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Biodegradation of phenol by free and immobilized *Acinetobacter* sp. strain PD12

WANG Ying[1,2], TIAN Ye[1], HAN Bin[3], ZHAO Hua-bing[1], BI Jian-nan[1], CAI Bao-li[1,2,*]

1. Department of Microbiology, Nankai University, Tianjin 300071, China. E-mail: caibaoli@nankai.edu.cn
2. Key Laboratory of Bioactive Materials, Ministry of Education, Nankai University, Tianjin 300071, China
3. College of Enviromental Science and Engineering, Nankai University, Tianjin 300071, China

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Abstract

A new phenol-degrading bacterium with high biodegradation activity and high tolerance of phenol, strain PD12, was isolated from the activated sludge of Tianjin Jizhuangzi Wastewater Treatment Facility in China. This strain was capable of removing 500 mg phenol/L in liquid minimal medium by 99.6% within 9 h and metabolizing phenol at concentrations up to 1100 mg/L. DNA sequencing and homologous analysis of 16S rRNA gene identified PD12 to be an *Acinetobacter* sp. Polyvinyl alcohol (PVA) was used as a gel matrix to immobilize *Acinetobacter* sp. strain PD12 by repeated freezing and thawing. The factors affecting phenol degradation of immobilized cells were investigated, and the results showed that the immobilized cells could tolerate a high phenol level and protected the bacteria against changes in temperature and pH. Storage stability and reusability tests revealed that the phenol degradation functions of immobilized cells were stable after reuse for 50 times or storing at 4°C for 50 d. These results indicate that immobilized *Acinetobacter* sp. strain PD12 possesses a good application potential in the treatment of phenol-containing wastewater.

Key words: phenol; biodegradation; *Acinetobacter* sp. PD12; immobilized bacterium

Introduction

Phenols are distributed either as natural or artificial mono-aromatic compounds in various environmental sites as major pollutants. Their existence in wastes from industrial processes such as oil refineries, coking plants, wastewater treatment plants, petroleum-based processing, and phenol resin industry manufacturing and plants, has been well established (Ahmed *et al*., 1995). Phenol and its derivatives are among the most frequently found pollutants in rivers, industrial effluents, and landfill runoff waters. Biodegradation of phenol and its derivatives by bacteria has been extensively studied and a large number of phenol-degrading bacteria have been isolated and characterized at the physiological and genetic level (Koutny *et al*., 2003; Shen *et al*., 2004; Polymenakou and Stephanou, 2005). However, little information on bacteria with a high phenol tolerance and high metabolizing activity is available (El-Sayed *et al*., 2003). Therefore, there is still the need to isolate new phenol-degrading bacteria that can aerobically grow at elevated concentration of phenol.

Among various methods available, biodegradation is environmental friendly and cost effective for pollution remediation in the environment. Biological treatment of phenol contamination has therefore gained an increasing attention in pollution prevention. However, the use of free bacterial cells for wastewater treatment in activated sludge processes creates problems such as solid waste disposal. Immobilized microorganisms have been showed to be effective to treat phenol-containing wastewater with little sludge production and have been receiving increasing attention (Gonzalez *et al*., 2001; Pazarlioglu *et al*., 2005; Wu *et al*., 2005).

In this study, a bacterial strain that can degrade elevated concentration of phenol, *Acinetobacter* sp. strain PD12, was isolated and characterized. Moreover, polyvinyl alcohol (PVA) was used to immobilize the strain PD12. Factors affecting phenol biodegradation process and operational and store stability of immobilized cells were investigated.

1 Materials and methods

1.1 Chemicals

Phenol with greater than 99% purity was obtained from Tianjin University Chemical Factory. PVA with an average degree of polymerization of 2400–2500 was purchased from Shanghai Chemical Factory.

1.2 Medium and culture conditions

The phenol-degrading strain PD12 used in this study was isolated by enrichment for growth on phenol as the sole carbon source from activated sludge of Tianjin

Project supported by the Undergraduate Research Foundation of Nankai University (2004). *Corresponding author.
E-mail: caibaoli@nankai.edu.cn.

Jizhuangzi Wastewater Treatment Facility, China. This strain was grown aerobically at 30°C in minimal medium (MM) containing (per liter) 0.9 g KH_2PO_4, 6.5 g $Na_2HPO_4 \cdot 12H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.4 g $(NH_4)_2SO_4$, and 1 ml of trace element solution (Scholtz *et al*., 1988). Phenol was added to the sterilized medium to a final concentration of 500 mg/L.

1.3 Immobilization of bacteria

PVA solid powder was heated to dissolve in KH_2PO_4-Na_2HPO_4 buffer (25 mmol/L, pH 7.2), and kept overnight at room temperature. The bacterial cells were collected by centrifugation at 12000 r/min for 5 min, then washed with KH_2PO_4-Na_2HPO_4 buffer twice. The concentrated cells were added to the mixture containing 14% PVA (the final bacterial concentration was 24.6 mg wet cells/L), and stirred to be uniform. The mixture was frozen in a refrigerator at –20°C for at least 24 h and then thawed at 4°C. The freezing and thawing process was repeated for two additional cycles to increase the mechanical strength of immobilized cells (Cao *et al*., 2002). Finally, PVA gel was cut into 2×2×2 (mm) cubes, then washed thoroughly with KH_2PO_4-Na_2HPO_4 buffer and kept at 4°C.

1.4 Analytical methods

Equal amounts of the harvested cells were transferred into the bulk solution or entrapped in the PVA cubes, then inoculated to the flasks containing MM medium and incubated at different cultural conditions. 3 ml samples were taken at 1–2 h intervals over 12 h. Biomass was determined turbidometrically at 600 nm and converted into dry cell weight with a standard conversion curve. The quantities of phenol in liquid medium degraded by bacteria were estimated by measuring the absorbance of the culture solution at 270 nm (Bastos *et al*., 2000).

1.5 Identification of strain PD12 by 16S rDNA sequence

16S rDNA was amplified with the primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CG GYTACCTTGTTACGACTT-3′). PCR reaction was done as originally described by Cai *et al*. (2003). The PCR product was cloned into the pMD 18-T vector (TaKaRa Biotechnology Co.) following the manufacturer's instruction. 16S rDNA was sequenced by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. The final sequence of 1500 bp was submitted to the GenBank under accession number AY673994. The sequence was submitted to a BLAST search of the NCBI GenBank database to identify the organism.

1.6 Enzyme assays

The strain PD12 was grown overnight in MM medium containing 500 mg of phenol per liter. For the preparation of crude extract, the collected cells were washed with potassium phosphate buffer (33 mmol/L, pH 7.0), then resuspended in the same buffer, and sonically disrupted. The broken cells were centrifuged at 15000 r/min for 30 min at 4°C. The enzyme activities were spectrophotometrically determined in 50 mmol/L potassium phosphate buffer (pH 7.0) containing 0.04 mmol/L EDTA, 0.4 mmol/L phenol as the substrate. After adding 50 μl of the crude extract, catechol 1,2-dioxygenase (C12O) or catechol 2,3-dioxygenase (C23O) activities were estimated by measuring the reaction products *cis*-*cis*-muconic acid at 260 nm or 2-hydroxymuconic semialdehyde at 375 nm, respectively (El-Sayed *et al*., 2003).

2 Results and discussion

2.1 Isolation and characterization of *Acinetobacter* sp. strain PD12

By enrichment culture, 48 bacterial isolates capable of growth on phenol as the sole carbon and energy source were isolated. Each isolate was tested for their ability to utilize phenol at concentrations from 100 mg/L to 1100 mg/L. The strain PD12 was selected for detailed studies because of its high phenol-degrading rate. This strain was capable of removing 500 mg phenol/L in liquid minimal medium by 99.6% in 9 h (Fig.1) and metabolizing phenol at concentrations up to 1100 mg/L. By PCR amplification, a 1500-bp 16S rRNA gene fragment of strain PD12 was obtained and submitted for sequencing. The blast search of this sequence indicated that PD12 was matched at 99.39% to *Acinetobacter seohaensis*. Thus, PD12 was identified to be an *Acinetobacter* sp.

In general, the aerobic metabolism of phenol is known to be initiated by two different pathways, either the *ortho*- or the *meta*-pathway. Both *meta*- and *ortho*-pathways are distinguishable by measuring their characteristic enzyme activities, that is C23O for the *meta*-pathway and C12O for the *ortho*-pathway. The enzyme assays proved that the crude extract from strain PD12 only showed C12O activity, which indicated that the strain PD12 metabolized phenol in the *ortho*-pathway.

2.2 Effect of phenol concentration, pH and temperature on phenol degradation by free and immobilized cells

PVA as a gel matrix was used to immobilize *Acinetobacter* sp. strain PD12 by repeated freezing and thawing. Factors affecting phenol degradation of immobilized cells such as phenol concentration, pH and temperature were investigated. To determine the effect of phenol concentration on phenol degradation, free and immobilized cells

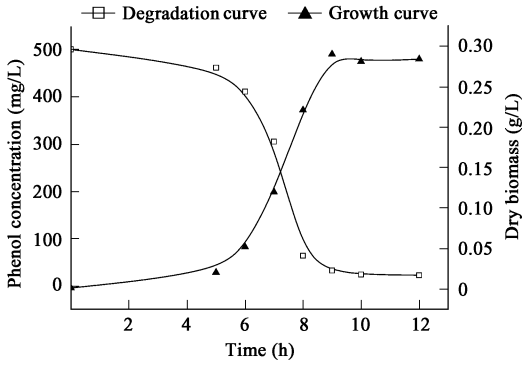

Fig. 1 Phenol-degrading and growth curves of strain PD12.

are grown in liquid MM medium (pH 7.2) at 30°C with different initial phenol concentration. Comparing with free cells, immobilized cells exhibited a lower specific degradation rate when phenol concentrations were below 300 mg/L. However, when phenol concentrations were over 300 mg/L, immobilized cells displayed better phenol-removing efficiencies (Fig.2). The toxicity of phenol at high concentrations could inhibit the growth of free cells and result in a lower removal efficiency of phenol. Under the same condition, the carrier material of the immobilized cell could act as a protective shelter against the toxicity of phenol. Activity enhancement of immobilized viable cells has also been reported by other researchers. It has been demonstrated that immobilization of viable cells could alter their physiological features in metabolism such as enhanced enzyme induction, reduced specific cell growth and cell yield (Chung *et al*., 2003).

The pH optimum experiments for phenol degradation by immobilized cells was performed at 30°C with phenol concentration of 500 mg/L and the results are shown in Fig.3. The immobilized cells have higher degradation rate constant and wider pH range than that of free cells. The changes of the degradation rate constant of both free and immobilized cells were not sharp when pH was in the range of 7.2–10. When pH fallen bellow 7.2 the degradation rate constants of the immobilized and free cells were lower. Especially, when pH was at 5.5, phenol-degrading activity of free cells was completely inhibited. However, immobilized cells still maintained an acceptable degradation rate of 0.66 h^{-1} at the same pH. All those indicated that tolerance ability to acid conditions of immobilized cells was much better than that of free cells.

To confirm that immobilized cells can also tolerate a wider temperature changes, phenol degradation experiments were performed at a temperature range from 20 to 45°C at pH 7.2 with phenol concentration of 500 mg/L. Fig.4 shows the effect of temperature on phenol degradation of free and immobilized cells. Although the optimal temperature was the same in the both cases (30°C), the degradation rates were 0.9696 and 1.249 h^{-1} for free and immobilized cells, respectively. In the range of 20 to 35°C, the immobilized cells showed a higher value of

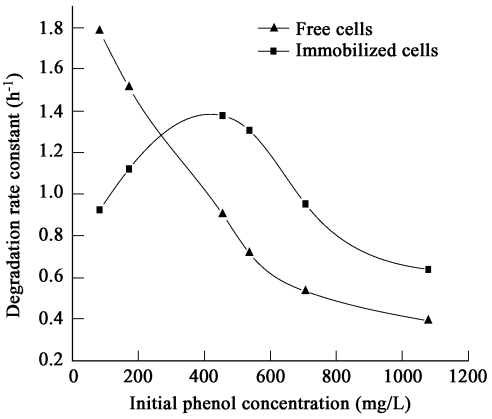

Fig. 2 Effect of initial phenol concentration on phenol degradation of free and immobilized cells.

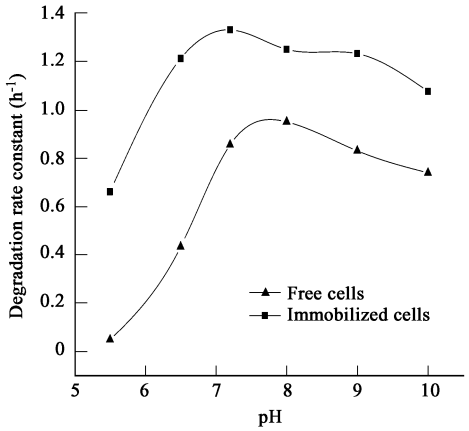

Fig. 3 Effect of pH on phenol degradation of free and immobilization cells.

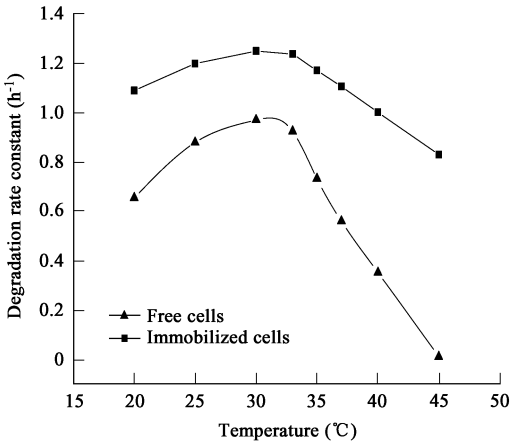

Fig. 4 Effect of temperature on phenol degradation of free and immobilized cells.

degradation rate constants than that of free cells. Especially when the temperature reached 32°C and over, with the increase of the temperature, the curve of free cells declined sharply, but the curve of immobilized cells fell slowly. And even when the free cells nearly lost all activity at 45°C (degradation rate of 0.011 h^{-1}), the immobilized cells still maintained a degradation rate of 0.829 h^{-1}. These results verified that temperature has a less effect on immobilized cells than that of free cells, because immobilization increased the thermal stability of the cells under the protection of PVA carrier.

2.3 Storage stability and reusability of immobilized *Acinetobacter* sp. PD12

The stability during long-term storage and operation is an essential factor for practical application of immobilized cell system. In order to identify the storage stability of immobilized cells, free and immobilized cells that stored for 0, 10, 20, 30, 40, and 50 d at 4°C were tested for phenol degradation rate. As shown in Fig.5, with the extension of storage time, immobilized cells hold a stable phenol degradation rate and could remove 99% phenol after being stored for 50 d at 4°C, while the degradation rate of free cells decreased after storing for 20 d at 4°C, and nearly lost all of its activity after storing for 50 d at 4°C. These

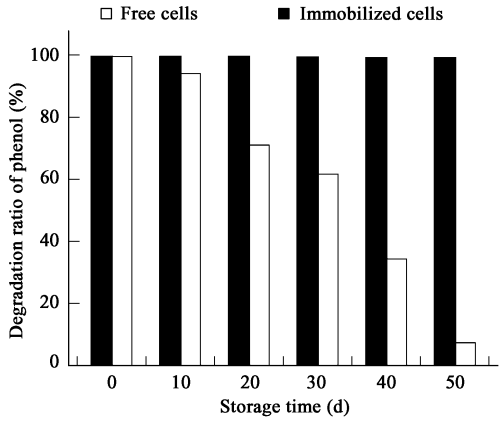

Fig. 5 Comparison of storage stability at 4°C between immobilized and free cells.

indicated that immobilized cells have stronger storage stability than that of free cells.

Futhermore, PVA immobilized cells were tested in several consecutive phenol-degradation processes to determine if there was deactivation of cells after repeated use. The results showed the phenol-degrading ability only decreased by 0.8% after the immobilized cells of *Acinetobacter* sp. strain PD12 were used for fifty cycles (incubated for 6 h each time). This indicated that immobilized cells could be reused for at least fifty cycles. In addition, we found that the PVA cubes had a good flexibility and did not expand after fifty cycles. The medium remained clear after the final cycle, which demonstrated that PVA carrier cubes retained a high mechanical strength. These results are significantly better than previous report of immobilized *Ralstonia metallidurans* CH34 entrapped in sodium alginate (Wu *et al*., 2005).

3 Conclusions

This study investigated the biodegradation of phenol by free and immobilized *Acinetobacter* sp. strain PD12 and the following conclusions were drawn: (1) As new phenol-degrading bacterium with high biodegradation activity and high tolerance of phenol, *Acinetobacter* sp. strain PD12 was isolated from the activated sludge. This strain was capable of removing 500 mg phenol/L in liquid minimal medium by 99.6% in 9 h and metabolizing phenol at concentrations up to 1100 mg/L. (2) Polyvinyl alcohol (PVA) was used as a gel matrix to immobilize the strain PD12. The immobilized cells have higher degradation rate constant and wider pH range than that of free cells. Temperature has a less effect on immobilized cells than that of free cells. (3) The immobilized cells possess better storage stability and can be reused for at least fifty cycles.

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