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Cyclodextrin-linked alginate beads as supporting materials for *Sphingomonas cloacae*, a nonylphenol degrading bacteria

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Abstract

Calcium alginate beads covalently linked with α -cyclodextrin (α -CD–alginate beads) were prepared and examined for their ability to serve as a supporting matrix for bacterial degradation of nonylphenol, an endocrine disruptor. Column chromatographic experiment using α -CD–alginate beads with diameter of $657 \pm 82 \mu\text{m}$ and with degree of CD substitution of 0.16 showed a strong affinity for nonylphenol adsorption. Although addition of α -CD (2.7–27 mM) to the culture broth of *Sphingomonas cloacae* retarded nonylphenol degradation, the immobilized bacteria on the CD–alginate beads were effective for the degradation. Batch degradation tests using the immobilized bacteria on α -CD–alginate-beads showed 46% nonylphenol recovery after 10-day incubation at $25 \pm 2 \text{ }^\circ\text{C}$, and the recovery reached to about 17% when wide and shallow incubation tubes were used to facilitate uptake of the viscous liquid of nonylphenol on the surface of the medium. Scanning electron microscopic photographs revealed that multiplied bacteria was present both on the surface and inside the beads and the matrix of CD–alginate was stable and suitable during 10-day incubation.

Keywords: Cyclodextrin; Alginate bead; Nonylphenol; Inclusion complex; *Sphingomonas cloacae*

1. Introduction

Nonylphenol is one of the organic pollutants found in aquatic environments as a consequence of the biodegradation of nonylphenol polyethoxylate (NPnEO), a non-ionic surfactant contained in industrial cleaning products and in household detergents (Ahel et al., 1993). Although the parent surfactant itself is less toxic, NPnEO released to the environment is rapidly decomposed to form nonylphenol. Since nonylphenol is relatively hydrophobic (log

K_{ow} 4.8 – 5.3) and its water solubility is extremely low (5.43mg/l at 20 °C) (Ying et al., 2003; Kim et al., 2005), it accumulates in sediments, groundwater, and sewage sludge. Nonylphenol was reported to have an estrogenic activity at a tissue concentration of 1 μ M *in vitro*, although the activity was 10^{-6} times less than 17 β -estradiol (E2), the main natural estrogen, (Jobling and Sumpter, 1993). Substantial evidences have been demonstrated showing that nonylphenol causes various disorders of the male reproductive system, including reduced testicular size and sperm production in rainbow trout and other marine animals (Granmo et al., 1989; Ekelund et al., 1990). Moreover, *in vitro* experiments also revealed that it induced proliferation of the human breast cancer cell line (Verma and Goldin, 1998). Although nonylphenol is recalcitrant in nature, its bioavailability has triggered extensive studies on its bio-remediation by use of some yeast and gram-negative bacteria. *Candida aquaetexoris* was reported to decompose the alkyl chain of nonylphenol to give 4-acetylphenol (Vallini et al., 2001), while several bacteria such as *Sphingomonas xenophaga* (Gabriel et al., 2005), *Sphingomonas cloacae* (Fuji et al., 2000), and *Sphingobium amiense* (Ushiba et al., 2003) metabolized its aromatic moiety to the corresponding C9 alcohols.

Cyclodextrin (CD), a naturally occurring cyclic oligosaccharides composed of several α -D-glucopyranoses, has been considered as having one of the major roles in increasing the stability action, encapsulation, and adsorption of contaminants by the formation of a inclusion complex (Dodziuk, 2006). Utilizing CDs, various methods for nonylphenol removal from wastewater have been studied in order to minimize its release into the environment (Kawasaki et al., 2001; Fukazawa et al., 2005; Kim et al., 2005). Polysaccharide-based CD derivatives were synthesized by coupling of CD and chitosan, a polycationic biopolymer prepared by de-Nacetylation of a natural mucopolysaccharide chitin (Furusaki et al., 1996; Tanida et al., 1998; Tojima et al., 1998). This grafting of CD onto chitosan had an inclusion ability and showed cumulative effect for the affinity chromatographic separation of *p*-nitrophenolates and bisphenol A (Tojima et al., 1999; Nishiki et al., 2000). CD–alginate was synthesized by activation of the secondary hydroxyl groups of sodium alginate with cyanogen bromide and subsequent coupling with 6-amino- α -CD, and the degree of CD substitution (DS 0.05–1.58) depended on α -CD and alginate ratio and on reaction conditions (Pluemsab et al., 2005). The inclusion ability of α -CD–alginate was confirmed by spectroscopic examination with *p*-nitrophenol as a model guest compound. The objective of this work was to investigate the possibility of bead formation and the applicability of CD–alginate for immobilization of microbial cells for bio-remediation application of nonylphenol contaminated water.

2. Methods

2.1. Materials

Nonylphenol and sodium alginate (viscosity of 1% aqueous solution – 300–400 cP at 20 °C) were purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). Nonylphenol was

a mixture of isomers and it was used without further purification. All other chemicals used were of reagent grade. *S. cloacae* (Fuji et al., 2000) was purchased from Japan Collection of Microorganisms, Riken Bioresource Center (Saitama, Japan); catalog number 10874. The stock cultures were stored at 4 °C in nutrient agar. Water used in this study was prepared by a Millipore pure water apparatus Elix 3-1 equipped with both ion-exchange and reverse-osmosis membranes.

2.2. Instruments

HPLC analyses were performed with a Hitachi instrument equipped with an ODS-silica column, Mightysil RP-18GP (4.6 x 150mm; Kanto Chemical Co., Inc.). Hitachi L-7400 UV detector was equipped and recorded at 275nm. Samples were injected via a 1 µl-injection loop and eluted isocratically with acetonitrile–water (75:25 v/v) at a flow rate of 1.0ml/min. Centrifugation was carried out with TOMY EX-125. Photographs of scanning electron microscopy (SEM) were taken on a Hitachi S-800 instrument after the samples were dried with Hitachi HCP-1 critical point dryer. Incubation was performed on a reciprocal shaker, Advantec TBK202AA. Optical density (OD) was recorded with a Jasco ETC-505S UV–vis spectrometer.

2.3. Preparation of α -CD–alginate beads and its adsorption property

Aqueous sodium alginate solution (2% w/v) was dropped through a needle into an aqueous calcium chloride solution (2% w/v) with air pressure to control the size of beads (diameter size, $930 \pm 93 \mu\text{m}$), stirred for 24 h, and then washed with water twice to give white gel beads. The bead diameters measured by a micro-scale under a microscope were $657 \pm 82 \mu\text{m}$. The resulting alginate beads (0.1 g dry weight) were surface modified by treatment with CNBr (60mg) in an aqueous suspension (10 ml) at pH 10.0–11.0 controlled by 6 M NaOH until 1 h. The resulting beads were washed with water twice and treated with 6-amino- α -CD (0.5 g) for 24 h in CaCl_2 solution (2% w/v, 10 ml) to give α -CD–alginate beads. The data of elemental analysis were C, 34.68%; H, 4.86% and N, 1.10%. α -CD–alginate beads (3 ml) and CD-microbeads (total volume, 3 ml) were packed in a glass column with a diameter of 1 cm, 10% aqueous methanol solution (10 ml) containing nonylphenol (300 µg) was applied and allowed to stand for 5 min, and eluted with water and then methanol as described earlier (Tojima et al., 1999). Elution was performed at the flow rate of 0.5 ml/min and fractions (5 ml) were collected. The amount of nonylphenol in each fraction was determined by UV absorption at 270 nm.

2.4. Nonylphenol biodegradation

2.4.1. Culture conditions

The inoculum of *S. cloacae* was enriched in 5 ml-nutrient broth for two days and then transferred into 100ml-nutrient broth (Difco, Japan), which was incubated at $25 \pm 2 \text{ }^\circ\text{C}$ for 48

h in a reciprocal shaker (100 strokes per min). Cells were harvested by centrifugation at 3000g at 4 °C for 30 min and washed twice with physiological saline solution at pH 7.0. The precipitated bacterial mass was diluted with 10ml of NaCl solution (0.85% w/v) and used as a bacterial suspension in the following experiments.

2.4.2. Degradation of nonylphenol with immobilized *S. cloacae* and free-suspension cells

The bacterial suspension (OD = 27 at 550 nm, 0.1 ml) was inoculated into freshly sterilized aqueous solutions (0.9 ml) containing 2% sodium alginate, or 2 or 4% w/v CD–alginate (DS 0.18: prepared according to [Pluemsab et al., 2005](#)). Each suspension was dropped into 2% aqueous calcium chloride solution (100ml) through a syringe, and stirred for 3 h until complete gel formation. The beads were washed twice in 0.85% aqueous NaCl solution and then incubated in narrow, or wide-diameter tubes (4 x 13cm and 2.5 x 13 cm, respectively) of 5 ml yeast nitrogen base (YNB; Difco) broth that contained nonylphenol at a concentration of 600 mg/l. All tubes were shaken at 25 ± 2 °C on a reciprocal shaker (100 strokes per min) for 10 days. Free-suspension cells (0.1 ml cell suspension) were cultured in the same conditions as described above, and the initial cell concentration was approximately 4.6 g dry cell/l. Each treatment was carried out in duplicate. The effect of -CD in culture broth was examined by addition of α -CD (1, 5, and 10 molar equivalents against nonylphenol) into the free-suspension cell culture broths.

2.4.3. Determination of nonylphenol content and microbial growth

After 10-day incubation, the beads contained in each culture medium were dissolved by adding 2ml of 0.1M sodium citrate solution (pH 7.2), and the nonylphenol in whole cultured broth was extracted with dichloromethane (2 ml). The organic layer was collected by centrifuging at 3500 rpm. The samples were subjected to HPLC to determine the concentration of the nonylphenol by comparing the peak area with that of the authentic sample. The number of viable cells in the freely-suspended, or immobilized cells were determined by a colony formed on nutrient agar. The CD–alginate beads immobilized with the cell were dissolved in a test tube containing 9 ml of 0.1 M sodium citrate buVer (pH 7.2) and stirred until the gel beads were completely dissolved. The cell suspension was appropriately diluted and spread on nutrient agar plates. After cultivation at 25 ± 2 °C for four days, the colonies formed on the nutrient agar plates were counted and the value for CFU/ml was determined. These growth and extraction tests were carried out in duplicate.

2.4.4. SEM observation

The cross-sections of α -CD–alginate beads with *S. cloacae* were observed by SEM. The bead samples grown on 600mg/l nonylphenol at 25 °C for 0 and 10 days were photographed. The beads were washed with water and mixed by 2% glutaraldehyde for 5 h at 5 °C and 2% osmium tetroxide at room temperature for 2 h. After fixation, the samples were washed twice

with ice-cold 0.1 M cacodylate buffer for 10 min. The mixed beads were subsequently dehydrated in aqueous ethanol stepwise increasing the concentration of 50%, 60%, 70%, 80%, 90%, and 95%, being left for 10 min in each concentration. Finally, dehydration was done twice in 100% ethanol. The beads were then suspended in isopentyl acetate and dried under liquid CO₂ and left for 5 min to replace ethanol gradually in a critical point dryer. The critical temperature and pressure of CO₂ were 31 °C and 70 bar, respectively, and this step was repeated eight times. Subsequently, the resulting beads were coated under vacuum with Pt/Pd and photographed by SEM.

3. Results and discussion

3.1. Adsorption ability of CD–alginate beads

Due to the highly hydrophobic property of nonylphenol, it was reported to be entrapped into the cavities of CD by hydrophobic interaction (Aoki et al., 2003; Fukazawa et al., 2005). The large surface area of calcium alginate beads was expected to possess an ability to adsorb nonylphenol after coupling of CD. Thus, the alginate beads with a diameter of $657 \pm 82 \mu\text{m}$ were successively treated with cyanogen bromide and 6-amino- α -CD to introduce α -CD residues. The resulting beads were packed in a glass column, charged with aqueous suspension of nonylphenol at the flow rate of 0.5 ml/min, allowed to stand for 5 min for a complete adsorption, and then eluted with water and subsequently with methanol. All fractions were monitored by UV absorption at 270 nm as shown in Fig. 1. It was found that nonylphenol was strongly retained in the CD–alginate beads during elution with water and that it was washed out with methanol. These results indicated that CD–alginate beads possessed sufficient ability to adsorb nonylphenol.

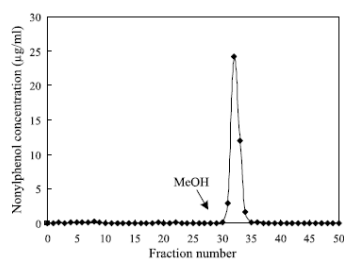


Fig. 1. Elution profiles of nonylphenol on a column of α -CD–alginate beads (DS 0.16)

3.2. Nonylphenol degradation by the freely-suspended and immobilized cells

As shown in Fig. 2, the remaining amount of nonylphenol was time-dependently decreased in the culture medium and the cell numbers increased during 6–7 days. These results suggested that the strain was suitable for the degradation of nonylphenol using α -CD–alginate beads.

Immobilization of the living cell on CD–alginate beads involved some difficulties, since the above-mentioned method for the coupling reaction used a severe toxic agent (cyanogens bromide). Therefore, a homogeneous solution of α -CD–alginate (DS 0.18) (Pluemsab et al., 2005) was mixed with the living cells and then solidified with calcium chloride solution to give a bead with diameters of 1.82 ± 0.11 mm. The typical HPLC chromatograms of before and after 10-day incubation by α -CD–alginate immobilized cells are shown in Fig. 3, in which the major peak of nonylphenol isomers at retention time of 8.30min significantly decreased. The results of nonylphenol biodegradation are summarized in Fig. 4. It showed that *S. cloacae* immobilized on α -CD–alginate beads could degrade nonylphenol more efficiently than that immobilized on alginate beads; the remaining amounts of nonylphenol were 46% and 69%, respectively. These results suggested that CD–alginate was a more efficient material for immobilization of the bacteria and degradation of nonylphenol. Surprisingly, free bacteria in the culture medium rapidly decomposed nonylphenol without any beads.

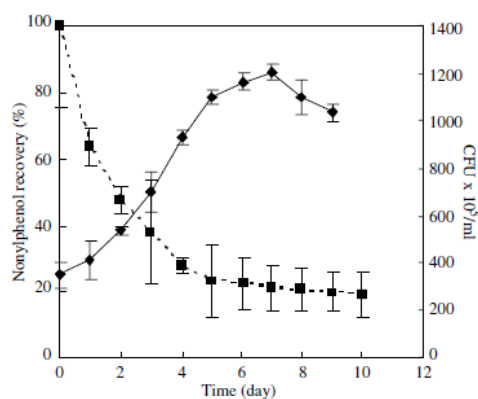


Fig. 2. Percentage nonylphenol remaining undegraded (■) and cell number (◆) in freely-suspended cell cultivation of *Sphingomonas cloacae* after 10-day incubation at 25 ± 2 °C.

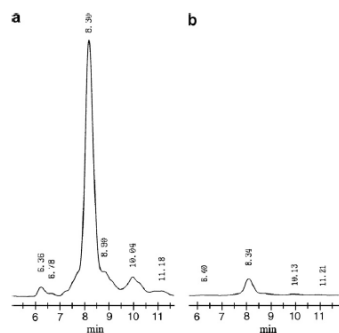


Fig. 3. Typical HPLC chromatogram of nonylphenol extracted from culture media of *S. cloacae* immobilized in α -CD–alginate beads (4% w/v) before (a) and after 10 days (b). Column: Mightysil RP-18GP column (4.6 x 150mm); solvent: acetonitrile 75%; flow rate: 1.0 ml/min; detection wavelength 275 nm.

3.3. Effect of CD on nonylphenol biodegradation

As shown in Fig. 4, addition of 1, 5, and 10 molar equivalents of α -CD to nonylphenol (which referred to 2.7, 13.5, and 27 mM α -CD, respectively) into cultured broths retarded nonylphenol consumption. These data suggested that formation of inclusion complexes of α -CD-nonylphenol in solution negatively effected nonylphenol biodegradation.

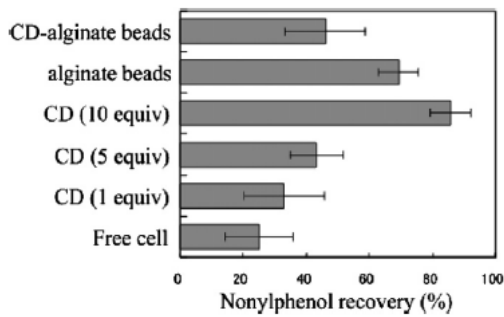


Fig. 4. Nonylphenol remaining undegraded after a 10-day incubation with freely-suspended cells, α -CD–alginate immobilized cells, alginate-immobilized cell and the presence of α -CD.

3.4. Accumulation of nonylphenol on α -CD–alginate beads

The above-mentioned results showed that nonylphenol degraded by immobilized cells was slower than freely-suspended cells, which coincided with the results of Fuji et al. (2000). Apparently the poor solubility and low density (0.95 g/cm^3) of nonylphenol, which seemed like the viscous oil on the surface of the culture medium, was difficult for diffusion into aqueous phase. Thus, the bacterial cells entrapped in the alginate-beads could not decompose nonylphenol efficiently compared to free cells. Hence, the diameter of cultivated tubes changed from 2.5 to 4 cm by maintaining the other incubation conditions, *i.e.*, volume of the culture broth and shaker speed. This resulted in the improved degradation by *S. cloacae* immobilized beads (Table 1). Nonylphenol recovered from the culture media and the beads remaining percentages were 16.6% and 12.3% with α -CD–alginate concentrations of 2% and 4% (w/v), respectively (Table 1).

3.5. CD–alginate morphology

The SEM images of the cross-section views of α -CD–alginate bead before and after 10-day incubation are shown in Fig. 5. Evidently highly microporous network structure was made by fiber-like CD–alginate, where *S. cloacae* cells grew even after incubation of 10 days. Noticeably, these morphological structures of the beads were still neither changed, nor broken. The significantly increased amount of bacterial cells inside the bead after 10-day incubation (Fig. 5D) was one of the important evidences that α -CD–alginate was stable and suitable for use as supporting material for cell immobilization.

In addition, the cell leakage from CD–alginate beads was not detected from the viability checking of culture medium on nutrient agar plates as well as the culture of immobilized alginate beads. The results indicated that CD–alginate was a good matrix for nonylphenol biodegradation. The inclusion of nonylphenol at α -CD cavity of CD–alginate matrix could provide the positive effect of the biodegradation and could reduce the toxicity of high nonylphenol concentration compared to freely-suspended cells.

Table 1 Nonylphenol biodegradation^a by freely-suspended, alginate and α -CD–alginate^b immobilized *S. cloacae* cells

	Alginate (%)	Nonylphenol recovery (%)	Bacterial growth (CFU/ml)
Freely-suspended cells	0	25 ± 4.3	1.3 x 10 ⁸
Alginate (2%)-immobilized cells	2	18.4 ± 0.7	4.0 x 10 ⁷
CD–alginate (2%)-immobilized cells	1.05	16.6 ± 9.0	5.1 x 10 ⁷
CD–alginate (4%)-immobilized cells	2.10	12.3 ± 3.5	2.7 x 10 ⁸

^a Initial cell concentration was $1.75 \pm 0.6 \times 10^6$ CFU/ml. The cultivations were done in wide-diameter tubes (4 x 13 cm) of 5ml yeast nitrogen base broth supplemented with 600mg/l nonylphenol at 25 ± 2 °C on a reciprocal shaker (100 strokes per min) for 10 days.

^b α -CD–alginate (DS 0.18) was used for α -CD–bead preparation.

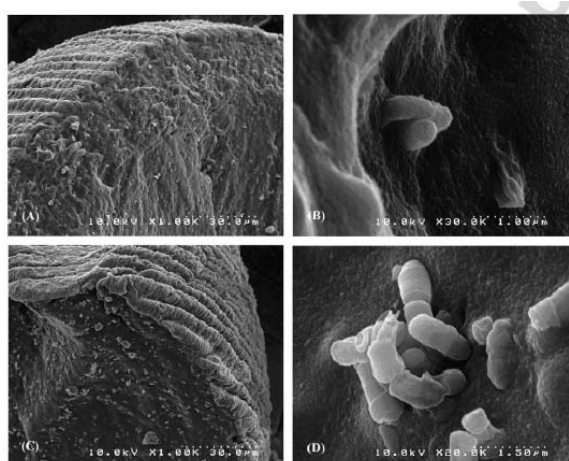


Fig. 5. α -CD–alginate-immobilized *S. cloacae*: (A) surface before incubation, (B) cross-section before incubation, (C) surface after 10-day incubation, (D) cross-section after 10-day incubation.

4. Conclusions

From the study, it could be concluded that the α -CD–alginate matrix showed the strong affinity to nonylphenol as well as the immobilized cells in CD–alginate beads showed more efficient degradation than those in alginate beads. The effect of α -CD on nonylphenol degradation resulted in the inclusion complex formed outside of the beads and retarded the degradation whereas the bead sphere showed the encouragement. α -CD–alginate beads showed the microporous network structures, which resulted from α -CD cross-linking. Significantly increased amount of bacterial cells adhered inside of the bead layer after 10-day incubation compared to initial incubation was one of the evidences that α -CD–alginate was suitable for use as supporting material for cell immobilization.

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