Biodetoxification of Coumaphos Insecticide Using Immobilized *Escherichia coli* Expressing Organophosphorus Hydrolase Enzyme on Cell Surface

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Abstract Recently, we reported an improved technology for the degradation of organophosphate nerve agents using whole cells of genetically engineered *Escherichia coli* that anchored and displayed the enzyme organophosphorus hydrolase on the cell surface. In this paper we report the immobilization of these cells on highly porous sintered glass beads and the subsequent application of the immobilized cell in a continuous-flow packed bed bioreactor for the biodetoxification of a widely used insecticide, coumaphos.

Keywords: OPH, pesticides, nerve agents, bioreactor, porous sintered glass beads

INTRODUCTION

Neurotoxic organophosphates (OPs), amongst the most toxic compound known, are widely used as pesticides, insecticides and chemical warfare agents. In the United States annually over 40 million kg of organophosphate pesticides are consumed [1] while another 20 million kg are produced for export [2]. Chlorpyrifos (lorsban) and parathion are two of most popular pesticides used for agriculture crop protection with more than 13 and 7 million pounds consumed in 1995, respectively [3]. Organophosphates such as diazinon, chlorpyrifos (dursban), and malathion are also used heavily for industrial and home applications. The use of these pesticides, though very important to the success of the agricultural industry, affects the environment. There is now growing concern regarding (1) the safe disposal of large volume of organophosphate wastes generated from the washing of pesticide holding tanks and application machinery used by farmers; (2) the safety of the workers coming in contact with these chemicals, whether in everyday use or when thrust upon in response to emergency resulting from spills, fires or illegal misuse; and (3) cleanups forced upon by the illegal use for extermination of indoor pests by unscrupulous exterminators.

One of the most serious decontamination problems in the US involves the use of the organophosphorus insecticide coumaphos by the US Department of Agriculture (USDA) [4]. The Veterinary Services Branch of the USDA's Animal and Plant Health Inspection Service (APHIS) operates a Tick Eradication Program designed to prevent the re-introduction of Cattle Fever into the United States through ticks (*Boophilus microplus* and *Boophilus annulatus*) on cattle imported from Mexico. The primary tool used in the eradication program is a series of dipping vats placed at the border crossing points. Cattle coming in from Mexico must be dipped in vats before entering the US. Currently, coumaphos is the pesticide of choice for this application. The operation on the US side employs 42 vats each containing about 15,000 liters of coumaphos at a level of about 2,000 mg/L. Annually, these operations generate about 460,000 L of high-level coumaphos wastes that must be properly disposed.

The current disposal method for spent coumaphos dips is to pump them into evaporation pits or waste lagoons located adjacent to the dip vats. Since many of the pits are unlined, this resulted in contamination of the underlying soils with high concentrations of coumaphos. Incineration, which is only EPA-approved method for destruction, has met serious public opposition because of potentially toxic emissions and is economically restrictive. Clearly, there is a grave need for alternative, economical, and reliable methods for detoxification/destruction of coumaphos wastes. These coumaphos-containing dip wastes are ideal for disposal by biodegradation since these wastes are concentrated, contained, and have no other toxic compounds.

Biological detoxification of OP nerve agents based on organophosphorus hydrolase (OPH) have been studied as a potential means of "environmentally friendly" decontamination. Systems using immobilized purified OPH and immobilized *Escherichia coli* cells harboring the *opd* gene expressing OPH intracellularly have been investigated [5,6]. While the immobilized enzyme sys-

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tems are economically restrictive due to the cost of purifying the enzyme and its instability in purified form, the immobilized cell system is limited by the mass transport of the substrate and product across the cell wall membrane. In order to alleviate the above problems, we recently anchored and displayed active OPH on the cell surface of *E. coli* using an Lpp-OmpA (46-159) fusion system [7]. Cultures with surface-expressed OPH degraded parathion and paraoxon very effectively without the diffusional limitation observed in cells expressing OPH intracellularly, and also exhibited a very long shelf-life, retaining 100% activity over a period of one month [8]. Cells with active OPH on the surface were subsequently immobilized on non-woven polypropylene fabric and applied for hydrolysis of a number of OP pesticides and insecticides in sequence batch reactor [9]. This paper report the immobilization of the recombinant *E. coli* cells with OPH on the cell surface in porous sintered glass beads and the application of the immobilized cells for detoxification of coumaphos in a continuous-flow packed bed reactor.

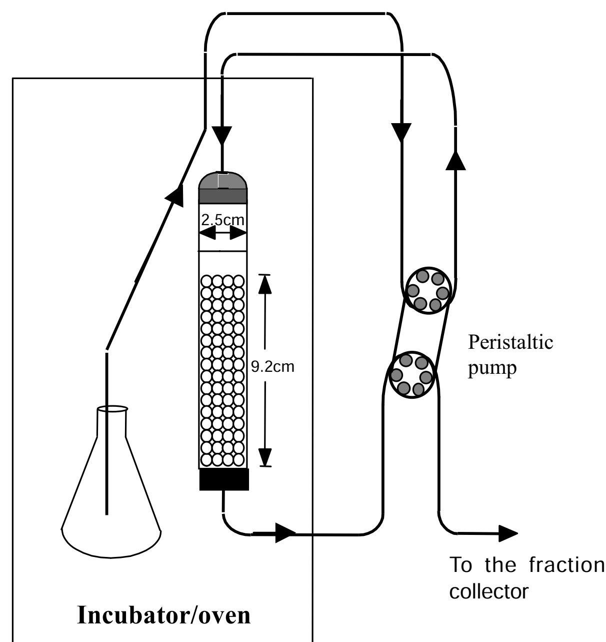

Fig. 1. Schematic diagram of the experimental set-up for the immobilized cell bioreactor.

MATERIALS AND METHODS

Reagents and Materials

Luria-Bertani (LB) media, potassium monobasic phosphate, sodium citrate, potassium dibasic phosphate and cobalt chloride were purchased from Fisher Scientific (Tustin, CA, USA). Paraoxon, polyoxyethylene 10 lauryl ether (POLE) were obtained from Sigma Chemical Co. (St. Loius, MO). Coumaphos was a gift from Bayer Co. SIRAN, 60-300 μm pore size and 2-3 mm diameter were obtained from Jaeger Biotech Engineering Inc. (Costa Mesa, CA).

Bacterial Strains and Plasmids

E. coli strain XL1-Blue (*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17 (r_k^-, m_k^+), *supE*44, *relA*1, *lac*[*F' proAB, lacI*^{q} ZDM15, Tn10 (Tet^r)]) carrying plasmid pOPK132 was used for expressing Lpp-OmpA-OPH on the cell surface [5]. Expression of OPH is tightly regulated by a *tac* promoter due to the presence of the *lacI*^{q} gene on the plasmid.

Growth Conditions and Cell Preparation

Strain bearing plasmid pOPK132 was grown in 50 mL LB media buffered to pH 7.0 with 0.017 M KH_2PO_4 and 0.072 M K_2HPO_4, supplemented with 100 μg/mL ampicilin in 250-mL Erlenmeyer flask at 37°C and 300 rpm on an orbital incubator-shaker (Innova 4000, New Brunswick Scientific, Edison, NJ, USA). Once the OD_{600} of cell suspension reached 0.5, the culture was induced for the expression of OPH on the cell surface with 1 mM IPTG. One mM $CoCl_2$ was added to the culture 24 h after induction. The cells were harvested 48 h after growth by centrifugation at 5,000 × g for 10 min, washed with pH 8.0, 50 mM citrate-phosphate + 100 mM NaCl + 0.05 mM $CoCl_2$ buffer (henceforth designated as buffer A) twice and resuspended in buffer A.

Preparation of Immobilized Cell Bioreactor

One and a half gram (wet weight) of cells suspended in 70 mL of buffer A was circulated overnight through a 2.5 × 20 cm column (Kimble, Vineland, NJ), packed with 20 g of SIRAN glass beads (bed height of approximately 9 cm) at room temperature and a flow rate of 23 mL/h using a peristaltic pump (EVA Pump, Eppendorf, Milwaukee, WI, USA). The OD_{600} of cell suspension was measured before and after cell loading as an indicator of cell loading. After draining the cell suspension from the column, glutaraldehyde solution of different concentration (in buffer A) was circulated through the column for 2 h at room temperature and 23 mL/h flow rate to crosslink the cells. The column was then washed overnight at room temperature and a flow rate of 23 mL/h. The column was conditioned to the working temperature of 37°C for 2 h prior to detoxification experiments.

Detoxification of Coumaphos in Continuous Flow Immobilized Cell Packed Bed Bioreactor

Fig. 1 shows the schematic of the experimental set-up used in the research. Detoxification experiments were started by introducing a feed from the top of the buffer filled bioreactor at a desired rate by a precision flow multihead peristaltic pump (EVA Pump, Eppendorf, Milwaukee, WI, USA) and withdrawing the effluent

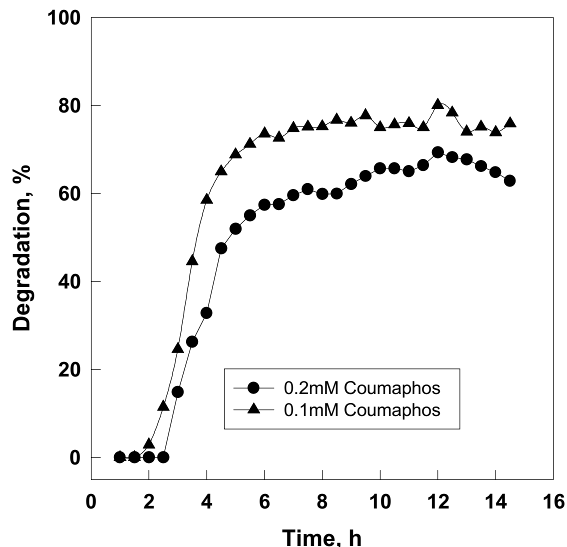

Fig. 2. Degradation of different concentrations of coumaphos prepared in buffer A supplemented with 5% methanol by the immobilized cell bioreactor. Operating conditions: Flow rate 10 mL/h; temperature: 37°C.

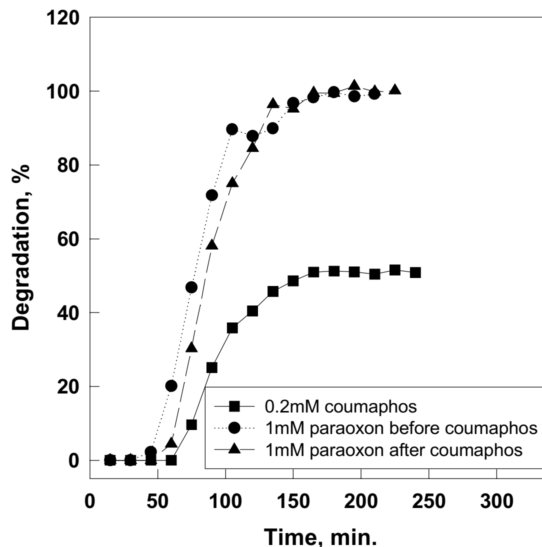

Fig. 3. Evaluation of the stability of the immobilized cells. Operating conditions: Buffer A plus 5% methanol; flow rate: 23 mL/h; temperature: 37°C.

stream at the same rate from the bottom by the second head of the same multihead peristaltic pump. The effluent stream, collected in 2 mL aliquots using a fraction collector (Pharmacia LKB, Upsala, Sweden), was subsequently analyzed for the product of hydrolysis/degradation by measuring the absorbance spectrophotometrically (Cary 1E, Varian, Melbourne, Australia) at 410 nm for *p*-nitrophenol and 348 nm for chlorferon, hydrolysis products of paraoxon and coumaphos, respectively [10].

RESULTS AND DISCUSSION

The degradation of higher pesticide concentration solutions is advantageous, as it will reduce the volume of wastewater to be processed. Since organophosphate pesticides are not very soluble in aqueous solution, organic solvent is generally added to increase their solubility in aqueous medium. In the first attempt to evaluate the hydrolysis of coumaphos by the immobilized cells in a continuous flow bioreactor, up to 0.2 mM coumaphos solution in pH 8.5 citrate-phosphate buffer with 5% methanol at a flow rate was used. As the data in Figs. 2 and 3 show, at steady-state only 66% and 50% of coumaphos was degraded at the flow rates of 10 mL/h (residence time 1 h) and 23 mL/h (residence time 0.435 h), respectively. When compared to paraoxon degradation (100% for a 1 mM feed concentration at similar flow rates), these degradation numbers were very low. The reduction of the enzyme activity due to exposure to higher concentration of the coumaphos in the feed or the chlorferon produced during hydrolysis was suspected to be the reason for this low degradation efficiency. In order to investigate this possibility, paraoxon degradation before and after coumaphos degradation was evaluated. As shown in Fig. 3, the enzyme activity for paraoxon hydrolysis was not affected by the exposure to coumaphos and chlorferon. A much closer look of the experimental setup during the course of the coumaphos hydrolysis revealed a white powder coating the walls of the tubing connecting the feed vessel to the bioreactor. The white powder coating, subsequently associated to coumaphos precipitation because of the low solubility, limited the supply of the coumaphos to the immobilized cells and therefore the estimated coumaphos degradation.

Surfactants have been used in many applications to increase the solubility of hydrophobic organic compounds [11,12]. Ionic surfactants such as SDS improve the solubility but are detrimental to the cell walls and enzymes. Nonionic surfactants are less severe on the cell wall and the enzyme. One such surfactant, polyoxyethylene 10 lauryl ether (POLE), was demonstrated to be useful in increasing the solubility of biphenyl without damaging the cell integrity [13,14]. Effect of different concentration of this surfactant on the *E. coli* cells and the OPH enzyme was investigated. As shown in Fig. 4 up to 2% of POLE added to the buffer did not affect the steady-state paraoxon hydrolysis by the immobilized cell bioreactor. However, there was a very small effect on the overall rate of hydrolysis over the control (0% POLE) at 2% POLE. Therefore, in future studies POLE at 1% in conjunction with 5% methanol was added to the buffer to improve coumaphos solubility. Using these additives to the buffer we were able to additives dissolve up to 0.3 mM of coumaphos in the buffer without any precipitation on the tubing walls.

The dynamic and steady-state degradation of 0.1 to 0.3 mM coumaphos at a flow rate of 23 mL/h using immobilized cell bioreactor is shown in Fig. 5. Ap-

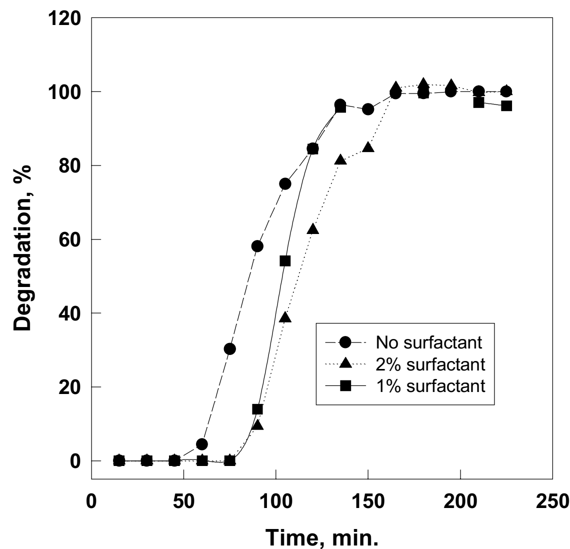

Fig. 4. Effect of POLE concentration on the biodetoxification of paraoxon using the immobilized cell bioreactor. Operating conditions: Buffer A plus 5% methanol and appropriate POLE amount; flow rate: 23 mL/h; temperature: 37ºC.

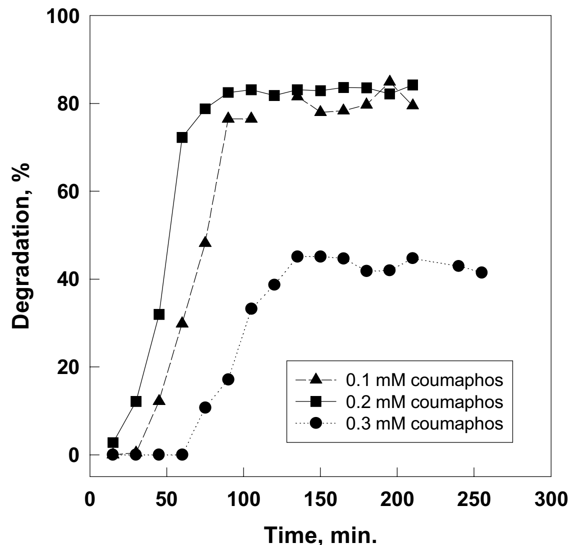

Fig. 5. Degradation of different concentration of coumaphos using immobilized cell bioreactor. Operating conditions: Buffer A plus 5% methanol and appropriate POLE amount; flow rate: 23 mL/h; temperature: 37ºC.

proximately 80% of coumaphos was hydrolyzed up to 0.2 mM which dropped down to 45% when the concentration was increased to 0.3 mM. The degradation efficiency of the immobilized cell bioreactor can be improved by either lowering the flow rate in order to increase the residence time, increasing the loading of the cells in the column to increase the catalytic capacity or improving the catalytic efficiency (increasing V_{max} and/or lowering K_M) of the enzyme expressed on the cell surface. Research to improve the maximum catalytic rate and lowering the enzyme Michaelis-Menten constant is presently being conducted in our laboratory.

In conclusion, *E. coli* cells expressing the organophosphate hydrolase on the cell surface was immobilized on highly porous glass beads and used in a continuous flow packed bed reactor to detoxify coumaphos pesticide. A nonionic surfactant, polyoxyethylene lauryl ether at 1% in conjunction with 5% methanol was used to increase the solubility of coumaphos in aqueous medium to treat higher coumaphos concentration solutions.

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