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The phenol-degradation performance and immobilization of *Rhodococcus ruber* SD3 mutant M1

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ABSTRACT

Rhodococcus ruber SD3 mutant M1 was almost completely degrade phenol of 1.5 g/L for 72h when its inoculation dosage ranged from 2% to 4%. The suitable temperature for phenol-degradation ranged from 30°C to 35°C. The optimum pH was from 7.0 to 8.0. The degradation of phenol occurs mainly in the logarithm phase of growth. Cells of *Rhodococcus ruber* SD3 mutant M1 was immobilized by using alginate calcium and polyvinyl alcohol complex. The optima of alginate sodium concentration, polyvinyl alcohol concentration and bead size were 1%, 1% and 6mm, respectively. The immobilized cells degrade over 98% of phenol (2.0 g L^{-1}) for 72 hours during five cycles use. These results paved a road for the bioremediation of wastewater containing phenol.

KEYWORDS

Phenol biodegradation;
Immobilization;
Rhodococcus ruber;
Mutant.

INTRODUCTION

Phenol is a main kind of pollutants in industrial wastewaters, which is a raw material or an intermediate in petroleum, paper and pharmaceutical industry. It has severe toxicity to animals, plants and human[1]. In China, phenol is put in the priority pollutants blacklist and the permissible concentration of phenol in potable water is 0.001 mg/L. Therefore, removal of phenol from industrial effluents with convenient, robust and cost effective ways arouses great interest among researches. To date, there are a number of physical, chemical and biological methods which have developed to treat the phenol in the wastewater. Compared with traditional physical and chemical methods, biodegradation is an environmentally friendly and cost effective way[2]. Many researches have been performed on the screening of microorganisms with high removal efficiency of phenol. Several phenol-degrading microorganisms such as *Pseudomonas putida, Bacillus brevis, Candida maltosa, Candida tropicalis and Alcaligenes faecalis* are tolerant of the toxicity of the phenol at concentrations above 1000mg L^{-1}[3-7]. Moreover, the efficiency of phenol degradation can be improved by cell immobilization. Some strain have been reported to be successfully immobilized on various carried for enhancing phenol degradation[6,8-12].

To our knowledge, *Rhodococcus* genus such as *Rhodococcus erythropolis* can degrade phenol with high efficiency[13-16]. However, there are few reports on the degradation of phenol with *Rhodococcus ruber.* In our previous study, *Rhodococcus ruber* SD3 showed high phenol-degrading capacity and the mutant M1 of *Rhodococcus ruber* SD3 exhibited remarkable phenol-degrading capacity[17]. The objective of the paper

FULL PAPER

is to study the overall performance of phenol- degrading with the mutant M1 of *Rhodococcus ruber* SD3. To achieve elevated performance of phenol-degrading, the mutant was encapsulated in calcium alginate and PVA complex. Reuse of the immobilized cells to degrade phenol was attempted to study the potential in wastewater bio-remediation.

MATERIALS AND METHODS

Materials and medium

Tryptone, yeast extract and agar were biological reagents. All other chemicals were of analytical grade and were obtained from sinopharm chemical reagent Co., Ltd. Mineral salt medium contained 1 g /L NaClÿ1 g /L NH_4Clÿ3 g/L $MgSO_4 \cdot 7H_2O$ÿ1.5 g/L K_2HPO_4 and 0.5 g/L KH_2PO_4 in distilled water.

The effects of inoculation dosage of *Rhodococcus ruber* SD3 mutant M1 on phenol degradation

A loop of *Rhodococcus ruber* SD3 mutant M1 was innoculated into LB medium. Mutant M1 was grown at 35°C till OD_{600} reached 0.8-1.0. Then cultures with different inoculation dosage (1%, 2% and 4%) were seeded into mineral salt medium containing phenol 1.5%. Mutant M1 was grown at 35°C and 200 rpm. The residual phenol content in the medium was assayed with the method of spectrophotometry after mutant M1 was grown for 60h and 72h.

The effects of growth temperature of *Rhodococcus ruber* SD3 mutant M1 on phenol degradation

To study the effects of growth temperature of *Rhodococcus ruber* SD3 mutant M1 on phenol degradation, the mutant was grown at different temperature (19°C, 22 °C,25°C, 28°C, 30°C, 33°C, 35°C) and 200 rpm. The residual phenol content in the medium was assayed with the method of spectrophotometry after the mutant was grown for 72h.

The effects of initial pH of mineral salt medium on phenol degradation

To study the effects of initial pH of mineral salt medium on phenol degradation, mutant M1 was grown in mineral salt medium containing phenol 1.5% with various initial pH (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5) at 35°C and 200 rpm. The residual phenol content in the medium was assayed with the method of spectrophotometry after the mutant was grown for 72h.

The relationship between the growth of mutant M1 and phenol degradation

Mutant M1 was grown in mineral salt medium (pH 7.5) containing phenol 1.5% at 35°C and 200 rpm. The culture was withdrawn periodically for determining the residual phenol content and optical density at 600nm.

Optimization of immobilization of *Rhodococcus ruber* SD3 mutant M1 and reuse of the immobilized cells to degrade phenol

Alginate sodium and polyvinyl alcohol were mixed in distilled water and sterilized by autoclaving. *Rhodococcus ruber* SD3 mutant M1 (10 g wet weight) was added to 10 mL of the solution above. The resulting mixture was filled into syringe and instilled into the mixture of boric acid and calcium chloride solution to form immobilized cells, then stored at 4°C for 24 h. The entrapped cells were washed with distilled water and placed into to 50ml mineral salt medium containing phenol 1.5 g L^{-1}. The immobilized cell was incubated in 35°C (200 rev min^{-1}) for 72 h. The residual phenol was analyzed according to the method of spectrophotometry[1].

Three immobilization parameters were optimized for phenol degradation. To determining the optimum concentration of alginate sodium and polyvinyl alcohol, different concentrations (1%, 2%, 3% and 4%) of alginate sodium and different concentrations (1%, 2%, 4% and 8%) of polyvinyl alcohol were used. To determining the optimum bead size, the beads with various diameters (2mm, 4mm, 6mm and 8mm) were used.

After each use, the immobilized cells was separated from culture medium, rinsed three times with distilled water, and used for next cycle. The steps were repeated five times.

Analysis of phenol and calculation of phenol degradation rate

Each of the 1 mL broth samples was centrifuged at 10,000 rpm for 2min. The supernatant was then diluted by 100 times volume with water. The diluted supernatant was distilled to obtain 50 mL of distillates. 0.5 mL of 20% NH_4Cl in ammonia buffer solution, 1.0 mL of 8% potassium ferrocyanide solution, 1.0 mL of 2% 4-

aminoantipyrine solution were mixed with 50mL of distillates. The mixture was allowed to react for 10 min and absorbance was measured at 510 nm with the distilled water as the control[1]. The phenol degradation rate was calculated according to the following equation.

Phenol degradation rate (%) = (1-Phenol concentration after culture /Initial phenol concentration) ×100%

Statistical analysis

Experimental date were presented as mean ± SD. Statistical analysis Was performed using SPSS 11.5 software.

RESULTS AND DISCUSSION

The effects of inoculation dosage of *Rhodococcus ruber* SD3 mutant M1 on phenol degradation

Rhodococcus ruber SD3 mutant M1 have shown higher phenol degradation for 72h than for 60h as indicated in TABLE 1. The phenol degradation rate increased with the inoculation dosage. *Rhodococcus ruber* SD3 mutant M1 completely degraded phenol (1.5 g/L) for 72h when its inoculation dosage ranged from 2% to 4%. The strain AKG2 requires 60 h to degrade 100% phenol when its initial concentration is 500 mg/L[18].

TABLE 1 : The effects of inoculation on phenol degradation with *Rhodococcus ruber* SD3

inoculation dosage	1 %	2%	4%
phenol degradation rate for 60 h (%)	82.11	91.12	99.87
phenol degradation rate for 72 h (%)	87.20	99.76	99.92

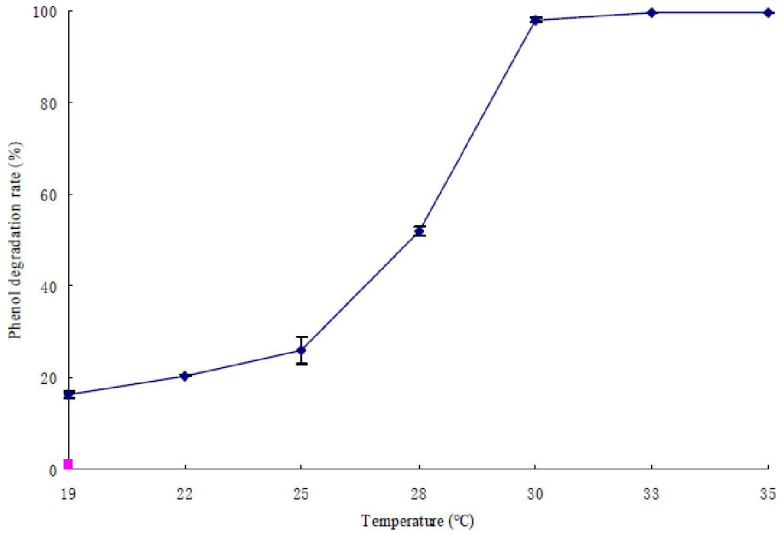

Figure 1 : The effects of temperature on phenol degradation with *Rhodococcus ruber* SD3

The effects of growth temperature of *Rhodococcus ruber* SD3 mutant M1 on phenol degradation

The phenol degradation rate increased with the growth temperature (Figure 1) and the optimum temperature for phenol degradation ranged from 30°C to 35°C, with phenol degradation rate being over 99%. This was due to the growth temperature affecting stability, enzyme activity and the fluidity of cell membrane.

The effects of initial pH of mineral salt medium on phenol degradation

Phenol degradation by *Rhodococcus ruber* SD3 mutant M1 was inhibited in acidic condition. The optimum initial pH of mineral salt medium was 7.5 and phenol degradation rate was 99.72% (Figure 2). Organic acids were formed during the degradation process of phenol. If mineral salt medium was slightly alkaline, it can neutralize organic acids. However, when the initial pH of mineral salt medium reached 8.5, the phenol degradation rate decreased to 18.49 %. The reason for the result was that further increase in initial pH gave rise to the change of surface charge on cells. Accordingly, the absorption of phenol by cell was affected. In a study by Shourian et al., the optimum pH value of phenol degradation by *Pseudomonas* sp. SA01 was found to be 6.5[19].

The relationship between the growth of mutant M1 and phenol degradation

Phenol degradation rate increased with the growth

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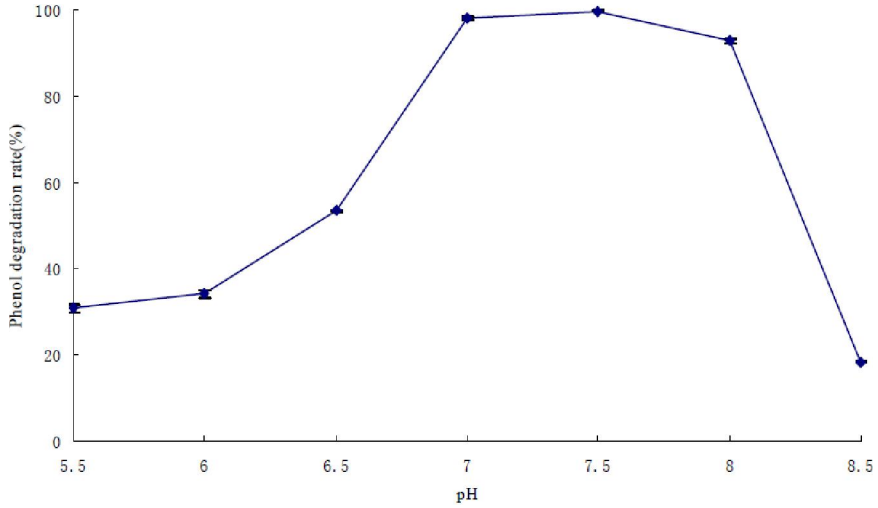

Figure 2 : The effects of initial pH value on phenol degradation with *Rhodococcus ruber* SD3 mutant M1

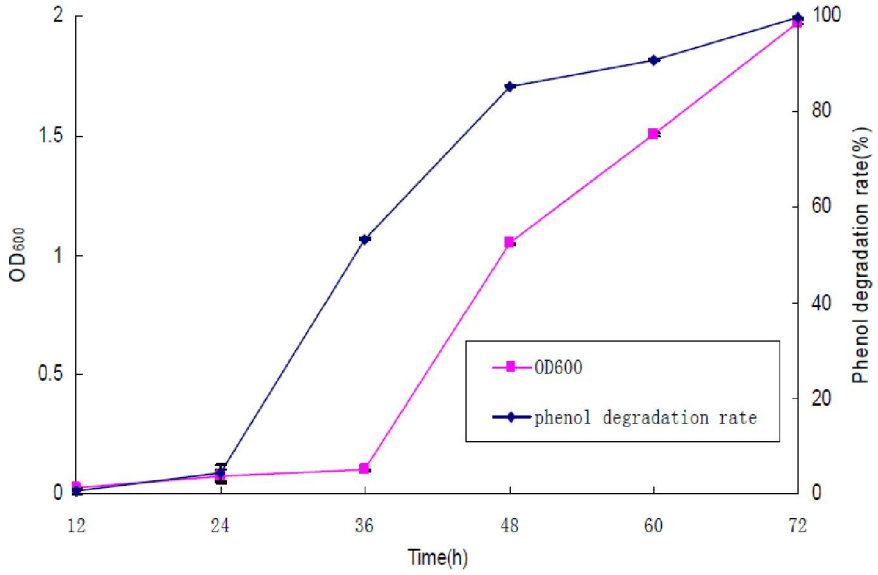

Figure 3 : The relationship between the growth of *Rhodococcus ruber* SD3 mutant and phenol degradation

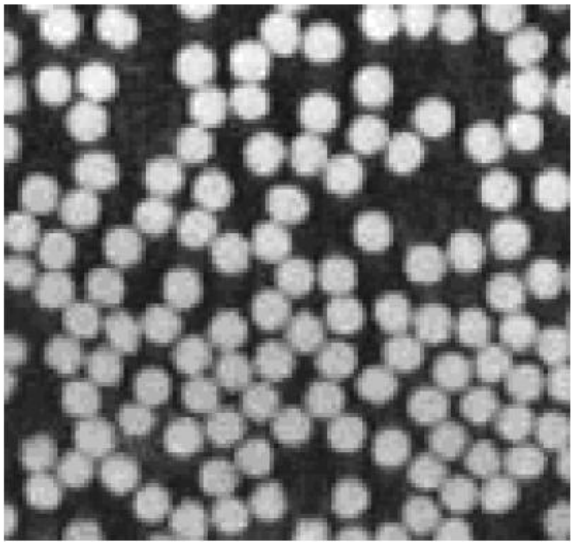

Figure 4 : The beads which cells were immobilize

of *Rhodococcus ruber* SD3 mutant M1. When mutant M1 was in the adaptation phase, phenol degradation rate was relatively low. Phenol degradation rate was in rapid increased during the logarithmic phase. Complete phenol degradation was achieved when the mutant reached the stationary phase (Figure 3). While *Pseudomonas* sp. SA01 started to degrade 0.7 g/l of phenol after an initial very short lag phase, and phenol decomposition was then rapidly completed within 30 h[19].

Optimization of immobilization of *Rhodococcus ruber* SD3 mutant M1 and repeated use of immobilized Cells in shaken flasks

Use of immobilized cells has attracted considerable and increasing interest since this strategy allows

process economically viable. In this study, cells of *Rhodococcus ruber* SD3 mutant M1 was immobilized by using polymeric entrapment matrices (Alginate calcium and polyvinyl alcohol complex) (Figure 4). The optima of alginate sodium concentration, polyvinyl alcohol concentration and bead size were 1%, 1% and 6mm, respectively (data no shown).

The immobilized cells were repeatedly used as the biocatalyst to degrade phenol in batch. Figure 5 showed that the immobilized cells degrade over 98% of phenol (2.0 g L^{-1}) for 72 hours during five cycles use. Compared with free cells, the immobilized cells enhanced the biodegradation of phenol. Several technological approaches and a number of materials have been used to immobilize microorganisms for wastewater depollution[6,8,20]. To date, entrapment of viable biomass was widely used in cell immobilization. Calcium alginate was the common immobilization matrix because of its low price, simple immobilization procedure and no toxicity to the cells[10]. However, calcium alginate was fragile and could easily be broken down by microorganism. If PVA was added into calcium alginate to form complex, it will improve the mechanical and thermal stability of gel beads[6]. The results in the Figure 5 indicated the feasibility and suitability of the cell entrapped in the calcium alginate and PVA complex for the phenol degradation. In similar studies, the coated *C. glutamicum* cells were capable of degrading 50 ppm phenol for 8d at 30°C and they could completely degrade phenol during the first 2 cycles, and retain 60% activity of phenol degradation for the third and four cycles[12].

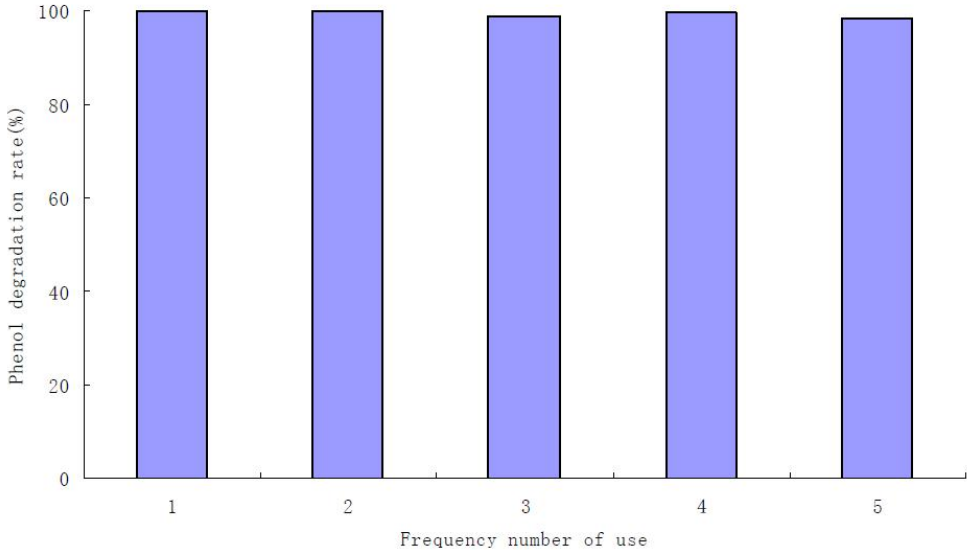

Figure 5 : Reuse of immobilized cells to degrade phenol

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FULL PAPER

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