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Biodegradation of coumaphos, chlorferon, and diethylthiophosphate using bacteria immobilized in Ca-alginate gel beads

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ABSTRACT

Calcium-alginate immobilized cell systems were developed for the detoxification and biodegradation of coumaphos, an organophosphate insecticide, and its hydrolysis products, chlorferon and diethlythiophosphate (DETP). Optimum bead loadings for bioreactor operation were found to be 200 g-beads/L for chlorferon degradation and 300 g-beads/L for DETP degradation. Using waste cattle dip (UCD) solution as substrate, the degradation rate for an immobilized consortium of chlorferon-degrading bacteria was five times greater than that for freely suspended cells, and hydrolysis of coumaphos by immobilized OPH$^+$ *Escherichia coli* was 2.5 times greater. The enhanced degradation of immobilized cells was due primarily to protection of the cells from inhibitory substances present in the UCD solution. In addition, physiological changes of the cells caused by Ca-alginate immobilization may have contributed to increased reaction rates. Degradation rates for repeated operations increased for successive batches indicating that cells became better adapted to the reaction conditions over time.

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1. Introduction

Immobilization of microorganisms offers several advantages over freely suspended cells in bioreaction systems, including easy separation of cells from the reaction medium, repeated use of cells, and avoidance of cell washout during continuous fermentation at high dilution rates (Willaert and Baron, 1996). In addition, higher volumetric reaction rates may be obtained with immobilized cells as a result of their higher local cell concentration (Lee et al., 1994; Rhee et al., 1996; Manohar et al., 2001) or altered cell permeability that allows better transfer of substrate into the cell (Manohar et al., 2001; Kim et al., 2002). Immobilized cells may have enhanced tolerance to toxic compounds as compared to purified enzyme or other processed biological materials (Lee et al., 1994; Dwyer et al., 1986; Keweloh et al., 1989; Westmeier and Rehm, 1985). Calcium-alginate cross-linking is one of the most commonly used immobilization methods because the procedure is simple, relatively mild and does not have toxic effects on the cells; however, the material is susceptible to biodegradation and has relatively low mechanical stability.

Coumaphos ('Co-Ral' formulation) is an organophosphate insecticide used predominantly by the US Department of Agriculture Animal and Plant Health Inspection Services (USDA-APHIS) for its tick eradication program along the US-Mexico border. Cattle imported from Mexico or coming from the quarantine zone in south Texas are dipped in vats at 42 different locations that contain approximately 15 m^3 of coumaphos solution in which the concentration is about 1500 mg/L (Karns et al., 1995). This process generates approximately 500 m^3 of concentrated waste annually that must be removed, treated, and discarded as "detoxified waste".

Coumaphos can be hydrolyzed to chlorferon and DETP by organophosphate hydrolase (OPH, EC 3.1.8.1) enzyme. Kim et al. (2002) previously investigated coumaphos hydrolysis using recombinant *Escherichia coli* as freely suspended cells and immobilized in PVA cryogel. The degradation rate with immobilized *E. coli* was twice that of free cells using the waste cattle dip solution as substrate. Isolation and enrichment of mixed cultures responsible for degrading chlorferon and DETP have been reported elsewhere (Ha et al., 2007). The objectives of the current study were to develop systems for degradation of chlorferon and DETP using the enriched cultures immobilized in Ca-alginate gel beads, and to compare the results for hydrolysis of coumaphos using a recombinant *E. coli* strain immobilized in alginate with results reported previously for the same strain immobilized in PVA cryogel (Kim et al., 2002).

2. Methods

2.1. Cattle dip vat solution and chemicals

Untreated cattle dip (UCD) waste solution was obtained from the APHIS dipping vats in Laredo, TX. Analytical grade chlorferon and coumaphos were gifts from Bayer Corporation (Animal Health Division, Merriam, KS). Chlorferon solution for degradation studies

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was also produced by hydrolyzing the coumaphos in UCD solutions using a recombinant *E. coli* strain containing the OPH enzyme immobilized in Ca-alginate beads. Hydrolysis of coumaphos was done at the optimum conditions (biomass concentration 70 g/L, reaction pH 8, and temperature 30 °C) found by Kim et al. (2002). The potassium salt of DETP (DETP–K) was obtained from Sigma and used as a standard for DETP. Sodium alginate (65–70% guluronic acid and 25–35% mannuronic acid) was obtained from Sigma.

2.2. Sample analysis method

Chlorferon and DETP were analyzed by HPLC using C-18 and C-8 columns, respectively. The mobile phase was HPLC grade acetonitrile with 0.75 mM phosphoric acid (80:20, v/v) at a flowrate of 1 ml/min. Absorbance was measured at 320 nm for chlorferon and 210 nm for DETP.

2.3. Cultivation of microorganisms and cell immobilization method

Microbial consortia responsible for degrading either chlorferon or DETP were enriched from UCD solution as described by Ha et al. (2007). The enriched culture solutions served as inocula to produce biomass. Cells were grown in a rich medium (glutamate–yeast extract) containing the target compound in a rotary shaking incubator (200 rpm) at 30 °C. Biomass was harvested during the late exponential phase by centrifugation at 4000 rpm at 4 °C for 15 min. The pellet obtained from centrifugation was washed with potassium phosphate buffer (50 mM, pH 7.2) and centrifuged again at the same conditions. The resulting pellet was then resuspended in reaction medium containing the target compound, 10 mg/L of chlorferon or 100 mg/L of DETP, for acclimation and incubated in a rotary shaking incubator (200 rpm) at 30 °C until the target compound was completely degraded. After complete degradation, biomass was harvested again as described above and immobilized for degradation studies. Without cell acclimation, a week-long lag period was required before degradation was initiated. A recombinant *E. coli* strain containing the native OPH enzyme (Dave et al., 1993) was used to produce biomass for coumaphos degradation according to the procedure of Kim et al. (2002).

Biomass was prepared separately for each target compound and was resuspended in 2% (w/v) sodium alginate solution to a final cell concentration of 80 g/L for chlorferon-degrading organisms, 60 g/L for DETP-degrading organisms, or 70 g/L for OPH^+ *E. coli* which were the optimum biomass concentrations for freely suspended cells determined in previous studies (Ha et al., 2007; Kim et al., 2002). The alginate-cell suspensions were then added dropwise to well-stirred, sterilized 2% (w/v) $CaCl_2$ solutions using a syringe. Calcium-alginate gel beads having a spherical shape (1.65–1.7 mm in radius) were formed instantly. Beads were left in $CaCl_2$ solution with gentle stirring for 4 h to allow them to harden. They were then washed with DI water. Before use for degradation studies, bead surfaces were blotted dry with paper towels. Because of the instantaneous formation of cross-linked alginate on the surface of the droplets, all cells were assumed to be trapped within the beads and no measurements were taken to determine if any cell loss occurred.

2.4. Reaction system

Degradation studies using immobilized cells were performed in a 1.25 L BioFlo II fermentor system (New Brunswick Scientific Co., Inc., Edison, NJ) with a working volume of 0.3 L at the optimum conditions determined for each compound. Temperature was maintained at 30 °C. The reaction medium was agitated at 200 rpm by a flat bladed turbine impeller. Aeration was not provided to the system.

2.5. Degradation of chlorferon and DETP using immobilized cells

Experiments were conducted to determine optimum bead loading using loadings of 100, 200, 300, 400, and 500 g-beads/L. Bead loading greater than 500 g-beads/L could not be evaluated because the beads were not completely suspended in the liquid medium. For chlorferon degradation, the reaction medium was prepared by diluting hydrolyzed UCD solution, which contained chlorferon at a concentration of about 600 mg/L, with mineral salt (MS) medium to provide a final chlorferon concentration of 50 mg/L. The lower concentration was needed to prevent inhibition of the organisms. For DETP degradation studies, MS medium containing 10 mg/L of yeast extract (MSYE) with 250 mg/L of DETP was used. DETP produced from hydrolysis of coumaphos in UCD solution could not be used for this study because DETP degradation was inhibited by the chlorferon present. Reaction pH was adjusted to 7.5 for both compounds.

2.6. Hydrolysis of coumaphos using immobilized OPH^+ *E. coli*

Studies for coumaphos hydrolysis using OPH^+ *E. coli* were conducted using freely suspended cells and cells immobilized in Ca-alginate beads to compare with the results for immobilization in PVA cryogel reported by Kim et al. (2002). Experiments were conducted using optimum conditions found by Kim et al. (2002). The reaction medium was either UCD solution or pure coumaphos dissolved in methanol added to CHES buffer solution (100 mM, pH 8). Initial coumaphos concentrations for experiments in pure solution and UCD solution were 500 mg/L and 1800 mg/L, respectively. Experiments using free cells were performed under batch reactor conditions in 250 ml Erlenmeyer flasks with a working volume of 50 mL. Biomass concentration in the suspension was 70 g/L. Experiments using immobilized cells were conducted in the BioFlo II fermentor system described above. Cell concentration in the beads was 70 g/L, and a bead loading of 200 g-beads/L was used.

3. Results and discussion

3.1. Degradation of chlorferon and DETP using immobilized cells

The optimum bead loadings for degradation of chlorferon and DETP were determined as the bead loadings giving both a high volumetric reaction rate (defined as the mass degradation rate of the target compound per unit total reaction volume) and a high specific reaction rate (defined as the mass degradation rate per unit mass of biomass). Optimum loadings were found to be 200 g-beads/L for chlorferon (Fig. 1a) and 300 g-beads/L for DETP (Fig. 1b).

The volumetric reaction rate for chlorferon degradation at the optimum bead loading of 200 g-beads/L was 2.45 mg/L h. At this loading, the concentration of immobilized chlorferon-degrading organisms was 15 g-biomass/L in the total working volume. From data reported elsewhere (Ha et al., 2007), the volumetric reaction rate for freely suspended cells at this biomass concentration was estimated to be 0.49 mg/L h; therefore, the volumetric reaction rate for chlorferon degradation was about five times greater with immobilized cells (Fig. 2a). Similar results were found in comparing specific reaction rates for free cells (0.034 mg/g-biomass h) and immobilized cells (0.163 mg/g-biomass h). The beads were prepared using the optimum concentration determined for free cells reported elsewhere (Ha et al., 2007) as the concentration within the beads.

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