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Biodegradation of quinoline by gel immobilized *Burkholderia* sp.

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Abstract

Burkholderia sp, a Gram-negative, rod-shaped, aerobe, capable of degrading quinoline was immobilized in calcium alginate gel beads and used for degradation of quinoline in aqueous solution in the reactor. The optimal conditions for immobilization of the microorganism, such as alginate concentration, calcium ion concentration, initial cell loading, hardening time and bead size, were determined with a view to improving the quinoline degradation rate. The characteristics of quinoline degradation by immobilized microbial cells were investigated. The repeated use of immobilized cells for quinoline degradation was performed and the results revealed that the bioactivity of immobilized cells was stable over 100 h in the repeated batch cultivation for quinoline degradation.

Keywords: *Burkholderia* sp.; Immobilization; Quinoline; Biodegradation

1. Introduction

In recent years, there has been increasing concern over the public health threat presented by the introduction of N-heterocyclic compounds into the environment (Stuermer et al., 1982; Zachara et al., 1984; Sims and Oloughlin, 1989). Little is known about the environmental fate of N-heterocycles.

Quinoline, a heterocyclic compound, is found in coal tar, mineral oil and bone oil. In the chemical industry it serves as a solvent and is the starting material for the synthesis of quinoline dyes and pharmaceuticals. Quinoline and some of its derivatives were reported to be toxic, carcinogenic and mutagenic (Minako et al., 1977; Sideropoulos and Secht, 1984). The widespread use of quinoline and its derivatives entails that these compounds, together with many other environmental chemicals, are distributed in the environment thus polluting soil and water.

Conventional biological processes (activated sludge, trickling filters) can destroy a large fraction of biodegradable organic compounds found in wastewater, and can remove additional materials by adsorption. Moreover, the biological treatment cost is much lower than that of physical and chemical methods. However, many hazardous compounds are poorly removed in conventional biological processes due to their toxicity, recalcitrance or inhibition. Most of these compounds pass through conventional wastewater treatment facilities unaltered. In addition, they also have adverse impact on the composition and activities of microorganism communities in activated sludge flocs, thus reducing the overall performance of these facilities. The removal of these compounds is a real challenge for waste treatment engineers and scientists.

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One response to this problem is to utilize specific microbial cultures that are highly active in degrading these recalcitrant wastes. The problem then is proper control of culture conditions in an appropriate reactor over a prolonged period.

There have been several reports on the bacterial degradation of quinoline (Grant and Al Najjar, 1976; Dzumedzei et al., 1982; Bennett et al., 1985; Shukla, 1986). Investigations on the taxonomy of the quinoline-degrading bacteria showed that in most cases they were members of the genus *Pseudomonas*. *Pseudomonas* are characterized by their ability to use a wide range of organic compounds as sole sources of carbon and energy. Among them are also aromatic and heterocyclic substances which occur naturally or are synthetic products.

However, the ability to degrade quinoline does not appear to be confined to *Pseudomonas*. A *Moraxella* species, also a Gram-negative, aerobic bacterium, and a *Nocardia* species were described showing these degrading properties (Grant and Al Najjar, 1976; Shukla, 1986). In our laboratory, the degradation of quinoline is being studied by *Burkholderia* sp., an aerobic, rod shaped and Gram-negative bacterium (Han et al., 2000).

Immobilization of microbial cells have received increasing interest in recent years (Wang et al., 1995; Wang et al., 1997; Wang and Qian, 1999). It offers a promising potential for the improvement of efficiency of bioprocesses. Compared with free cell, immobilized cell has several advantages. The main advantages in the use of immobilized cells are their higher operational stability, their ease of use in a continuous reactor and their ability for scale-up.

Immobilized cell systems have the potential to degrade toxic chemicals faster than conventional wastewater treatment systems since high densities of specialized microorganisms are used in immobilized cell systems. One of the most widely used techniques for cell immobilization is cell entrapment. Carriers such as biopolymer beads can increase the ease of inoculant storage and application and also reduce the chance of bioaerosol formation (Cassidy et al., 1996). Bead structure may also protect the immobilized microbial cells from environmental stresses such as freezing/thawing (Leung et al., 1995), wet/dry cycles (Trevors et al., 1993) and species-specific phages (Smith et al., 1996). Amendments added to beads, such as clay and skim milk, may enhance establishment of introduced microorganisms and expression of biodegradative activity (Cassidy et al., 1996). Applying the microbial cell in a biopolymer bead may also provide increased protection from concentrations of recalcitrant organics that are toxic to free cells (Hu et al., 1994). The carrier for cell immobilization must meet the requirements of strong consistency, durability and high cell viability as well as low cost.

Immobilized cells have been widely used for treatment of numerous toxic compounds such as pentachlorophenol (OReilly and Crawford, 1989), 4-chlorophenol (Balfanz and Rehm, 1991; Wang and Qian, 1999), benzene derivatives and chlorobenzoates (Sahasrabudhe et al., 1988), 2,4-dichlorophenoxy acetic acid (2,4-D) (Shreve and Vogel, 1993) and phthalic acid ester (Wang et al., 1997).

However, there is no single ideal method for immobilization of all types of microorganisms. In fact, it is necessary to choose one method and some particular conditions for each type of cell. In this study, among the various cell immobilization methods that are available, entrapment in calcium alginate gel beads has been chosen for its ease of use, low economic cost, and low toxicity.

The objective of this study was to optimize the immobilization conditions of quinoline-degrading microorganism and investigate the microbial degradation of quinoline by immobilized cells.

2. Materials and methods

2.1. Microorganism

The microorganism used in this study was a pure strain of quinoline-degrading isolate, which was isolated from the activated sludge of wastewater treatment plant by enrichment shaking culture at 30°C. The strain was purified by successive streak transfers agar-plate medium and maintained as slant cultures on tryptone–glucose extract agar. It was identified as *Burkholderia* sp.

2.2. Medium

The medium used in this study is given in Table 1. Quinoline was used as the sole source of carbon and nitrogen in all experiments.

2.3. Analytical method

Quinoline concentrations of all samples in this work were analyzed by HPLC system (Hewlett-Packard

Table 1
Composition of stock nutrient medium[a]

Component	Concentration
Quinoline	100–1000 mg/l
Na_2HPO_4	4.26 g/l
KH_2PO_4	2.65 g/l
$CaCl_2 \cdot 2H_2O$	0.02 g/l
$MgSO_4 \cdot 7H_2O$	0.2 g/l
$MnSO_4 \cdot 7H_2O$	0.002 g/l
$FeSO_4 \cdot 7H_2O$	0.01 g/l

[a] *Note*. The pH of the medium was adjusted to 7.2–7.8.

model 5050 with an UV detector). Samples were prepared by centrifugation and filtration, 20 μl of which was injected. Separation was carried out in a C^{18} reverse-phase column, 250 × 4.6 mm, 5 μm (Hewlett-Packard Zorbax SB-C^{18}, USA). The elution solvent, which consisted of a mixture of methanol and water (60:40, v/v), was introduced to the column at a flow rate of 1 ml/min. Quinoline was detected at 275 nm wavelength.

2.4. Biomass cultivation

Before an experiment, organism was cultured in a 500 ml Erlenmeyer flask containing 200 ml medium that had been autoclaved at 121°C for 15 min. After 36 h, 5 ml of the culture was transferred to two 1000 ml flasks, each containing 400 ml of medium and 200 mg quinoline per liter. After 36 h, the biomass were harvested, washed once, and separated by centrifugation. The centrifuged cells were suspended in 300 ml medium containing 10 mg quinoline per liter to allow utilization of endogenous materials. After 36 h, the biomass were harvested, washed three times with sterile distilled water, and suspended in 20 ml medium. Approximately 1 ml of the biomass suspension was transferred to 250 ml flasks containing 50 ml of medium. The resulting biomass concentration were approximately 10^7 cells/ml.

Flasks containing the same amount of medium without inoculum served as control for abiotic processes. All flasks were incubated at 30°C in a temperature-controlled shaker.

2.5. Cell immobilization in alginate gel beads

Entrapment of cells using sodium alginate was performed as follows. Centrifuged cells were mixed thoroughly with 20 ml of sterile sodium alginate. The mixture was extruded as drops into a solution of calcium chloride. Bead size was controlled by the gauge number of the hypodermic needle used during extrusion. The beads were cured in the same solution at room temperature for 1 h, then washed with sterile distilled water to remove excess calcium ions and unentrapped cells.

2.6. Enumeration of viable cells

The alginate gel beads of a known weight were washed twice in sterile physiological saline and suspended in 10 ml of 0.1 M phosphate buffer (pH 7.3). The suspension was stirred at room temperature for 15 min. After the alginate gels had completely dissolved, the cell suspension obtained was diluted and spread on nutrient agar plates. The colonies were counted after the incubation of plates for 48 h at 30°C. All counts were performed in triplicate.

2.7. Batch degradation

For batch degradation, free and immobilized cells were cultivated in 250 ml shake flasks containing 50 ml medium. Degradation was carried out over 20 h at 30°C and shaking at 200 rpm.

In all experiments for optimization of immobilizing conditions, the quinoline concentrations were 200 mg/l.

The repeated batch degradation was carried out as above, after each 20 h the immobilized cells were aspetically removed from the medium and then washed 3–4 times with sterile distilled water. They were used again, together with fresh medium, in the same experimental conditions.

3. Results and discussion

3.1. Optimization of immobilization in Ca-alginate gel beads

Calcium alginate gel beads are easy to produce on a large scale without any sophisticated equipment, they have been extensively used for the immobilization of enzymes and cells. However, there does not seem to be any uniformly in the conditions of entrapment, so there are a number of immobilization parameters that used to be optimized. In this study, different conditions of immobilization, such as alginate concentration, calcium ion concentration, initial cell loading, hardening time and bead size, were investigated with a view to improving the biodegradation rate of quinoline.

3.2. Effect of sodium alginate concentration

Different concentrations of sodium alginate solution were sterilized and used for the immobilization of cell suspension, to give the final alginate concentration in gel beads to be 10, 20, 30 and 40 g/l. Quinoline was degraded by these immobilized cells, and the experimental results (shown in Table 2). The degradation time was defined as the time taken to degrade quinoline to non-detect level. Table 2 indicated that the highest degradation rate was achieved when the alginate concentration was 20 g/l. The lower concentration gave the lower strength of the alginate matrix, making the gel beads more fragile and causing release of the microbial

Table 2
Effect of sodium alginate concentration on degradation time

Sodium alginate concentration (g/l)	Degradation time (h)
10	20
20	19
30	23
40	26

cells during the degradation. The higher concentration of alginate may cause a higher mass-transfer resistance to substrates.

3.3. Effect of cell loading in the beads

Cell concentrations varying from 0.25 to 2.0 g/20 ml gel were entrapped in alginate gel beads and applied for the degradation of quinoline. The effect of initial biomass concentration on quinoline degradation is shown in Table 3.

It was evident that quinoline degradation was affected by the initial cell loading. The optimal initial cell loading of the beads was found to be 0.75 g biomass/20 ml gel. It was also found that higher initial concentration of cells did not improve the degradation rate. When the initial biomass was raised to 2.0 g biomass/20 ml gel, a drastic reduction of degradation rate was recorded. It has been reported by Gosmann and Rehm (1986) that with increasing cell concentration in gels, oxygen was consumed faster than it could diffuse into the beads. At this point, diffusion became limited factor. However, when the initial cell concentration was 0.25 g biomass/20 ml gel, the lowest biodegradation rate of quinoline was observed. This suggested that the optimal concentration of cells in gel beads was about 0.75 g biomass/20 ml gel.

3.4. Effect of calcium ion concentration

The effect of $CaCl_2$ concentration was investigated. Different concentrations of $CaCl_2$ (0.05, 0.1, 0.2, 0.3 M) in the curing solution were used to formulate the gel beads. The results showed that 0.3 M $CaCl_2$ caused the lowest degradation rate, indicating that the microbial activity was inhibited. So the use of an excess of the hardening reagent could therefore cause damage to the cells. When the $CaCl_2$ concentration was 0.05 M, the formulated gel beads were not strong enough, some of them ruptured during the application, suggesting that the amount of calcium ions required for the gelation of the beads was too small. 0.2 M $CaCl_2$ concentration gave the highest degradation rate, so for further experiments, a concentration of 0.2 M $CaCl_2$ was used (see Table 4).

It has been reported that calcium alginate gel is unstable in the presence of phosphates and certain cations such as Mg^{2+} or K^+, which are major nutrients of living microbial cells. However, the solubilizing effect of these agents can be overcome by supplementing the growth medium with $CaCl_2$. Table 5 showed the effect of concentration of calcium chloride in the medium on the biomass growth and the quinoline degradation by immobilized cells.

Table 3
Effect of initial biomass concentration entrapped in alginate gel on degradation of quinoline

Wet weight of the cells/20 ml gel	Degradation time (h)
0.25	25
0.5	20
0.75	18
1.0	21
2.0	22

Table 4
Effect of calcium ion concentration on degradation time

Calcium ion concentration (M)	Degradation time (h)
0.05	20
0.1	21
0.2	19
0.3	23

Table 5
Effect of calcium chloride concentrations on biomass growth and quinoline degradation with alginate beads

$CaCl_2$	Viable cells/20 beads	Degradation time (h)
Control	6.9×10^9	21
0.01	7.1×10^9	22
0.02	6.8×10^9	23
0.03	7.2×10^9	20
0.05	5.6×10^9	24

Table 5 revealed that addition of calcium salts up to 0.03 M slightly reduced the degradation rate without much affecting the immobilized cells. At 0.05 M concentrations, an inhibition in the number of viable cells, and hence a increase in degradation time, were noted.

3.5. Effect of hardening time

The formulated gel beads were immersed in $CaCl_2$ solution for different time (0.5, 1.0, 1.5, 2.0 h) to investigate the effect of time of exposure of the beads to the hardening solution. The results demonstrated that hardening time had no significant impact on bioactivity of immobilized cells. In other words, longer contact time did not cause lower degradation rate, indicating that contact with the hardening solution could not cause loss in cell viability. Further exposure to the hardening solution was not necessary because one hour is enough for gelation completed. However, too short contact time caused incomplete gelation so that the mechanical strength of the gel was too low.

3.6. Effect of bead size

Beads of three diameters (2, 3, and 5 mm) were prepared and used to degrade the quinoline. The results indicated that a maximum degradation rate was obtained using 3 mm diameter beads and a considerable

reduction (about 30%) of degradation rate was observed with 5 mm beads.

3.7. Biodegradation of quinoline by immobilized cells

The microbial cells were entrapped in calcium alginate gel beads and used for the degradation of quinoline. The time course of microbial growth and quinoline degradation by immobilized cell was investigated. The results are shown in Fig. 1.

The biomass concentration increased with quinoline degradation, which demonstrated that the microorganisms isolated in this study were capable of utilizing quinoline as the sole source of carbon and nitrogen. The final densities reached about 1.0×10^{12} cells/l in immobilized cell systems.

The effect of initial quinoline concentration on degradation rate by immobilized cells was investigated. Fig. 2 gives the data from the experiments with different concentrations of quinoline.

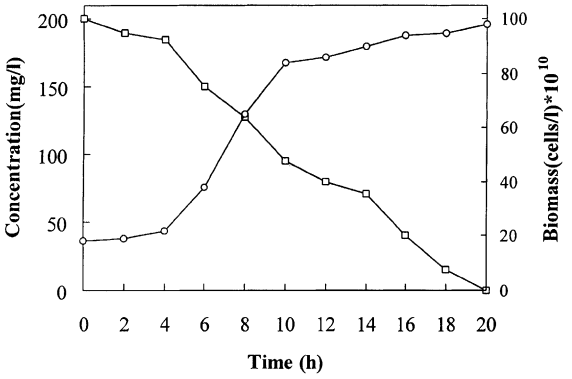

Fig. 1. Characteristics of quinoline degradation and microbial growth in immobilized cell system.

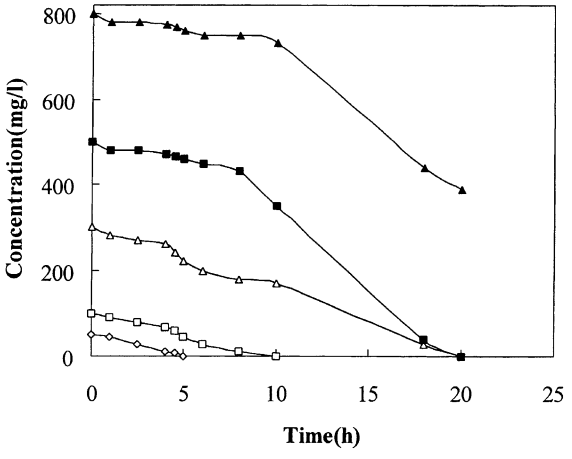

Fig. 2. Effect of initial quinoline concentration on degradation rate by immobilized cells.

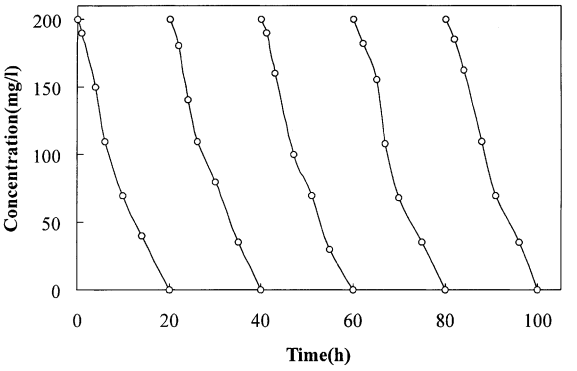

Fig. 3. Degradation of quinoline by immobilized cells during repeated batch cultivation.

It can be seen from Fig. 2 that the lag-phase duration of quinoline degradation prolonged with an increase in the initial quinoline concentration, that is to say, the microorganisms exhibited extended lag time at higher initial quinoline concentration.

3.8. Repeated use of immobilized cells

The entrapped cells were cultivated for 100 h in 5 batches and each batch lasted for 20 h to investigate the successive reuse of immobilized cells for the degradation of quinoline, after every 20 h the beads were washed with sterile distilled water and re-suspended in fresh medium. The results are shown in Fig. 3.

From Fig. 3, we can see that the bio-activity of immobilized cells was stable over 100 h in the repeated batch cultivation. We can also observe that from third to fifth batch, leakage of cells scarcely increased during repeated cultivation (data not shown). The weakening phenomenon of the immobilization matrix was not observed in the experimental condition.

4. Conclusions

The calcium gel was used as a carrier for immobilization of microbial cells. The immobilization conditions were optimized as follows: sodium alginate concentration, 20 g/l; initial cell loading in the bead, 0.75 g biomass/20 ml gel; calcium ion concentration, 0.2 M $CaCl_2$; hardening time 1 h; bead size, about 3 mm in diameter. The degradation experiments were performed in 500 ml shaking flasks to treat the synthetic wastewater containing quinoline as the sole source of carbon and energy. The results demonstrated that the microorganism immobilized calcium alginate gel can degrade quinoline rapidly. The immobilized cell can be repeatedly used for 5 batches without significant loss of their bio-activity. From the viewpoint of the stability of the cells in re-

peated use for quinoline degradation, it seems that this immobilization method is effective.

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