Degradation of Carbazole by Microbial Cells Immobilized in Magnetic Gellan Gum Gel Beads

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Polycyclic aromatic heterocycles, such as carbazole, are environmental contaminants suspected of posing human health risks. In this study, we investigated the degradation of carbazole by immobilized Sphingomonas sp. strain XLDN2-5 cells. Four kinds of polymers were evaluated as immobilization supports for Sphingomonas sp. strain XLDN2-5. After comparison with agar, alginate, and κ-carrageenan, gellan gum was selected as the optimal immobilization support. Furthermore, Fe₃O₄ nanoparticles were prepared by a coprecipitation method, and the average particle size was about 20 nm with 49.65-electromagnetic-unit (emu) g⁻¹ saturation magnetization. When the mixture of gellan gel and the Fe₃O₄ nanoparticles served as an immobilization support, the magnetically immobilized cells were prepared by an ionotropic method. The biodegradation experiments were carried out by employing free cells, nonmagnetically immobilized cells, and magnetically immobilized cells in aqueous phase. The results showed that the magnetically immobilized cells presented higher carbazole biodegradation activity than nonmagnetically immobilized cells and free cells. The highest biodegradation activity was obtained when the concentration of Fe₃O₄ nanoparticles was 9 mg ml⁻¹ and the saturation magnetization of magnetically immobilized cells was 11.08 emu g⁻¹. Additionally, the recycling experiments demonstrated that the degradation activity of magnetically immobilized cells increased gradually during the eight recycles. These results support developing efficient biocatalysts using magnetically immobilized cells and provide a promising technique for improving biocatalysts used in the biodegradation of not only carbazole, but also other hazardous organic compounds.

The pollution of soil, river sediments, and ground water by hazardous organic compounds has been gaining increasing attention in the field of environmental remediation. Carbazole and its derivatives are common nitrogen compounds found in environments contaminated by coal tar, crude oil, and creosote (13). When these nitrogen compounds are combusted, nitrogen oxides, NOₓ, are released into the atmosphere, which causes acid rain and air pollution (23). Moreover, carbazole and its derivatives have been found to be toxic (24, 34) and mutagenic (10, 27), and they readily undergo radical chemistry to generate the more poisonous hydroxynitrocarbazoles (1). Soil, river sediments, and ground water polluted by carbazole have become a great threat to the environment. Therefore, it is necessary to establish effective methods to clean up carbazole and its derivatives to protect the environment.

Many researchers have focused their studies on the isolation and identification of carbazole-degrading microorganisms, such as Pseudomonas (7, 25), Sphingomonas (32), Ralstonia (29), Bacillus (14), Gordonia (28), and Mycobacterium and Xanthomonas (8). Our laboratory has also isolated and constructed several bacteria that can degrade these heterocyclic compounds (6, 18, 19, 20, 43, 44). The isolates degrade carbazole by following similar pathways, in which carbazole is initially attacked at the angular position by dioxygenation, followed by spontaneous conversion of the dihydroxylated intermediate to 2'-aminobiphenyl-2,3-diol. The extradiol dioxygenase attacks the hydroxylated ring at the meta position to give 2-hydroxy-6-(2-aminophenyl)-6-oxo-2,4-hexadienoic acid. This meta-cleavage product is hydrolyzed to produce anthranilic acid, which is then mineralized (42). Nevertheless, current studies are mostly focused on the pathways of such chemical metabolisms, as well as the genes and enzymes involved, and rarely on the development of an immobilization method for bioremediation.

The use of immobilized microorganisms rather than free cells in biotransformation is advantageous to enhance the stability of the biocatalyst and to facilitate its recovery and reuse. These advantages have encouraged researchers to investigate the application of immobilized cells in the biodegradation of toxic compounds, such as phenol, pyridine, dibenzothiophene, and quinoline (5, 15, 17, 37, 38). However, mass transfer limitation involved in substrate diffusion to the reaction system is still the major drawback in the application of an entrapment technique.

Nanoparticles represent a new generation of environmental-remediation technologies that could provide cost-effective solutions to some of the most challenging environmental clean-up problems. There are two factors that contribute to the capabilities of nanoparticles as an extremely versatile remediation tool. First, the size (1 to 100 nm) that characterizes nano-
particles provides them with large specific surfaces and high specific energies. Second, their flexibility makes them versatile both in situ and ex situ (12, 45). Recent laboratory research has largely established nanoparticles as an effective method for removing a broad range of environmental contaminants, such as chlorinated ethenes, heavy metal ions, dibenzothiophene, and polycyclic aromatic hydrocarbons (PAHs) (16, 26, 30, 31, 35). Therefore, the exploitation of nanoscale technology in environmental applications appears very promising.

In this study, we demonstrate a new process for carbazole biodegradation employing magnetically immobilized cells. An improved and simple method for the immobilization of Sphingomonas sp. strain XLDN2-5 in magnetic gellan gel beads was developed, and the stability and activity of the biocatalyst for the degradation of carbazole were also evaluated.

MATERIALS AND METHODS

Microorganism and cultivation. Sphingomonas sp. strain XLDN2-5, which can use carbazole as the sole source of carbon, nitrogen, and energy, was cultivated in mineral salts medium (MSM) as previously described (6). Cells were harvested in the exponential phase (the optical density was about 0.68 to 0.70 at 620 nm) by centrifugation at 12,000 × g for 10 min. The pellet was washed twice with distilled water and then resuspended in distilled water. The inactive controls were made using heat-killed cells (autoclaved at 115°C for 20 min).

Chemicals. Analytical grade carbazole was purchased from Sigma-Aldrich (St. Louis, MO). Gellan gum was prepared as previously described (39, 40). All other chemicals were of analytical grade and commercially available.

Preparation of Fe3O4 nanoparticles. The Fe3O4 nanoparticles were prepared by the conventional coprecipitation method (21) with some modifications: 23.5 g FeCl3 · 6H2O and 8.6 g FeCl2 · 4H2O were dissolved in 600 ml distilled water at 30°C. Before reaction, N2 gas flowed through the reaction medium to prevent possible oxidation. NH4Cl · H2O (8 M) was then slowly injected into the mixture of FeCl3 and FeCl2 with vigorous stirring until the pH reached 10. After precipitation, the Fe3O4 nanoparticles were repeatedly washed until the pH was constant and then lyophilized for 48 h under vacuum to form powder. Fe3O4 powder (1.5 g) was put into 10 ml distilled water to form the Fe3O4 particle suspension. After ultrasonic disruption (25 KHz; 10 min; BRU25-96; BRANSON) of the suspension, the Fe3O4 nanoparticles were well dispersed in distilled water to form a stable suspension, which we called the magnetic suspension. The average diameter of the Fe3O4 particles was about 20 nm (Fig. 1a), and their saturation magnetization was 49.65 electromagnetic units (emu) g−1. The magnetic nanoparticles were evaluated as immobilization supports for carbazole-degrading Sphingomonas sp. strain XLDN2-5. Adsorption experiments showed that the highest adsorption of carbazole was presented by magnetic gellan gel beads (24.10 mg g−1), followed by gellan gel beads (12.15 mg g−1), agar (4.44 mg g−1), and κ-carrageenan (0.77 mg g−1) at equilibrium. In contrast, all calcium alginate gel beads were broken. In order to investigate the most suitable immobilization support, the physical properties (size, formation, and breakage) of gel beads were also studied. As shown in Table 1, all gel beads were spherical and homogeneous. The gel beads of gellan gum, κ-carrageenan, and agar were more robust (breakages were zero) and presented higher breakage resistance than those of calcium alginate.

We also investigated the biodegradation activities of nonmagnetically immobilized Sphingomonas sp. strain XLDN2-5 in gellan gum, κ-carrageenan, and agar. Figure 2a shows that 3,340 μg carbazole could be degraded in 20 h by free cells. The equivalent amount of carbazole could be degraded in 36 h by immobilized cells when gellan gum served as the immobilization support. In contrast, when beads of immobilized inactive

Biodegradation experiments. In biodegradation experiments, the initial content of carbazole and the incubation conditions were the same as those of the adsorption experiments. Nonmagnetically immobilized cells and magnetically immobilized cells were added to MSM with carbazole as a biocatalyst. The controls were gellan gel beads, nonmagnetically immobilized inactive cells, and magnetically immobilized inactive cells incubated in MSM with carbazole. In the recycling experiments, after each biodegradation batch, the magnetically immobilized cells were collected by application of a magnetic field and then were washed once with MSM to remove the free cells. After the MSM was drained, 10 ml of fresh MSM containing carbazole was added to repeat the cycle. Additionally, the same amount of cells (the cell wet weight was 60 mg) was used in all batch biodegradation experiments, including the first cycle of reuse experiments. All experiments were performed in triplicate. The specific biodegradation rate was expressed as the amount of carbazole (in μg) consumed by 1 g (wet weight) of cells per hour.

Analytical methods. After each batch of biodegradation, the biodegradation mixture was filtered through glass wool to separate the gel beads from the supernatant. Then, 20 ml and 4 ml ethanol were added to the supernatant and the gel beads, respectively, followed by centrifugation (12,000 rpm for 20 min) and filtration. The residual carbazole contents were determined using high-performance liquid chromatography performed with an Agilent 1100 series (Hewlett-Packard) instrument equipped with a reverse-phase C18 column (4.6 mm by 150 mm; Hewlett-Packard). The mobile phase was a mixture of methanol and deionized water (90:10 [vol/vol]) at a flow rate of 0.5 ml min−1, and carbazole was monitored at 254 nm with a variable-wavelength detector.

The sizes and morphologies of the magnetic nanoparticles were determined by transmission electron microscopy (JEM-100cx II; JEOL, Japan). Each sample was prepared by evaporating a drop of properly diluted nanoparticle suspension on a carbon copper grid. The morphology of cells immobilized in gel beads was determined using a scanning electron microscope (SEM) (S-570; Hitachi, Japan). Magnetization curves for the magnetically immobilized cells were obtained with a vibrating sample magnetometer (MicroMag 2900/3900). The spectra of FTIR were obtained on a NEXUS 380 (Nicolet). To determine the average size of the beads, a direct measurement was carried out. The diameters of 30 randomly chosen beads were measured with a Vernier caliper. The breakage of beads was determined as the quotient of the number of broken beads divided by the total number of beads.

RESULTS

Selection of immobilization supports. Four kinds of polymers were evaluated as immobilization supports for Sphingomonas sp. strain XLDN2-5. Adsorption experiments showed that the highest adsorption of carbazole was presented by magnetic gellan gel beads (24.10 mg g−1), followed by gellan gel beads (12.15 mg g−1), agar (4.44 mg g−1), and κ-carrageenan (0.77 mg g−1) at equilibrium. In contrast, all calcium alginate gel beads were broken. In order to investigate the most suitable immobilization support, the physical properties (size, formation, and breakage) of gel beads were also studied. As shown in Table 1, all gel beads were spherical and homogeneous. The gel beads of gellan gum, κ-carrageenan, and agar were more robust (breakages were zero) and presented higher breakage resistance than those of calcium alginate.

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cells and beads without cells were used for the degradation reaction, no decrease in the total content of carbazole was detected (Fig. 2b). The activities of cells immobilized by κ-carrageenan and agar were lower, and the residual contents of carbazole were 2,381 g and 1,875 g after 48 h of incubation, respectively (Fig. 2c and d). Therefore, gellan gum was chosen as the most suitable support in the subsequent experiments.

**Biodegradation of carbazole by magnetically immobilized cells.** The effects of different concentrations of Fe₃O₄ nanoparticles (3, 6, 9, and 12 mg ml⁻¹) on the activity of immobilized cells were studied. The biodegradation of carbazole was conducted in MSM by free cells, nonmagnetically immobilized cells, and magnetically immobilized cells. Figure 3 shows that the highest biodegradation activity for carbazole was presented by the magnetically immobilized cells, and 3,340 g carbazole could be degraded completely in 16 h to 18 h. While the equivalent amount of carbazole could be degraded completely in 20 h by free cells, the residual carbazole content was 1,035 μg at 20 h with nonmagnetically immobilized cells. In contrast, no decrease of the total carbazole content was observed when magnetically immobilized inactive cells and nonmagnetically immobilized inactive cells served as biocatalysts.

**Reuse of magnetically immobilized cells and nonmagnetically immobilized cells for carbazole biodegradation.** The activities of magnetically immobilized cells (at the optimal Fe₃O₄ nanoparticle content of 9 mg ml⁻¹) and nonmagnetically immobilized cells were tested repeatedly. As shown in Fig. 4a, from the first to the fifth cycle, 3,340 μg carbazole was completely consumed by magnetically immobilized cells in 16 h; from the sixth to the eighth cycle, the same amount of carbazole was completely consumed in only 12 h. In contrast, from the first to the fifth cycle, the residual carbazole contents were

<table>
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<th>Polymer</th>
<th>Conc (%) (wt/vol)</th>
<th>Bead-forming procedure</th>
<th>Form</th>
<th>Bead size (mm)</th>
<th>Breakage (%)</th>
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<tr>
<td>Gellan gum</td>
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<tr>
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<tr>
<td>Alginate</td>
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<td>Ionotropic</td>
<td>Spherical beads</td>
<td>2.25 ± 0.09</td>
<td>100</td>
</tr>
<tr>
<td>κ-Carrageenan</td>
<td>2</td>
<td>Interphase</td>
<td>Spherical beads</td>
<td>2.93 ± 0.17</td>
<td>0</td>
</tr>
</tbody>
</table>

*Values are the means ± standard deviations of three separate determinations.*

**FIG. 2.** Carbazole contents of the supernatant and gel beads during biodegradation by immobilized cells in different supports. (a) Biodegradation by free cells. (b) Biodegradation by immobilized cells in gellan gum. (c) Biodegradation by immobilized cells in κ-carrageenan. (d) Biodegradation by immobilized cells in agar. The solid symbols, including free cells (▲), cells immobilized by gellan gum (●), cells immobilized by κ-carrageenan (■), and cells immobilized by agar (★), represent the supernatant; the open symbols, including gellan beads (○), κ-carrageenan beads (□), and agar beads (◇), represent the gel beads. Gellan gel beads without cells (►) and nonmagnetically immobilized inactive cells (▷) were controls. The same amounts of cells (the cell wet weight was 60 mg) were used in all experiments. The error bars represent standard deviations.
about 123 μg to 232 μg at 20 h with nonmagnetically immobilized cells, and from the sixth to the eighth cycle, the residual carbazole contents were about 210 μg to 324 μg (Fig. 4b).

SEM images of Sphingomonas sp. strain XLDN2-5 immobilized in gellan gel beads and magnetic gellan gel beads. SEM images of Sphingomonas sp. strain XLDN2-5 are shown in Fig. 5. The Sphingomonas sp. strain XLDN2-5 cells can be clearly observed on the surfaces of the gellan gel beads (Fig. 5a1), while inside the gellan gel beads, the sheets of gellan gum matrix were tightly bound together and bacterial cells were not clearly observed (Fig. 5a2). The SEM images of Sphingomonas sp. strain XLDN2-5 immobilized on the surfaces of magnetic gellan gel beads are almost the same as those of the surfaces of gellan gel beads (Fig. 5b1). However, the sheets of gellan gum matrix were loosely bound together inside magnetic gellan gel beads, and many pores existed between the sheets of gellan gum matrix (Fig. 5b2). Figures 5c1 and 5c2 are SEM images of Sphingomonas sp. strain XLDN2-5 immobilized in magnetic gellan gel beads after eight cycles of the biodegradation experiments. On the surfaces and inside of magnetic gellan gel beads, the amounts of cells evidently increased. Moreover, the sheets of gellan gum matrix were also loosely bound together inside magnetic gellan gel beads, and many pores also existed between the sheets of gellan gum matrix.

DISCUSSION

Microbial degradation of hydrophobic compounds, such as chlorophenols and PAHs, in soil and sediments is thought to be limited by their mass transfer to the aqueous phase (2, 9, 36). The bioavailability of such hydrophobic compounds is controlled by a number of physicochemical processes, such as adsorption and desorption, diffusion, and dissolution (2). Adsorption of these hydrophobic compounds to extracellular polymeric substances has been reported previously. For example, PAHs, benzene, toluene, and xylene have been adsorbed to exopolysaccharides to promote their bioavailability or to
remove them from water (11, 33). In addition, Dohse et al. reported that microbial polymers, acting as phenanthrene adsorbents and carriers, may partly facilitate the transport of phenanthrene in sand columns (4). Carbazole and its derivatives are hydrophobic aromatic compounds, and there are problems of mass transfer in their biodegradation processes in the aqueous phase. Considering this, we investigated the adsorption of carbazole by several polymers, including gellan gum, κ-carrageenan, agar, and alginate. The results showed that gellan gum presented the highest adsorption activity for carbazole at equilibrium. However, for immobilization supports, high adsorption of carbazole alone is not enough to ensure a high biocatalyst activity. Therefore, the biodegradation activities of *Sphingomonas* sp. strain XLDN2-5 in immobilization supports were also investigated. The results indicated that the biodegradation activities of *Sphingomonas* sp. strain XLDN2-5 immobilized by κ-carrageenan and agar were very low (Fig. 2c and d). The reason may be that an oily phase was used during the gel bead preparation of κ-carrageenan and agar by the interphase technique, and the oil adsorbed by gel beads may also result in impeding of the degradation rates for carbazole. Moreover, the gel bead-forming procedure was complex and ineffective, which made them less suitable for industrial biodegradation processes. In contrast, the *Sphingomonas* sp. strain XLDN2-5 cells immobilized by gellan gum presented a high specific biodegradation rate (1,546 μg g cell wet weight$^{-1}$ h$^{-1}$) (Fig. 2b). The carbazole contents of the supernatant and gellan gel beads could not be detected at the same time, which may be due to the immediate consumption of carbazole adsorbed by gellan gel beads in the biodegradation process. Additionally, no decrease of carbazole content was observed when nonmagnetically immobilized inactive cells and gellan gel beads without cells served as biocatalysts, which confirmed that the removal of carbazole was due to biodegradation by the *Sphingomonas* sp. strain XLDN2-5 cells. It is clear that gellan gum was the optimal immobilization support due to high carbazole adsorption, superiority in maintaining the high biodegradation activity of the biocatalyst, and a simple gel bead-forming procedure. Moreover, gellan gum produced by *Sphingomonas paucimobilis* ATCC 31461 is considered natural, nontoxic, and compatible with the environment (39, 40). These results were also consistent with previous reports that the semicolloid gellan gum may enhance the aqueous solubility of fluoranthene, which would in turn lead to increased mineralization rates (11). The specific biodegradation rate of cells immobilized by gellan gum was still lower than that of free cells (1,546 μg g cell wet weight$^{-1}$ h$^{-1}$ to 2,783 μg g cell wet weight$^{-1}$ h$^{-1}$) (Fig. 2a and b). This may be due to a mass
transfer limitation of immobilized cells, which may have some-what reduced the bioavailable concentration in the inner spaces of the beads in contrast to that in the bulk liquid.

Nanoscale particles have large specific surfaces and high surface reactivity, which gives them the potential to address some of the challenges of environmental remediation. Recently, there have been reports that the remediation of Cr(VI) and Pb(II) was carried out by nanoscale zero-valent iron (26) and that engineered polymeric nanoparticles were used in remediation of soil contaminated with PAHs (35). Figure 1a and b1 show that the average particle diameter of Fe3O4 nanoparticles was about 20 nm, and their saturation magnetization was 49.65 emu g-1, which provides the particles with superparamagnetic properties so that the Fe3O4 nanoparticles could be easily separated and recycled by an external magnetic field. In the biodegradation processes, the specific biodegradation rate of nonmagnetically immobilized cells was only 1,761 g g cell wet weight-1 h-1 (Fig. 3). The reason may be that bacterial cells were tightly trapped in the gellan gum matrix, which resulted in impeding the mass transfer of substrate from the environment to the central reaction site (Fig. 5a2). In contrast, high specific biodegradation rates (from 3.092 g cell g wet weight-1 h-1 to 3.479 g cell g wet weight-1 h-1) were obtained when magnetically immobilized cells served as the biocatalyst, which may be supported by the existence of nanoparticles, the loose binding of the sheets of gellan gum matrix, and the existence of many pores between the sheets of gellan gum matrix (Fig. 5b2). When nonmagnetically immobilized inactive cells and magnetically immobilized inactive cells were incubated as biocatalysts, no degradation of carbazole was observed, which also confirmed that the removal of carbazole was not due to adsorption but to biodegradation by Sphingomonas sp. strain XLDN2-5. Among the different concentrations of Fe3O4 nanoparticles, the biodegradation rate was slightly higher at an Fe3O4 nanoparticle concentration of 9 mg ml-1, especially in the period from 4 h to 8 h, and the saturation magnetization of magnetically immobilized cells was 11.08 emu g-1 (Fig. 1b2), which made it possible to solve the problem of recovering immobilized cells with a magnetic field. Additionally, the saturation magnetization of magnetic gellan gel beads increased with the number of Fe3O4 nanoparticles added, while the resistance to breakage of magnetic gellan gel beads decreased. These results revealed that the biodegradation activity of the immobilized Sphingomonas sp. strain XLDN2-5 cells was significantly enhanced by adding Fe3O4 nanoparticles, which may be due to the reduction or elimination of mass transfer problems.

In an industrial bioremediation process, the recycling of the biocatalysts could be an important factor that determines the effectiveness of degradation over time. From the first to the eighth cycle, magnetically immobilized cells presented higher biodegradation activity (the specific biodegradation rates increased from 3.479 µg g cell wet weight-1 h-1 to 4.638 µg g cell wet weight-1 h-1), as shown in Fig. 4a. Nevertheless, the specific biodegradation rates of nonmagnetically immobilized cells decreased from 2,680 µg g cell wet weight-1 h-1 to 2,513 µg g cell wet weight-1 h-1 during the recycling processes (Fig. 4b). The high biodegradation activity may be supported by the good growth of cells in the magnetic gellan gel beads, as shown in Fig. 5c1 and c2. The increase of biomass was most pronounced on the surfaces of the magnetic gellan gel beads, and the concentration of cells inside the magnetic gellan gel beads also increased. These results also confirmed that the removal of carbazole was due to biodegradation by Sphingomonas sp. strain XLDN2-5, because Sphingomonas sp. strain XLDN2-5 could grow with carbazole as the sole source of carbon, nitrogen, and energy. These results were also consistent with a previous report that the growth of cells in cellite beads was considered to have enhanced the desulfurization rate in the subsequent batch (3).

In conclusion, magnetically immobilized cells were evaluated as a novel aspect of the industrialization of cell immobilization. Gellan gum, as an immobilization support, required simple gel bead-forming procedures and presented high carbazole adsorption, which led to an increased mineralization rate. Moreover, magnetic (Fe3O4) nanoparticles, as one component of the magnetic immobilization support, have a large specific surface and superparamagnetic properties, which not only reduced the mass transfer resistance of traditional immobilization processes, but also facilitated the recovery of immobilized cells in the reuse processes. Additionally, the recycling experiments demonstrated that the degradation activity of magnetically immobilized cells was still high after eight cycles. These results support the development of efficient biocatalysts using magnetically immobilized cells and provide a promising technique for improving the biocatalysts used in the biodegradation of not only carbazole, but also other hazardous organic compounds.

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