Detoxification of Organophosphate Nerve Agents by Immobilized *Escherichia coli* with Surface-Expressed Organophosphorus Hydrolase

Ashok Mulchandani, Irina Kaneva, Wilfred Chen

Department of Chemical and Environmental Engineering, University of California, Riverside, California 92521; telephone: 909-787-6419; fax: 909-787-3188; e-mail: adani@engr.ucr.edu

Received 17 June 1998; accepted 14 October 1998

Abstract: An improved whole-cell technology for detoxifying organophosphate nerve agents was recently developed based on genetically engineered *Escherichia coli* with organophosphorus hydrolase anchored on the surface. This article reports the immobilization of these novel biocatalysts on nonwoven polypropylene fabric and their applications in detoxifying contaminated wastewaters. The best cell loading (256 mg cell dry weight/g of support or 50 mg cell dry weight/cm^2 of support) and subsequent hydrolysis of organophosphate nerve agents were achieved by immobilizing nongrowing cells in a pH 8, 150 m*M* citrate-phosphate buffer supplemented with 1 m*M* Co^{2+} for 48 h via simple adsorption, followed by organophosphate hydrolysis in a pH 8, 50 m*M* citrate-phosphate buffer supplemented with 0.05 m*M* Co^{2+} and 20% methanol at 37°C. In batch operations, the immobilized cells degraded 100% of 0.8 m*M* paraoxon, a model organophosphate nerve agent, in approximately 100 min, at a specific rate of 0.160 m*M* min^{-1} (g cell dry wt)$^{-1}$. The immobilized cells retained almost 100% activity during the initial six repeated cycles and close to 90% activity even after 12 repeated cycles, extending over a period of 19 days without any nutrient supplementation. In addition to paraoxon, other commonly used organophosphates, such as diazinon, coumaphos, and methylparathion were hydrolyzed efficiently. The cell immobilization technology developed here paves the way for an efficient, simple, and cost-effective method for detoxification of organophosphate nerve agents. *Biotechnol Bioeng* **63**: 216–223, 1999.
Keywords: OPH; pesticides; biodegradation; bioreactor

INTRODUCTION

Organophosphorus (OP) compounds, which are among the most toxic substances known, are used as pesticides, insecticides, and nerve gases (Chapalamadugu and Chaudhry, 1992; Compton, 1988; Donarski et al., 1989; FAO, 1989; USDA, 1992). These compounds act by inhibiting the activity of acetylcholinesterase (AChE), resulting in acetylcholine (AChE) accumulation, which interferes with muscular responses, and in vital organs produces serious symptoms and eventually death (Donarski et al., 1989).

In the United States over 40 million kilograms of organophosphate pesticides are consumed (FAO, 1989), while another 20 million kilograms are produced annually for export (USDA, 1992). Although the use of these pesticides is very important to the success of the agricultural industry, there is now growing public concern regarding their contamination in food products and water supplies, particularly the large quantity of pesticide wastes, such as: (1) excess or unused pesticide residues remaining in their original containers; and (2) dilute aqueous pesticide solution generated by the pesticide producer and consumer resulting from washing of storage tanks and spraying equipment (Munnecke, 1980). One of the more serious problems is the approximately 400,000 L of cattle dip wastes, containing approximately 1500 mg/L of the organophosphate insecticide, coumaphos, that are generated yearly along the U.S.–Mexico border under a USDA program designed to control the spread of cattle fever through ticks on disease-carrying cattle imported from Mexico or on cattle in the areas of the U.S. where there is a likely exposure to ticks from Mexico (Mulbury et al., 1996). Additionally, large stockpiles of extremely toxic organophosphate-based nerve gases such as sarin, soman, and VX, stored around the world, have to be destroyed by the year 2007.

Organophosphorus hydrolase (OPH) has been isolated and shown to effectively hydrolyze organophosphate pesticides (P—O bond hydrolysis) and degrade other organophosphates (P—F or P—CN bond cleavage), including those utilized in nerve gases (Dumas et al.,1989a, 1990). Enzymatic hydrolysis rates are 40 to 2450 times faster than chemical hydrolysis, and the activity of this enzyme has been reported to be stable at temperatures up to 45° to 50°C (Munnecke, 1979). The reaction products from hydrolysis by OPH are generally of greatly reduced toxicity relative to the parent compounds (Lai et al., 1994; Yakovlevsky et al., 1997).

Both native and recombinant OPHs, immobilized onto

Correspondence to: A. Mulchandani
Contract grant sponsors: U.S. EPA; NSF
Contract grant numbers: R823663-01-0; BES9731513

 CCC 0006-3592/99/020216-08

nylon (membrane, powder, and tubing) (Caldwell and Raushel, 1991a), porous glass, and silica beads (Caldwell and Raushel, 1991b), have been applied as enzyme reactors for the detoxification of organophosphate pesticides. These processes are economically restrictive because of the high cost associated with purifying OPHs. This problem can be eliminated if whole cells (either growing or nongrowing), rather than enzymes, are immobilized onto the support (such as in an immobilized-cell bioreactor). However, the use of immobilized cells in a bioreactor does have disadvantages. One serious potential problem is the mass transport limitation of substrates and products across the cell membrane, which can act as a permeability barrier and inhibit substrates from interacting with the enzymes residing within the cell. Several recent reports have shown that the uptake of OP nerve agents is indeed the rate-limiting step in the degradation of these pesticides by whole cells expressing OPH intracellularly (Elashvili and DeFrank, 1996; Elashvili et al., 1998; Hung and Liao, 1996; Richins et al., 1997). Similar observations of the resistance to mass transport of substrates for other enzymes present in the cytoplasm of *E. coli* have been reported by others (Martinez et al., 1996). The resistance to mass transport can be reduced by treating cells with permeabilizing agents such as EDTA, DMSO, tributyl phosphate etc. However, not all enzymes are amenable to this treatment, and immobilized viable cells cannot be subject to permeabilization.

Recently, active OPH was successfully anchored and displayed onto the cell surface of *E. coli* using an Lpp–OmpA (46–159) fusion system (Richins et al., 1997). 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 1 month in resting state in buffer without any nutrients (Chen and Mulchandani, 1998).

Practical application of cells expressing OPH on the cell surface necessitates that the cells be immobilized. Immobilized cell systems have the potential to degrade toxic chemicals faster than conventional wastewater treatment systems, because high densities of toxic-chemical degrading bacteria are used in immobilized cell systems, which are responsible for: (1) increased metabolic activity and metabolite production; (2) protection from toxic substances; and (3) increased plasmid stability (Cassidy et al., 1996). In the case of the recombinant *E. coli* expressing OPH on the cell surface, immobilization will also make the repeated usage of the same cells over a long period feasible. Chemical crosslinking using glutaraldehyde/polyglutaraldehyde is a widely used method for cell immobilization. However, this immobilization method when tried on *E. coli* cells displaying β-lactamase on the cell surface resulted in significant 84% to 87% (for glutaraldehyde) and 45% to 65% (for polyglutaraldehyde) reductions of original β-lactamase activity (Freeman et al., 1996). A similar significant reduction in OPH activity was also observed on crosslinking with glutaraldehyde (unpublished results). In this study, we report a very simple and efficient method for the immobilization of *E. coli* cells expressing OPH on the cell surface onto solid supports and demonstrate that the immobilization of these cells onto solid supports can provide an attractive and economical means for detoxification of organophosphate nerve agents in place of immobilized enzymes or immobilized whole cells expressing OPH intracellularly, affording no diffusional barrier, lowered labor cost, and the potential for easy regeneration.

MATERIALS AND METHODS

Reagents and Materials

Luria–Bertani (LB) medium, potassium monobasic phosphate, sodium citrate, potassium dibasic phosphate, cobalt chloride, and nichrome resistance wire were purchased from Fisher Scientific (Tustin, CA). Paraoxon was obtained from Sigma Chemical Co. (St. Louis, MO). Parathion, methyl parathion, and diazinon were obtained from Supelco Inc. (Bellefonte, PA, USA). Coumaphos was a gift from Bayer Corp. (Merriam, KS). Nonwoven fabric, 909 PP white, was a gift from Texel Inc. (St. Elzear, Canada).

Bacterial Strains and Plasmids

E. coli strains JM105 (*end*A1, *thi*, *rps*L, *sbc*B15, *hsd*R4, D(*lac-pro*AB), [F′, *tra*D36, *pro*AB, *lac*I^{q}ZDM15]) and XL1-Blue (*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_k^-, m_k^+), *sup*E44, *rel*A1, *lac*[F′ *pro*AB, *lac*I^{q}ZDM15, Tn10 (Tet^r)]) were used in this study.

Plasmid pOPK132 was used for expressing Lpp–OmpA–OPH on the cell surface (Richins et al., 1997). Expression of OPH is tightly regulated by a *tac* promoter due to the presence of the *lacI*^{q} gene on the plasmid.

Growth Conditions

E. coli cells XL1-Blue bearing plasmid pOPK132 were grown in buffered LB media (pH 7.0) supplemented with 100 μg/mL ampicillin at 37°C. After 48 h of growth, cells were harvested by centrifugation at 5000*g* for 10 min, washed with 150 m*M* NaCl twice, and then resuspended in 20 mL of pH 8, 150 m*M* citric acid–Na_2HPO_4 buffer. For the cells that were induced for OPH expression, 1 m*M* IPTG was added to the culture once the OD_{600} of cell suspension reached 0.5. Twenty-four hours after induction, $CoCl_2$ was added to 1 m*M* final concentration.

Cell Immobilization

Square (2.5 × 2.5 cm) pieces of 909 PP white-cell immobilization matrix were dried to constant weight at 105°C in an oven (Model 130DM, Thelco laboratory oven, Precision

Scientific). Each piece was then attached to a nichrome resistance wire (D24, Arcor), weighed, and suspended into a 500-mL Erlenmeyer flask containing 200 mL of either cell growth medium or pH 7 to 8.5, 150 m*M* citric acid –Na_2HPO_4 buffer with 0.05 to 1 m*M* $CoCl_2$ and sterilized by autoclaving at 121°C for 20 min. Flasks containing the growth medium were inoculated with a 4% (v/v) inoculum of the exponentially growing preinoculum, while the flasks with buffer were seeded with a suspension of washed cells to give a final concentration of 2 g wet weight/L. Cell immobilization was allowed to proceed for 24 to 48 h by incubating at 37°C and 110 rpm on the incubator shaker (Innova 4340, New Brunswick Scientific, Edison, NJ). The immobilized cell supports were subsequently used to either determine the dry weight of immobilized cells or for organophosphate degradation studies. To determine the dry weight of the immobilized cells, supports (with suspending wire) were removed from the flask, placed on a preweighed filter paper on a Büchner funnel, washed thoroughly with deionized water to remove entrapped salts and nutrients, and dried to a constant weight at 105°C overnight in an oven (Model 130DM Thelco laboratory oven).

Degradation of Pesticide by Immobilized Cells

Supports with immobilized *E. coli* cells were suspended in 100 mL of 50 m*M* (unless otherwise as stated) sterilized citric acid–Na_2HPO_4 buffer (pH 8.0) supplemented with 0.05 m*M* $CoCl_2$, 5% methanol (unless indicated) and known OP concentration, 1 m*M* paraoxon, 0.55 m*M* methyl parathion, 0.022 m*M* coumaphos, or 1.67 m*M* diazinon. The contents of the flask were incubated on an incubator shaker (Innova 4340, New Brunswick Scientific) at 37°C and 110 rpm. The hydrolysis/degradation of organophosphates was analyzed by measuring the absorbance of the hydrolyzed products spectrophotometrically (Cary 1E, Varian, Melbourne, Australia) at 410 nm for paraoxon and methyl parathion, 224 nm for diazinon, and 348 nm for coumaphos (Dumas et al., 1989b).

Reculturing of Cells Adsorbed on Support Matrix

For the reculturing of cells already immobilized onto the support by adsorption, nonwoven fabric with immobilized cells were placed into buffered LB media, supplemented with ampicillin, and incubated on a shaker (Innova 4340) for 1 day at 37°C and 110 rpm. IPTG was added to a final concentration of 1 m*M* 20 h after the start of culturing followed by 1 m*M* Co^{2+} 44 h after the start of reculturing. The cell loaded supports were subsequently either dried overnight to determine dry cell mass or placed in the citric acid–Na_2HPO_4 buffer solution for pesticide degradation.

Scanning Electron Microscopy

The 909 PP polyester mat with immobilized *E. coli* cells was washed with 0.1 *M* phosphate buffer (pH 7.0), and then soaked in 2% w/v glutaraldehyde solution overnight to fix the cells. It was washed five times with 0.1 *M* phosphate buffer (pH 7.0), followed by a secondary fixation with 1% w/v osmium tetroxide (1 h at 4°C) and consecutive washing with phosphate buffer. The fixed sample was dehydrated by successive treatments in 50%, 70%, 80%, 90%, 95% (% v/v) ethanol solutions for 10 min and twice for 60 min in 100% ethanol at room temperature to ensure complete dehydration of the sample. The ethanol was removed by a critical point dryer. The mat was then cut and placed on nickel grids and coated with gold using a gold sputtering instrument. The gold-coated samples were viewed in a scanning electron microscope.

RESULTS AND DISCUSSION

Cell Immobilization

Immobilization of whole cells onto a solid support by adsorption is a mild, simple, and nonspecific process. Inorganic carriers such as glass, brick, and diatomaceous earth or organic materials such as cellulose and ion-exchange resin have been used as supports (Bickerstaff, 1997). To provide a high surface area per unit bioreactor volume for cell immobilization, the aforementioned support materials must have highly porous morphology. Microorganisms are immobilized on these porous materials by lodging in the micropores. Reactant and product transport in these microporous supports encounter a strong pore diffusion. Because the use of such porous support would negate the benefit of presenting the OPH on the cell surface, it was decided to consider/evaluate only nonporous supports on which the cells would immobilize primarily on the surface as a biofilm. One such support material that has shown promise for surface immobilization of microorganisms, plant cells, and mammalian cells is nonwoven fabric (Archambault, 1995; Mulchandani et al., 1989; Naruse et al., 1996; Tokuda et al., 1997; Yahashi et al., 1996). These nonwoven fabrics are commercially available and come in different materials such as polyester, polypropylene, silk, etc., and also different fiber size, thickness, air permeability, and weight per unit area. Prior success in immobilization of microbial cells on these mats in the PI's laboratory (Mulchandani et al., 1989) was an additional reason for selecting the nonwoven fabric support in this research.

In a commonly used method of cell immobilization by adsorption technique, cells are cultivated/cultured in a complete nutrient medium in the presence of the support material. This method was found unsuitable for the immobilization of *E. coli* cells expressing OPH on the cell surface because: (1) the cells immobilized as a slimy film that was adsorbed/bound very weakly; (2) the slimy layer was easily disturbed/dislodged from the surface by mere lifting of the support from the medium; and (3) a majority of the cell growth was in the medium, as evident from the high turbidity of the medium.

Immobilization of Starving Cells

Starving/resting yeast cells, *Saccharomyces uvarum*, have been shown to bind strongly to immobilization supports (Bringi and Dale, 1985). Because our prior research with nongrowing (freely suspended in buffer in the absence of nutrients) *E. coli* cells expressing OPH on the cell surface demonstrated that these cells were extremely stable and retained 100% of the original OPH activity for >1 month (Chen and Mulchandani, 1998), it was decided to investigate how well precultivated and starved/rested cells immobilized on the nonwoven fabric support. Preliminary results indicated that the precultivated resting cells immobilized strongly and uniformly to the nonwoven fabric support. Unlike the adsorbed growing cells, starved immobilized cells adsorbed stongly/tightly to the support and did not dislodge from the support matrix easily. Therefore, this method was used for cell immobilization in this research.

Effect of pH and Surface Area on Starving Cell Immobilization

The surface charges on the cell and support play an important role in the cell–support interaction. Because the net surface charges are a function of the buffer pH, cell loading on the support should be affected by buffer pH (Mozes et al., 1987; Mulchandani et al., 1989). Cell immobilization on 909 PP white nonwoven fabric was a function of pH (data not shown), with the highest cell loading (0.256 ± 0.04 g of cell dry weight/g of fabric or 0.048 ± 0.002 g of cell dry weight per 12.5-cm^2 matrix) observed at pH 8.0. This pH is also favorable for subsequent organophosphate hydrolysis operation, because the activity of the free and immobilized OPH at pH 8 is approximately 80% of its maximum (Mulchandani et al., in press).

Cell immobilization was also a function of surface area, with increasing cell loading on larger support matrices (12 mg of cells on a 2.25-cm^2 matrix as compared with 50 mg on a 12.5-cm^2 matrix). The scanning electron micrograph of the cell support (Fig. 1) shows that the support matrix was covered completely and uniformly by the adsorbed cells.

Hydrolysis of Organophosphates

In the initial investigations of cell immobilization on the nonwoven fabric, cells were not induced to express OPH on the cell surface. Therefore, it was deemed necessary to determine whether cells that have OPH displayed on the cell surface will also immobilize similarly to those not displaying OPH on the cell surface. No difference in cell loading was observed for the two cases. The immobilized cells with active OPH on their cell surface hydrolyzed paraoxon at an initial rate of 0.02 m*M* min^{-1} (g cell dry wt)$^{-1}$. This rate of paraoxon hydrolysis was approximately 23-fold greater than that of control (1 m*M* paraoxon in pH 8, 50 m*M* citrate-phosphate buffer with 0.05 m*M* Co^{2+} and 5% methanol incubated with nonwoven fabric support at 37°C and 110 rpm). The method in which the cell cultivation and immobilization are separated is particularly beneficial if the degradative enzyme displayed on the cell surface was stable (see subsequent text). This will eliminate the need for main-

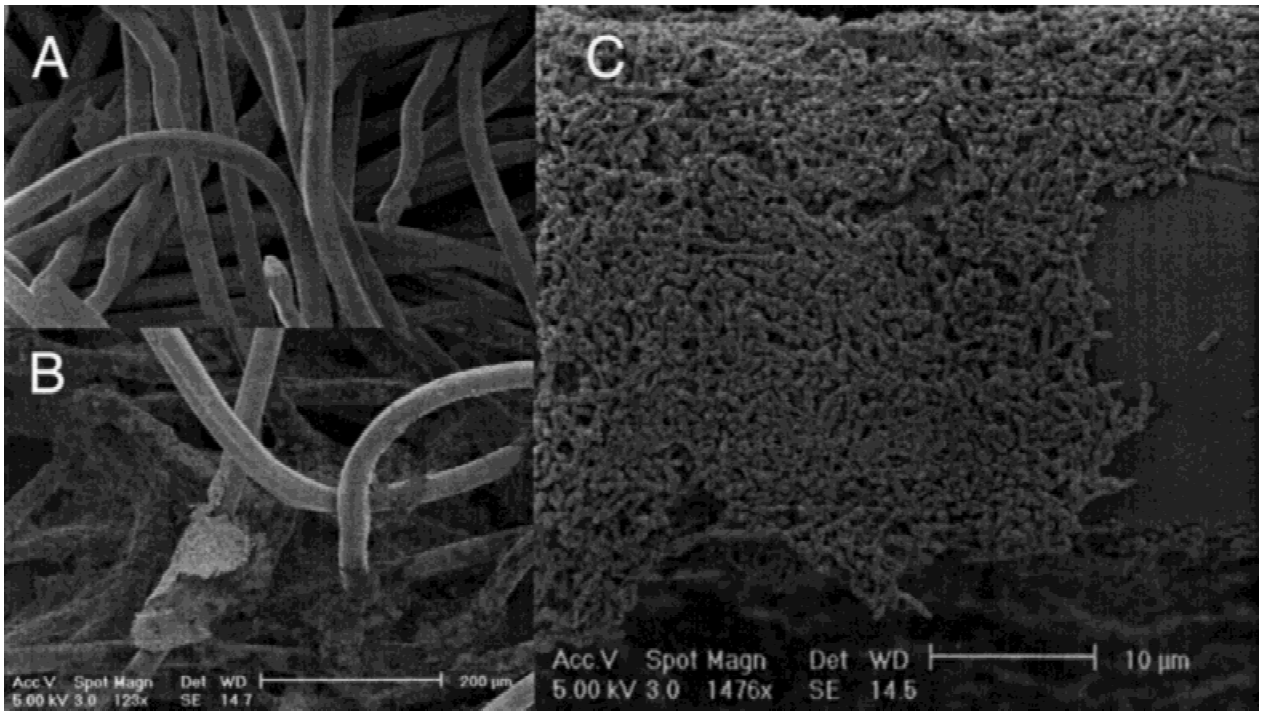

Figure 1. (A) Scanning electron micrograph of native support (original magnification 180×). (b) Cell loaded support matrix (original magnification 180×). (C) Cell-loaded support matrix (original magnification 2200×).

taining an aseptic environment after cell culturing, which can be done in a laboratory, during hydrolysis of organophosphates in the immobilized cell bioreactor.

Effect of Co^{2+}

Organophosphorus hydrolase contains a binuclear metal center that is actively involved in the catalysis (Lai et al., 1994). The native enzyme contains two Zn^{2+} ions per molecule (Dumas et al., 1989b). The Zn^{2+} ions can be replaced by a variety of divalent cations to alter the enzyme activity. The replacement of Zn^{2+} by Co^{2+} (1 m*M* Co^{2+} was added to the cell cultivation medium approximately 16 h after the induction of OPH synthesis) results in an approximately twofold enhancement of the OPH activity for paraoxon hydrolysis (Omburo et al., 1992). Experiments were done to determine the role of Co^{2+} cation on the efficiency of OP hydrolysis during cell immobilization and the subsequent use of the immobilized cells. Cells expressing active OPH on the cell surface were harvested, washed, and suspended in buffer with and without 0.05 m*M* Co^{2+} (the concentration of Co^{2+} in the buffer used in enzyme purification and activity measurement) during cell immobilization and subsequent paraoxon hydrolysis. The result showed that the presence of Co^{2+} did not affect the cell loading. The initial specific paraoxon hydrolysis rate, on the other hand, was affected by whether Co^{2+} was present or absent during hydrolysis; that is, 0.03 m*M* min^{-1}(g cell dry wt)$^{-1}$ when 0.05 m*M* Co^{2+} was present compared with 0.02 m*M* min^{-1} (g cell dry wt)$^{-1}$ when Co^{2+} was absent from the hydrolysis medium (in both cases, the cells were adsorbed from buffer supplemented with 0.05 m*M* Co^{2+}). Increasing the Co^{2+} concentration to 1 m*M* during the cell immobilization step resulted in an improvement of the initial specific paraoxon hydrolysis rate from 0.08 m*M* min^{-1} (g cell dry wt)$^{-1}$ in the absence of Co^{2+} during hydrolysis to 0.16 m*M* min^{-1} (g cell dry wt)$^{-1}$ in the presence of 0.05 m*M* Co^{2+} during hydrolysis. The presence of a very low concentration of Co^{2+} (0.050 m*M*) in the buffer during paraoxon hydrolysis also greatly prolonged paraoxon degradation by the immobilized cells (see ''Stability of Immobilized Cells'' subsection), probably by preventing the loss of cobalt from the OPH active center.

Effect of Methanol

The degradation of higher concentration of pesticides is advantageous as it will reduce the volume of wastewater to be processed. Because organophosphate pesticides are not very soluble in aqueous solution, organic solvent is generally added to increase their solubility in aqueous medium. Organic solvents, unfortunately, affect the activity and stability of many proteins and at high concentration can cause denaturation and precipitation. Additionally, biological materials immobilized by simple adsorption can be susceptible to desorption upon change in the environment and inevitably affect the stability (i.e., long-term operation) of the bioreactor. Therefore, it was considered necessary to study the effect of organic solvents on the activity and stability of the immobilized cells. As shown in Figure 2, there was no significant effect on the initial paraoxon hydrolysis rate by *E. coli* cells immobilized on nonwoven fabric with up to 20% methanol. The immobilized cells were stable over a 3-week period of repeated use (Fig. 3). Higher (30% and 40%) methanol concentrations, however, resulted in a moderate decline in paraoxon degradation rate. These results are in agreement with a study investigating purified OPH covalently immobilized onto nylon supports (Caldwell and Raushel, 1991b).

Effect of Temperature

Microorganisms, and the enzymes they carry, are rather labile and can be easily killed/inactivated at high temperatures. Although stability increases at low temperatures, the rates of the desired enzyme-catalyzed reactions are low. Temperature effect on paraoxon degradation by immobilized cells was investigated between 24° and 45°C. As for soluble OPH, the initial specific paraoxon hydrolysis rate increased from a value of 0.056 μM min^{-1} (g cell dry wt)$^{-1}$ at 24°C to 0.16 m*M* min^{-1} (g cell dry wt)$^{-1}$ at 45°C. Although increasing temperatures led to an enhanced initial paraoxon degradation rate, it is important to recognize that OPH is labile and undergoes a temperature-dependent inactivation (Rowland et al., 1991). Because the improvement in the initial specific paraoxon hydrolysis rate between 37° and 45°C was only marginal [0.14 compared with 0.16 m*M* min^{-1} (g cell dry wt)$^{-1}$] and the penalty in terms of enzyme stability rather severe, 37°C was selected for the future studies.

Effect of Organophosphate Concentration and Compounds

As stated earlier, degradation of higher concentration pesticides is advantageous because it reduces the volume of

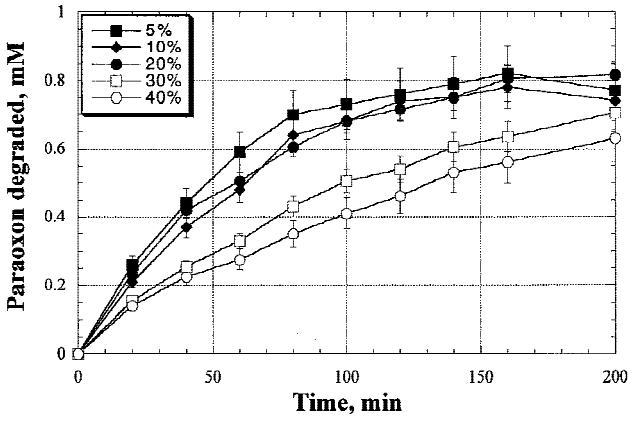

Figure 2. Effect of methanol on the degree of hydrolysis of 0.8 m*M* paraoxon by immobilized cells in pH 8, 50 m*M* citrate-phosphate buffer with 0.05 m*M* Co^{2+} at 37°C and 110 rpm.

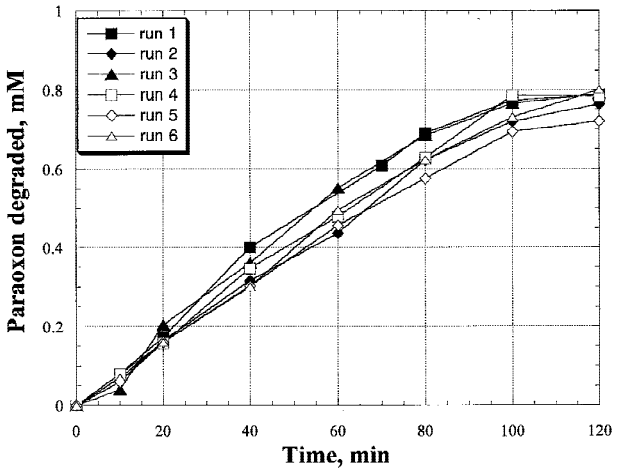

Figure 3. Effect of immobilized cell recycling on the degree of hydrolysis of 0.9 m*M* paraoxon after 2-h reaction in pH 8, 50 m*M* citrate-phosphate buffer with 20% methanol and 0.05 m*M* Co^{2+} at 37°C and 110 rpm.

wastewater to be processed. Figure 4 shows that immobilized *E. coli* cells expressing OPH on the cell surface hydrolyzed 100% of higher concentration paraoxon solutions very efficiently at a relatively high rate.

Organophosphorus hydrolase has broad substrate specificity. It is reported to hydrolyze a whole host of organophosphate-based nerve agents (Dumas et al., 1989b). The ability of the immobilized cells with OPH expressed on the cell surface to degrade various organophosphates was evaluated. Immobilized cells effectively degraded close to 100% paraoxon, coumaphos, and diazinon in less than 3.5 h, and methyl parathion in approximately 24 h (Fig. 5). The slower rate of methyl parathion hydrolysis compared with diazinon and coumaphos is an anomaly, considering that the reported k_{cat}/K_m for the free enzyme for methyl parathion is an order of magnitude greater than these organophosphates (Mason et al., 1997).

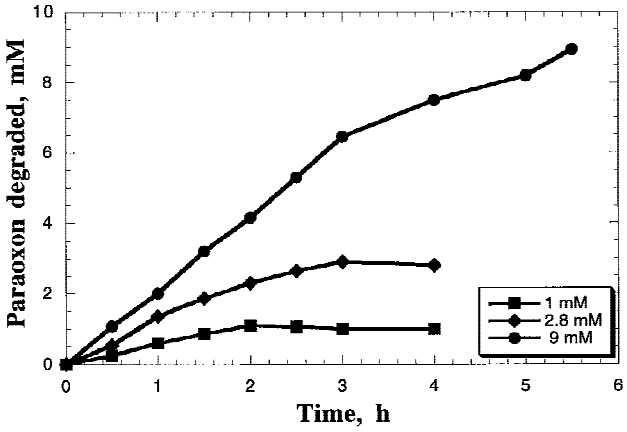

Figure 4. Effect of paraoxon concentration on its hydrolysis by immobilized cells in pH 8, 50 m*M* citrate-phosphate buffer with 20% methanol and 0.05 m*M* Co^{2+} at 37°C and 110 rpm.

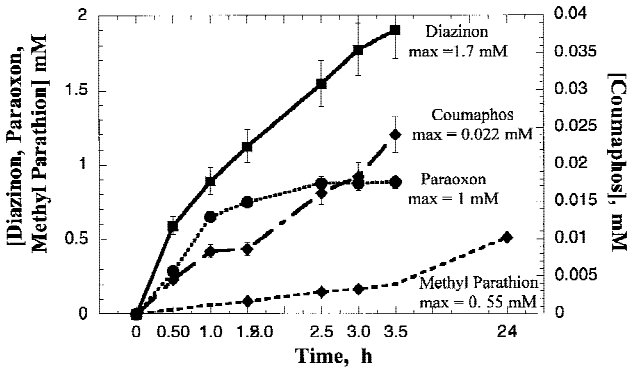

Figure 5. Hydrolysis of other organophosphate nerve agents by immobilized cells in pH 8, 50 m*M* citrate-phosphate buffer with 20% methanol and 0.05 m*M* Co^{2+} at 37°C and 110 rpm. Initial concentrations: 1 m*M* paraoxon; 0.55 m*M* methyl parathion; 1.7 m*M* diazinon; and 0.022 m*M* coumaphos.

Stability of Immobilized Cells

Immobilization of cells facilitates their recycle and reuse. Table I depicts the recycling/reuse ability of the immobilized *E. coli* cells with OPH on the cell surface. A very small decline in paraoxon hydrolysis performance of the immobilized cells from the initial level was detected after 12 cycles of paraoxon degradation over a 19-day period. Because earlier work in this laboratory has showed that suspended nongrowing (suspended in buffer in the absence of any nutrient) *E. coli* cells expressing OPH on the cell surface were stable up to 30 days (Chen and Mulchandani, 1998), the small decline in the paraoxon hydrolysis can be attributed to the detachment/loss of immobilized cells from the support.

Reculturing of Starved Immobilized Cells

The rate of hydrolysis/degradation of OPs can be improved by increasing the concentration of cells in the bioreactor. The amount of cells that can be immobilized by adsorption

Table I. Stability of immobilized cells on recycling.

Run number	Paraoxon degraded in 2 h (m*M*)
1	0.91
2	0.93
3	0.93
4	0.92
5	0.88
6	0.86
7	0.82
8	0.68
9	0.8
10	0.84
11	0.86
12	0.88

Degradation of 1 m*M* paraoxon in pH 8, 50 m*M* citrate-phosphate buffer with 20% methanol and 0.05 m*M* Co^{2+} at 37°C and 110 rpm. Immobilized cells were stored in pH 8, 50 m*M* citrate-phosphate buffer with 0.05 m*M* Co^{2+} at 37°C and 110 rpm.

alone, as has been done earlier, is, however, limited. Therefore, the strategy of cultivating/growing the cells after adsorption to increase the cell loading on the support was investigated. The results show that cells adsorbed (no Co^{2+} was present during adsorption) on nonwoven fabric could be further cultivated by suspending the support in the growth medium (buffered LB medium supplemented with ampicillin). The final cell loading after 48 h of cultivation was 144 mg dry weight/12.5 cm^2, which was threefold higher than the control. The immobilized cells could be induced to initiate the expression of active OPH on the cell surface by the addition of 1 m*M* IPTG and 1 m*M* Co^{2+} at 20 h and 44 h, respectively, after the start of recultivation. Subsequent to cell recultivation, the paraoxon hydrolysis capability of the cell-loaded support was tested in a pH 8, 50 m*M* citrate-phosphate buffer, without any nutrients for cell growth. As before, these immobilized cells were able to hydrolyze paraoxon effectively (Fig. 6). The initial rate of paraoxon hydrolysis by these immobilized cells was faster than before (11.2 vs. 8 μM min^{-1}). The higher initial rate can be attributed to the increased OPH activity resulting from the additional cell mass on the support. The immobilized cells could be cycled for successive hydrolysis cycles (Fig. 6) with no decrease in the hydrolysis rate over four cycles. Beginning the fifth cycle, however, the rate of hydrolysis decreased slightly from the initial value (Fig. 6). In comparison, over the same number of cycles (six) there was virtually no decrease in the rate of paraoxon hydrolysis for the cells that were not grown further after adsorption (Fig. 3). The larger decrease in the paraoxon hydrolysis rate, can be attributed to the weak binding of the newly formed cell layer to the support, resulting in a significantly greater loss of cells from this support.

CONCLUSIONS

Enzymatic degradation of organophosphorus nerve agents by organophosphorus hydrolase has been a subject of considerable attention during the past decade. However, practical applications of large-scale enzymatic degradation has always been limited by the cost and stability of OPH. Recently, we have successfully engineered *E. coli* cells that anchored and displayed active OPH on the cell surface. These cells degraded organophosphates very effectively, retaining 100% of their initial activity over 30 days without undergoing the diffusional resistance of the cell walls that has been observed in cells expressing OPH intracellularly. The availability of this organism has opened new avenues for developing bioprocesses based on immobilized whole cells.

In this article, we have developed a simple procedure for the immobilization of genetically engineered *E. coli* cells displaying OPH on their surface. Cells were immobilized by simple adsorption of nongrowing cells with the active OPH already displayed on the cell surface on nonwoven polypropylene fabric supports from a pH 8 buffer supplemented with 1 m*M* Co^{2+}. Unlike other methods (Freeman et al., 1996), no harsh chemicals, which effect the enzyme activity significantly, are used for cell immobilization. When used in batch operation, cells immobilized by this method efficiently and rapidly hydrolyzed almost of the 100% paraoxon, diazinon, coumaphos, and methyl parathion. The 8-μM min^{-1} rate of paraoxon hydrolysis by with 0.24 g of nonwoven fabric support with immobilized cells is a slight improvement over the rate of 6.25 μM min^{-1} obtained with 0.22 g of polyurethane-foam-entrapped OPH that was isolated from an *E. coli* strain expressing the enzyme intracellularly and purified to homogeneity (LeJeune et al., 1996). The immobilized cells retained close to 100% initial organophosphate hydrolysis activity for six repeated cycles, and 90% activity for 12 repeated cycles, over a period of 19 days without nutrient supplementation. This operational stability was significantly better than that observed for OPH immobilized within polyurethane foam matrix (LeJeune et al., 1997).

The method of cell immobilization developed in this research, in which the cell culturing was separated from the cell immobilization and detoxification process, has advantages, because there is no need for aseptic environment after culturing, which can be performed in the laboratory. A scale-up of the present bench-scale immobilized-cell reactor, whether operated in batch or continuous mode, is feasible using the reported designs (Archambault et al., 1990; Lewis and Yang, 1992). It is therefore expected that the procedures demonstrated in this study will pave the way to a cost-effective, simple, efficient technology for detoxification of organophosphate-nerve-agent-contaminated wastewaters and stockpile of nerve gases that have to destroyed by the year 2007 in accordance with the Chemical Weapons Treaty.

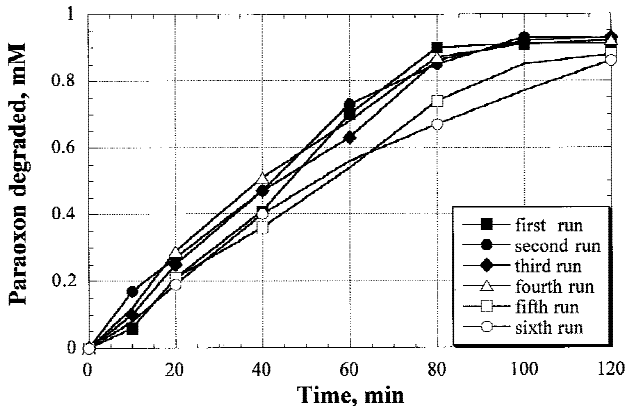

Figure 6. Hydrolysis of 0.9 m*M* paraoxon by recultured immobilized cells in pH 8, 50 m*M* citrate-phosphate buffer with 20% methanol and 0.05 m*M* Co^{2+} at 37°C and 110 rpm.

The authors thank Dr. Priti Mulchandani, Mr. Michael Howecker, and Mr. Seog Lee for their help in generating some of the data presented in this work.

References

Archambault J. 1995. Surface immobilization of plant cells. In: Hjortso MA, Roos JW, editors. Cell adhesion: fundamentals and biotechnological applications. New York: Marcel Dekker. p 111–134.

Archambault J, Volesky B, Kurz WGW. 1990. Development of bioreactors for the culture of surface immobilized plant cells. Biotechnol Bioeng 35:702–711.

Bickerstaff GF. 1997. Methods in biotechnology. Vol 1: Immobilization of enzymes and cells. Totowa, NJ: Humana Press.

Bringi V, Dale BE. 1985. Enhanced yeast *Saccharomyces uvarum* immobilization by nutrient starvation. Biotechnol Lett 7:905–908.

Chapalamadugu S, Chaudhry GS. 1992. Microbiological and biotechnological aspects of metabolism of carbamates and organophosphates. Crit Rev Biotechnol 12:357–389.

Caldwell SR, Raushel FM. 1991a. Detoxification of organophosphate pesticides using an immobilized phosphotriesterase from *Pseudomonas diminuta.* Biotechnol Bioeng 37:103–109.

Caldwell SR, Raushel FM. 1991b. Detoxification of organophosphate pesticides using a nylon based immobilized phosphotriesterase from *Pseudomonas diminuta.* Appl Biochem Biotechnol 31:59–73.

Cassidy MB, Le H, Trevors JT. 1996. Environmental applications of immobilized microbial cells: a review. J Indust Microbiol 16:79–101.

Chen W, Mulchandani A. 1998. The use of ''live biocatalysts'' for pesticide detoxification. TIBTECH 16:71–76.

Compton JA. 1988. Military chemical and biological agents. Clifton, NJ: Telford Press. p 135.

Donarski WJ, Dumas DP, Heitmeyer DP, Lewis VE, Raushel FM. 1989. Structure–activity relationships in the hydrolysis of substrates by the phosphotriesterase from *Pseudomonas diminuta.* Biochemistry 28: 4650–4655.

Dumas DP, Durst HD, Landis WG, Raushel FM, Wild JR. 1990. Inactivation of organophosphorus nerve agents by the phosphotriesterase from *Pseudomonas diminuta.* Arch Biochem Biophys 227:155–159.

Dumas DP, Wild JR, Raushel FM. 1989a. Diisopropyl fluorophosphate hydrolysis by a phosphotriesterase from *Pseudomonas diminuta.* Biotechnol Appl Biochem 11:235–243.

Dumas DP, Caldwell SR, Wild JR, Raushel FM. 1989b. Purification and properties of the phosphotriesterase from *Pseudomonas diminuta.* J Biol Chem 33:19659–19665.

Elashvili I, DeFrank JJ. 1996. Phosphonate transporter mutation enhances the utilization of diisopropylphosphate (DIPP) and diisopropyl fluorophosphate (DFP) in *Escherichia coli* K-12. Proceedings of 1996 US Army ERDEC Scientific Conference on Chemical Defense Research, U.S. Army ERDEC, Aberdeen Proving Ground, Aberdeen, MD.

Elashvili I, DeFrank JJ, Culotta VC. 1998. phnE and glpT genes enhance utilization of organophosphates in *Escherichia coli* K-12. Appl Environ Microbiol 64:2601–2608.

Food and Agricultural Organization of the United Nations. 1989. Rome: FAO. p 320.

Freeman A, Abramov S, Georgiou G. 1996. Fixation and stabilization of *Escherichia coli* cells displaying genetically engineered cell surface proteins. Biotechnol Bioeng 52:625–630.

Hung S-C, Liao JC. 1996. Effects of ultraviolet light irradiation in biotreatment of organophosphates. Appl Biochem Biotechnol 56:37–47.

Lai K, Dave KI, Wild JR. 1994. Bimetallic binding motifs in organophosphorus hydrolase are important for catalysis and structural organization. J Biol Chem 269:16579–16584.

LeJeune KE, Mesiano A, Bower SB, Grimsley JK, Wild J, Russell AJ. 1997. Dramatically stabilized phosphotriesterase-polymers for nerve agent degradation. Biotechnol Bioeng 54:105–114.

Lewis VP, Yang S-T. 1992. Continuous propionic acid fermentation by immobilized *Propionibacterium acidipropionici* in a novel packed-bed bioreactor. Biotechnol Bioeng 40:465–474.

Martinez MB, Flickinger MC, Nelsestuen GL. 1996. Accurate kinetic modeling of alkaline phosphatase in *Escherichia coli* periplasm: implications for enzyme and substrate diffusion. Biochem. 35:1179–1186.

Mason JR, Briganti F, Wild JR. 1997. Protein engineering for improved biodegradation of recalcitrant pollutants. In: Wild JR, Varfolomeyev SD, Scozzafava A, editors. Prespectives in bioremediation: technologies for environmental improvement. Dordrecht: Kluwer. p 107–118.

Mozes N, Marchal F, Hermesse MP, Van Haecht JL, Reuliaux L, Leonard AJ, Rouxhet PG. 1987. Immobilization of microorganisms by adhesion: interplay of electrostatic and nonelectrostatic interactions. Biotechnol Bioeng 30:439–450.

Mulbury WW, Del Valle PL, Karns JS. 1996. Biodegradation of organophosphate insecticide coumaphos in highly contaminated soil and in liquid wastes. Pestic Sci 48:149–155.

Mulchandani A, Luong JHT, LeDuy A. 1989. Biosynthesis of pullulan using immobilized *Aureobasidium pullulans* cells. Biotechnol Bioeng 33:306–312.

Mulchandani P, Mulchandani A, Kaneva I, Chen W. Biosensor for direct determination of organophosphate nerve agents. 1. Potentiometric enzyme electrode. Biosens Bioelectron. In press.

Munnecke DM. 1979. Hydrolysis of organophosphate insecticides by an immobilized-enzyme system. Biotechnol Bioeng 21:2247–2261.

Munnecke DM. 1980. Enzymatic detoxification of waste organophosphate pesticides. J Agric Food Chem 28:105–111.

Naruse K, Sakai Y, Nagashima I, Jiang GX, Suzuki M, Muto T. 1996. Development of a new bioartificial liver module filled with porcine hepatocytes immobilized on non-woven fabric. Int J Artif Org 19: 347–352.

Omburo GA, Kuo JM, Mullins LS, Raushel FM. 1992. Characterization of the zinc binding site of bacterial phosphotriesterase. J Biol Chem 267:13278–13283.

Richins R, Kaneva I, Mulchandani A, Chen W. 1997. Biodegradation of organophosphorus pesticides by surface-expressed organophosphorus hydrolase. Nature Biotechnol 15:984–987.

Rowland SS, Speedie MK, Pogell BM. 1991. Purification and characterization of a secreted recombinant phosphotriesterase (parathion hydrolase) from *Streptomyces lividans.* Appl Environ Microbiol 57: 440–444.

Tokuda H, Urata T, Nakanishi K. 1997. Hydrolysis of xylan by *Aspergillus niger* immobilized on non-woven fabrics. Biosci Biotechnol Biochem 61:583–587.

United States Department of Agriculture. 1992. Agricultural statistics. Washington, DC: United States Government Printing Office. p 395.

Yahashi Y, Hatsu M, Horitsu H, Kawai K, Suzuki T, Takamizawa K. 1996. D-Glucose feeding for improvement of xylitol productivity from D-xylose using *Candida tropicalis* immobilized on a non-woven fabric. Biotechnol Lett 18:1395–1400.

Yakovlevsky K, Govarhan C, Grimsley J, Wild J, Margolin A. 1997. Cross-linked crystals of organophosphorus hydrolase: an efficient catalyst for the breakdown of the O.P. compounds. Proceedings of the 213th American Chemical Society national meeting, April 13–17, San Francisco. Paper BIOT-0012.