degradation rate (Fig. 3, Fig. 4). The reason perhaps being that, under the same conditions, the embedding carrier material of the immobilized cells could act as a protective shelter against the toxicity of PNP.[21,22]

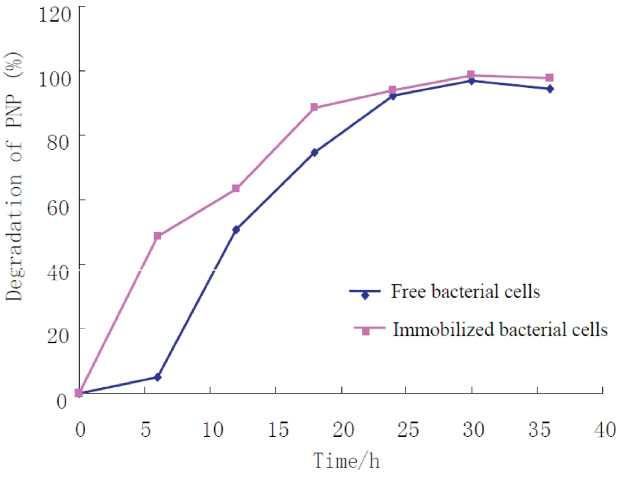

Fig. 3 – *Time courses of PNP degradation by free and immobilized bacterial cells*

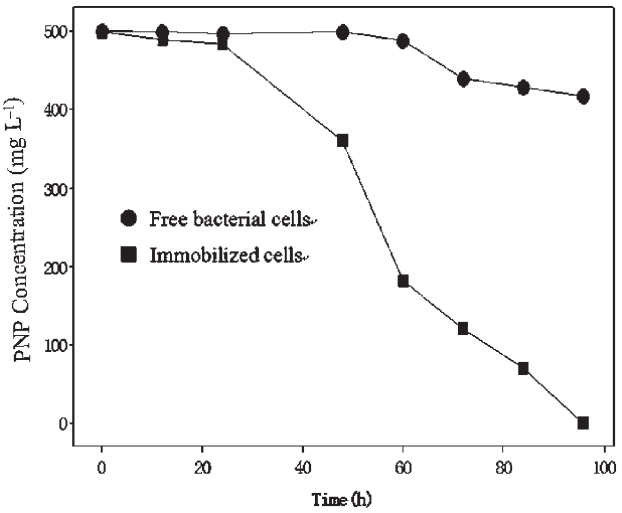

Fig. 4 – *Effect of initial PNP concentration on PNP degradation by free and immobilized bacterial cells*

Effect of inoculum concentration on PNP degradation by free and immobilized cells

To determine the effect of inoculum concentration on PNP degradation, free and immobilized cells are grown in a medium containing PNP (pH 7.0, 500 mg L^{-1} PNP) at 30 °C and shaken at 120 rpm for 24 hours. The initial inoculum concentration was set as 1 %, 2 %, 4 %, 6 %, 8 %, 10 %, respectively. An inoculum concentration of 1 % showed significantly lower PNP degradation rate, however, when the inoculum concentration increased from 2 to 8 %, the PNP degradation of all the cells increased from 75 % to 95 % (Fig. 5). When the inoculum concentration was above 8 %, the PNP removal efficiency improved only slightly.[23]

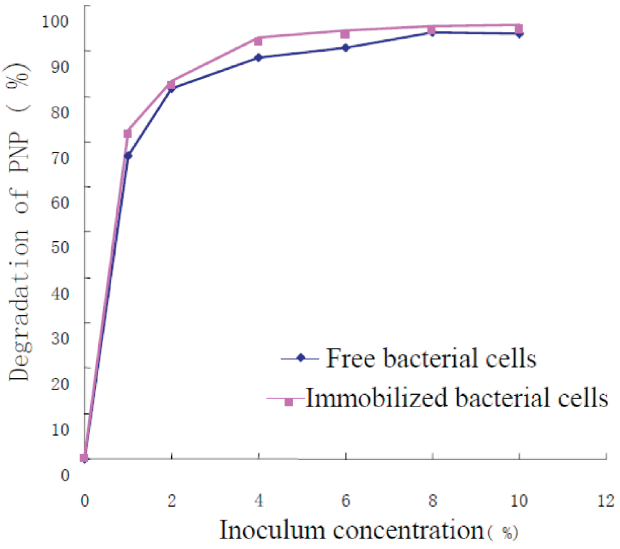

Fig. 5 – *Effect of inoculum concentration on the PNP degradation*

Effect of pH on PNP degradation by free and immobilized cells

The effect of pH on the PNP degradation rate was investigated (Fig. 6). The optimum pH range was broad, from 5.0–10.0 for all the cells. The degradation rate dropped when the pH fell below 5.0 and rose when above 10.0. Extremely high or low pH values generally result in complete loss of activity for most of the enzymes. The pH significantly

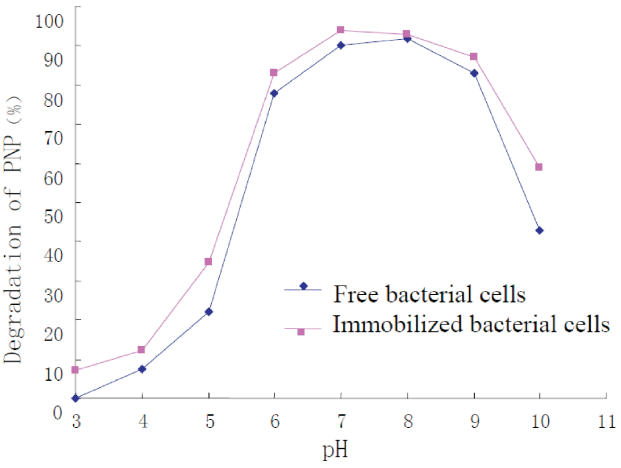

Fig. 6 – *Effect of pH on the PNP degradation*

affects the biochemical reactions required for phenol degradation. At the same pH, the immobilized cells possessed greater PNP-degrading ability, suggesting that the immobilized cells' tolerance ability to acid conditions (pH) was much better than that of the free cells.

Effect of agitation rate on PNP degradation by free and immobilized cells

The effect of agitation rate on the PNP degradation was also investigated. Experiments were performed at an agitation rate ranging from 40 to 180 rpm at 30 °C under pH 7.0 for 24 hours. In laboratory conditions, the shaker can provide the aerobic state for bacterial growth and phenol degradation. Since aerobic phenol degradation is preferred to anaerobic pathway, the dissolved oxygen concentration maybe a limiting factor of the phenol-degrading process. The dissolved oxygen saturation was achieved at agitation rate of 120–160 rpm, and there was no need to increase the agitation rate. An agitation rate of 120–160 rpm led to a maximal PNP removal efficiency for the free and immobilized cells (Fig. 7). The agitation rate of 120–160 rpm may have created a higher dissolved oxygen concentration, which thus led to better phenol degradation and growth environment for bacteria. At the same agitation rate, the degradation rate of the immobilized cells was always higher than that of the free cells. This indicated that the oxygen uptake of immobilized cells was higher than that of the free cells.[23–25]

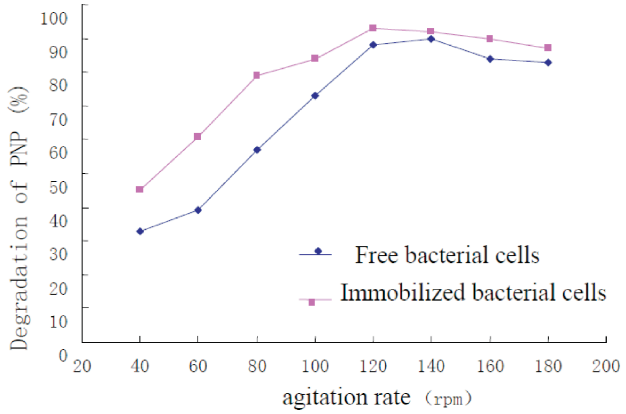

Fig. 7 – *Effect of agitation rate on the PNP degradation*

Effect of temperature on PNP degradation by free and immobilized cells

To assess the influence of the temperature changes, PNP degradation efficiency was compared under a temperature range from 20 to 40 °C at pH 7.0 with shaking at 120 rpm for 24 hours. At temperature below 30 °C, all the cells displayed increasing PNP removal efficiency (Fig. 8). When the temperature reached 30 °C and above, with the increase in temperature, the PNP-degrading curve of free and immobilized cells declined dramatically, and PNP degrading ability of strain Y-1 was optimized at 30 °C. Higher temperatures seemed to have a negative effect on the activity of the bacteria and hence hindered its biodegradation capabilities. It may have detrimental effect on the bacterial enzymes, which is the main step in the biological degradation process. Although the optimal temperature and degradation rates were the same in both cases (30 °C), in the range of 20 to 40 °C, the immobilized cells possessed a higher degradation rate than the free cells, illustrating that temperature had less effect on immobilized cells than on the free cells, because immobilization increased the thermal stability of the cells under the protection of PVA carrier.[10]

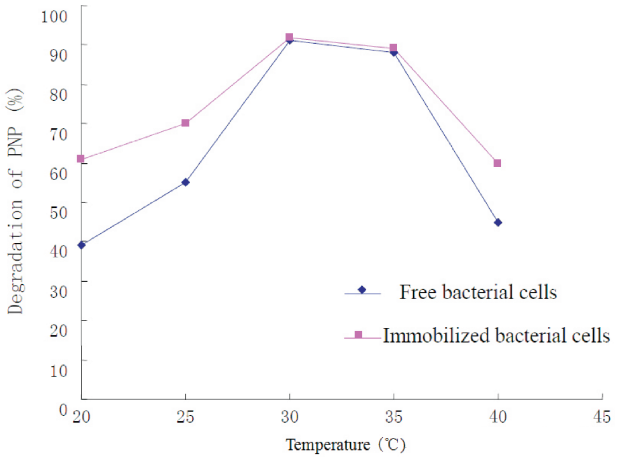

Fig. 8 – *Effect of temperature on the PNP degradation*

Reusability of immobilized cells

Stability during long-term operation is important for practical application of the immobilized cell system. In order to determine if there was deactivation of cells after repeated use, the immobilized cells were tested in eight consecutive PNP-degradation processes. The 12-hour reaction results showed that PNP-degrading ability only decreased slightly after the immobilized cells were reused in eight cycles (Fig. 9), demonstrating that the PVA and so-

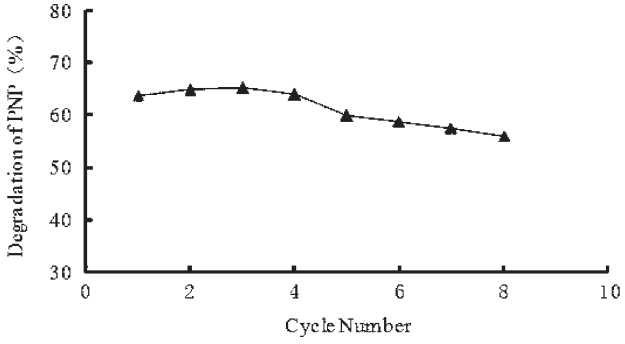

Fig. 9 – *Reusability of the immobilized bacterial cells*

dium alginate plus the Fe_3O_4 carrier retained high mechanical strength.

Conclusion

Generally, activated sludge is used for the treatment of PNP-contaminated wastewater, but it also takes a long time for microorganisms to degrade this compound. Activated sludge is basically a biomass containing mainly bacteria and protozoa. It is often possible to overcome the pollution problems in activated sludge using a variety of laboratory methods to obtain optimized microbial strains.

A new PNP-degrading bacterial strain Y-1 was isolated from the activated sludge. Cells of the strain were immobilized with PVA and sodium alginate using determined optimal conditions. The immobilized cells have a higher degradation rate the free cells, and can be reused for at least eight cycles. Detailed conclusions were drawn as follows:

1) Orthogonal matrix method was applied to investigate four variables such as concentration of PVA, sodium alginate, $CaCl_2$ and the ratio of the embedded reagent to bacterial culture (v:v) for optimal immobilization. A maximum degradation rate 98.53 % was obtained under the following conditions: 90 g L^{-1} PVA, 1.0 % sodium alginate, 3.0 % $CaCl_2$, with the ratio of the embedded reagent to bacterial culture of 3:1 (v:v).

2) Factors affecting PNP degradation of immobilized cells such as inoculum concentration, pH, agitation rate and temperature were investigated. The optimal condition for PNP degradation was determined as: 10 % inoculum concentration, at 30 °C, under pH 7.0 and shaken at 120 rpm. Under the same conditions, the immobilized cells had a higher degradation rate than the free cells, and their tolerance ability was much better.

3) Under optimal Fe_3O_4 amount (1 g), the immobilization retained mechanical strength for the cells under the protection of the carrier. The immobilized cells showed a degradation rate of around 100% and could be reused for at least eight cycles.

As an emerging technology in recent years, immobilized microorganisms have been attracting increased interest in the field of wastewater biological treatment. Immobilized microorganism technology is one kind of effective biotechnology for wastewater treatment, which fixes high densities of microorganisms on the carrier and maintains their biodegradation capabilities in optimal conditions, and could also be used repeatedly in order to meet the requirements of treatment processes. The immobilized microorganism technology has the following advantages: microorganisms can be enriched and maintained in small biological reactor, easy to implement solid-liquid separation, and capable of processing sewage discharges containing toxic or hazardous compounds. The degradation ability of immobilized cells has significantly increased, the possible reasons being: 1) The carrier plays a protective role and hinders the spread of organic pollutants, thus reducing the surface pollutant concentration on the immobilized cells. 2) After the cells were fixed to the carrier, a certain physical or chemical contact was established, increasing the stability of the cell membrane. 3) The microenvironment of immobilized cells and free cells is different; the changes in this microenvironment may lead to cell morphology, physiological characteristics and changes in metabolic activity.

The drawback of the free cell systems is that they are not viable for longer periods for purposes of effluent treatment or industrial applications. In this research, the procedures involving cell immobilization could be a better alternative. The use of immobilized cells offers several advantages over the free cells, such as retention of higher concentration of microorganisms, protection of cells from toxic substrates, and separation of suspended biomass from waste effluents. Our results indicated that immobilized *Rhodococcus* sp. strain Y-1 possessed a good potential for application in the treatment of wastewater containing PNP.

ACKNOWLEDGMENTS

We thank International Center of Papertrans (http://www.papertrans.cn/) for drafting and revision of the manuscript.

References

1. *Munnecke, D. M.,* Appl. Environ. Microbial. **32** (1976) 7.
2. *Perry, L. L., Zylstra, G. J.,* J. Bacteriol. **189** (21) (2007) 7563.
3. *Stephen, T. L. T., Benjamin, Y. P. M., Abdul, M. M., Joo, H. T.,* Appl. Microbiol. Biotechnol. (2005), Online 13 January.
4. *Khleifat, K. M.,* Process Biochem. **41** (2006) 2010.
5. *Jiang, Y., Wen, J., Lan, L., Hu, Z.,* Biodegradation **18** (2007) 719.
6. *Jones, K. H., Trudgill, P. W., Hopper, D. J.,* Arch. Microbiol. **163** (1995) 176.
7. *Karigar, C., Mahesh, A., Nagenahalli, M., Yun, D. Y.,* Biodegradation **17** (2006) 47.
8. *Sun, Z. J., Lv, G. J., Li, S.Y., Xie, Y. B., Yu, W. T., Wang, W., Ma, X. J.,* J. Biotechnol. **28** (2007) 150.
9. *Tsai, S. C., Tsai, L. D., Li, Y. K.,* Biosci. Biotech. Bioch. **69** (2005) 2358.
10. *Fernandes, K. F., Lima, C. S., Pinho, H., Collins, C. H.,* Process Biochem. **38** (2003) 1379.
11. *Caromori, S. S., Fernandes, K. F.,* Process Biochem. **39** (2004) 883.
12. *Cheng, J., Yu, S. M., Zuo, P.,* Water Res. **40** (2006) 283.

13. *Lai, Y. C., Lin, S. C.,* Process Biochem. **40** (2005) 1167.
14. *Shinozaki, Y., Kimura, N., Nakahara, T.,* J. Biosci. Bioeng. **93** (5) (2002) 512.
15. *Yang, H., Jang, Z., Shi, S., Tang, W. Z.,* Ecotox. Environ. Safe. **53** (3) (2002) 416.
16. *Santos, V. L., Linardi, V. R.,* Process Biochem. **39** (2004) 1001.
17. *Ramos, A. F., Gómez, M. A., Hontoria, E., González-López, J.,* J. Hazard. Mater. **142** (2) (2007) 175.
18. *Kulkarni, M., Chaudhari, A.,* Bioresource Technol. **97** (8) (2006) 982.
19. *Liu, Y. J., Zhang, A. N., Wang, X. C.,* J. Environ. Sci. **19** (2) (2007) 222.
20. *Harman, B. I., Koseoglu, H., Yigit, N. O., Beyhan, M., Kitis, M.,* Desalination **261** (2010) 27
21. *Arutchelvan, V., Kanakasabai, V., Nagarajan, S., Muralikrishnan, V.,* J. Hazar. Mater. **127** (1–3) (2005) 238.
22. *Chen, W. M., Chang, J. S., Wu, C. H.,* Res. Microbiol. **155** (8) (2004) 672.
23. *Godjevargova, T., Ivanova, D., Alexieva, Z., Dimova, N.,* Process Biochem. **38** (6) (2003) 915.
24. *Futamata, H., Nagano, Y., Watanabe, K., Hiraishi, A.,* Appl. Environ. Microbiol. **71** (2) (2005) 904
25. *Li, Q. Y., Li, Y., Zhu, X. K., Cai, B. L.,* Lett. Appl. Microbiol. **36** (5) (2003) 272.