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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 ± 82 μm 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 ± 2 °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 multiplicated 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 (5.43 mg/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 μM *in vitro*, although the activity was 10^{-6} times less than 17β-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 aquaetexotoris* 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.

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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-N-acetylation 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 × 150 mm; Kanto Chemical Co., Inc.). Hitachi L-7400 UV detector was equipped and recorded at 275 nm. Samples were injected via a 1 μl-injection loop and eluted isocratically with acetonitrile–water (75:25 v/v) at a flow rate of 1.0 ml/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 ± 93 μm), 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 ± 82 μm. The resulting alginate beads (0.1 g dry weight) were surface modified by treatment with CNBr (60 mg) 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 100 ml-nutrient broth (Difco, Japan), which was incubated at 25 ± 2 °C for 48 h in a reciprocal shaker (100 strokes per min). Cells were harvested by centrifugation at 3000*g* at 4 °C for 30 min and washed twice with physiological saline solution at pH 7.0. The precipitated bacterial mass was diluted with 10 ml 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 (100 ml) 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 × 13 cm and 2.5 × 13 cm, respectively) of 5 ml yeast nitrogen base (YNB; Difco) broth that contained nonylphenol at a concentration of 600 mg/l. All