Bioremediation of Organophosphate Neurotoxins Using Cryoimmobilized Matricies

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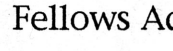
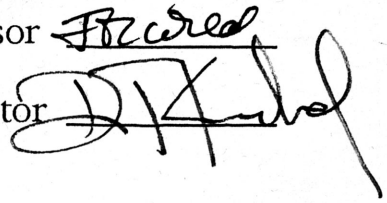
Fellows Advisor 

Honors Director 

ABSTRACT

E. coli cells expressing a recombinant organophosphate degrading gene (*opd*) were successfully encapsulated in polyvinyl alcohol (PVA) using cryoimmobilization. Gel matrices were composed of 10% cells in 10% PVA, and electron microscopy demonstrated that the cells were well distributed throughout the gel and that their morphology was preserved. . The beads were measured for activity with paraoxon, as a substrate, over a pH range of 4.0-10.0. Thermal stability was assayed by exposing beads to temperatures between 22 and 60°C, and for bioreactor simulation, a column of encapsulated cells was prepared and cheked for performance. Cryoimmobilization proved to be an effective technique for bioremediation.

INTRODUCTION

Synthetic organophosphates have become an environmental toxicity problem due to their wide spectrum of toxicity to many biological systems. They are in widespread use as neurotoxic pesticides for insects, and analogs of these chemicals have been developed as means of chemical warfare. These issues have created a need for microbial or enzyme-based systems that can degrade these organophosphates in an environmentally responsible manner. The organophosphate-degrading gene (*opd*) has been isolated from two different soil bacteria, *Pseudomonas diminuta* MG (pCMS1, 51kb) and *Flavobacterium sp.* ATCC 27551 (pSM55, 43kb) (Mulbry et al. 1986; McDaniel et al. 1988), that allow the bacterial degradation of a broad spectrum of organophosphates (Dumas et al. 1989). The organophosphate hydrolase (OPH) encoded for by the *Pseudomonas* *opd* gene has been expressed in *E. coli* and has been well characterized in terms of its post-translational processing (Miller 1992) and the enzymatic characteristics of its active site (Dumas et al. 1989; Caldwell and Raushel 1990; Lewis et al. 1988; Dave et al. 1993).

Bioremediation provides the potential for the natural degradation of hazardous chemical wastes without the environmental hazards of conventional methods like

incineration. The direct immobilization of an enzyme has the potential to degrade its substrate (Caldwell and Raushel 1991), but the immobilization of adequate amounts of pure protein to be practical is problematic, and the enzymes do not benefit from the protection of a cell wall from environmental contaminants. Whole cell immobilization does protect the enzyme and escape the need for purification of enzyme.

Immobilizing whole cells in a stable gel matrix has several potential advantages: 1) It protects the cells from the environment. 2) The biological systems can be efficiently controlled since there are potential hazards in releasing non-immobilized cells containing recombinant DNA constructions into the environment. 3) A matrix can provide a high density of cells with sustained enzyme activity in a biologically and mechanically stable environment.

The problem of costly techniques associated with immobilization may be avoided by using cryoimmobilization, a technology that was invented and pioneered by Dr. S.D. Varfolomeyev at Moscow State University (Varfolomeyev et al. 1992). The adaptation of this technique to *E. coli* has produced active encapsulated cells. Results presented in this study show that this method is capable of providing a practical way of degrading organophosphate neurotoxins in a matrix that is stable over varying pHs, temperatures, and flow rates, while maintaining the integrity of the cells.

MATERIALS AND METHODS

1. CHEMICAL MATERIALS

Transformed *E. coli* cells carrying the cloned gene on a plasmid were used in encapsulation. Chemically treated polyvinyl alcohol (PVA) was provided by Dr. E.I. Rainina and Dr. S.D. Varfolomeyev at Moscow State University. Paraoxon, 2-

(N-morpholino)-ethanesulfonic acid (MES), N-ethylmorpholine, diethanolamine 2-(N-cyclohexylamino) ethansulfonic acid (CHES), and ampicillin were obtained from Sigma Chemical Company. Yeast extract and bacto-tryptone were purchased from DIFCO Laboratories. Cobaltous chloride, glycerin, sodium chloride, and potassium phosphate monobasic and dibasic were obtained from Fischer Products Inc.

2. CELL CULTURE

Bacterial strains and manipulations of recombinant DNA vectors were developed by standard recombinant DNA techniques (Sambrook et al., 1989). A BamH1 restriction fragment containing the d2-29 *opd* open reading frame with the altered Shine Dalgarno sequence (Dave et al. to be submitted) was isolated from the M13 mp10 RF DNA. This 1170 bp fragment was subcloned into the BamH1 site of the *tac* promoter vector pPROK-1, obtained from Clontech Laboratories Inc., Palo Alto CA. The resulting construction (designated pKD2) was transformed into the *E. coli* strain DH5α [*sup* E44, ΔlacU169, (ϕ80, lacZΔM15), *hsd* R1, *rec* A1, *end* A1, *gyr* A96, *thi*- 1, *rel* A1].

Two 5 ml cultures of transformed *E. coli* were grown in a complex nutrient broth TYE composed of 10 g Bactotryptone, 5 g Yeast Extract, and 5 g NaCl per liter with 50 mg Ampicillin/l. The inoculum was transferred to one liter growth cultues of 'Terrific Broth,' containing 12 g Bactotryptone, 24 g Yeast extract, 4 ml Glycerin, 1 mM $CoCl_2$, 50 mg Ampicillin/l, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4, and cultured to late growth phase (200 Klett Units) in 36 hr at 27°C.

3. CELLULAR ENCAPSULATION

PVA was added to TYE in a 1:8 weight ratio and mixed for 12 hr in the absence of UV radiation (dark) and then sterilized. Harvested cells were added to this mixture

in a 1:9 weight ratio, added drop-wise to sterile Petri dishes, and frozen at -20°C to complete the cryostructuring process. The resulting gel matrices were submerged in a three component buffer system, Tripartate, of 0.1M 2-(N-morpholino)ethanesulfonic acid (MES), 0.51M N-ethylmorpholine, 0.51M diethanolamine 2-(N-cyclohexylamino), at pH 6.0 and stored at 4°C.

4. ENZYME ASSAY

In order to assay the immobilized cells, the beads were placed in a test tube of 5 ml containing assay mixture (1 mM paraoxon and 50 mM CHES) and vortexed for three minutes. As paraoxon was hydrolyzed to diethyl phosphate and p-nitrophenol, the changes in absorbance of p-nitrophenol was determined at 400 nm on a Gilford 300 N Micro-Sample Spectrophotometer. For determining absorbance specific activities, the values have been adjusted for the weight of the beads (Relative activity=ΔAbs_{400}/ mg immobilized gel matrix).

5. BIOREACTOR COLUMN

For column measurements, 1 g of beads were placed in a Kontes disposable column with a diameter of 7.0 mm and a bed volume of 1.0 ml. Figure 1 provides a schematic representation of the various components used in the biodegradation of paraoxon using these cryoimmobilized cells in our laboratory. (A) substrate reservoir, (B) pump, (C) column of beads, (D) fraction collector, (E) spectrophotometer. Five milliliter fractions were collected and with a Spectrum Spectra/Chrom Fraction Collector; the absorbance was then measured on a Gilford Response Spectrophotometer. Controlled flow rates of 0.6, 1.2, 1.8, 2.4 and 3.0 ml/min, were developed with an ISCO Wiz Pump, and higher rates (5, 10, 15, and 20 ml/min) required the use of a Pharmacia Pump P-50 was used to pump the assay mixture through the column.

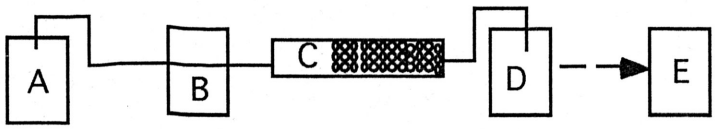


Figure 1. A general schematic for pump system. (A) substrate reservoir, (B) Pharmacia or ISCO pump, (C) column of beads, (D) Spectrum fraction collector, (E) Gilford Response® spectrophotometer

6. REACTION CHARACTERISTICS

The effect of varying pH and temperature studies on system stability were carried out on immobilized cells. For pH analysis, encapsulated cells were placed in 10 ml of appropriate buffer, stored at 4°C for 24 hr at standard assay conditions, and then assayed for activity. Citrate (0.1 M)- Potassium Phosphate buffer (0.1 M) was used to maintain pHs from 4-7, and Tripartate buffered in the range of 6-10. For temperature studies, beads were placed at similar conditions at temperature ranges from 22-60°C. Temperatures of 22- 40°C were controlled with Equatherm Water Baths from Curtis Matheson Scientific, Inc. 50°C and 60°C were maintained by American Scientific Multiblock Module Heaters. Enzyme activity was determined as described previously (section 4).

7. ELECTRON MICROSCOPY

PVA beads with immobilized *E.coli* were fixed in 3% glutaraldehyde in 100mM cocodylate buffer, pH 7.0. For transmission electron microscopy (TEM) beads were postfixed in 1% OsO_4 for 4 hr and dehydrated gradually. Following this, the beads were infiltrated with epoxy plastic (EM Sciences), polymerised at 70° C overnight, sectioned and stained with 2% uranyl acetate and Reynold's lead citrate. The sections were examined using the Philips 420 TEM. For scanning electron microscopy (SEM), beads fixed previously in 3% glutaraldehyde were rinsed with distilled water and then with 10% ethylalcohol. Following this, the beads were frozen solid with liquid nitrogen and fractured using pressure point, thawed in

100% ethylalcohol, placed in critical point dryer and dried. The fractured dried pieces were mounted on double stick tape, coated with gold-paladium alloy in iron-sputter coating device and examined on JEOL T330A SEM at 30 keV. (These studies were performed in the Center for Electron Microscopy at Texas A&M University.)

RESULTS AND DISCUSSION

The process of cryoimmobilization with polyvinyl alcohol was selected for its advantages in mechanical stability over a broad range of pHs and temperatures and for its proven ability to withstand the physical challenges of bioreactors. The encapsulated cells are permeable to substrates where the PVA residues have the environmentally positive quality of being 100% recyclable (Varfolomeyev et al. 1992). The *E. coli* strain DH5α expressing OPH was selected for its ability to degrade a broad spectrum of substrates, with hydrolysis of paraoxon approaching diffusion limits with a K_m of 0.05mM and a K_{cat} of 2100 sec^{-1} (Dumas et al. 1990). Besides this, the enzyme mechanism has been well defined (Lewis et al. 1988; Dumas et al. 1989; Caldwell and Raushel 1990).

In this study, the immobilized beads were subjected to a pH range of 4.0 to 10.0, Fig. 2. The optimum was found to be between 6.0 and 7.0 after 24 hr. The shoulder in the pH activity profile between pH 8.0 and 9.0 corresponds to the free enzyme's pK_a, which relates to the ionizable nature of an active site histidine (Courtesy of K. Lai). There was no observable change in the physical strength or shape of the beads at the varying pHs.

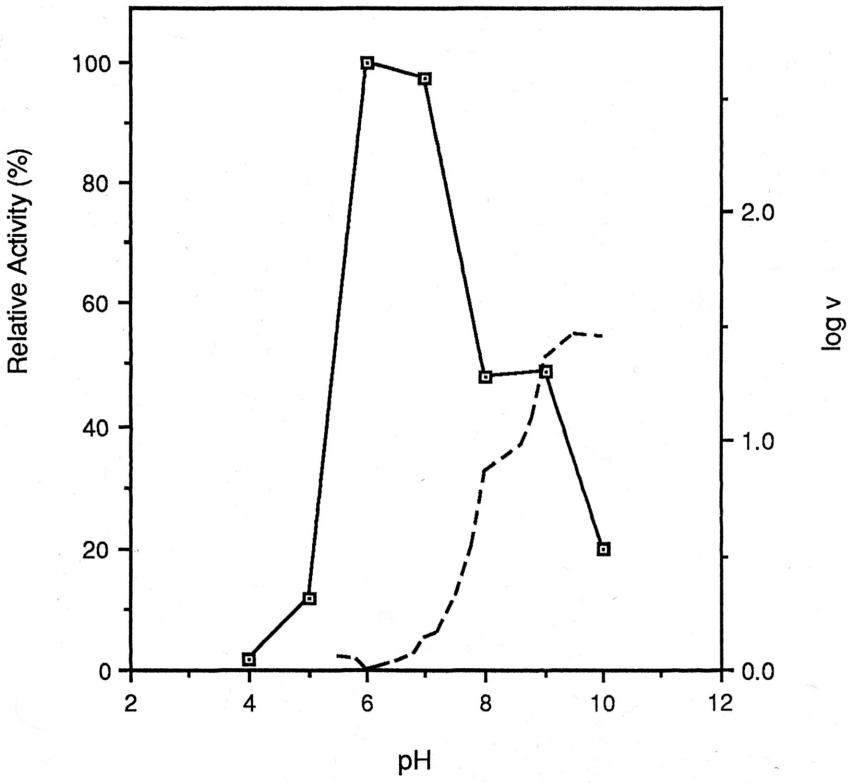


Figure 2. Beads were stored individually in 5 ml of buffer at 4C through the pH range of 4-10 for 24 hours and were then assayed under normal conditions. For pHs 4-7, beads were stored in Citrate-Phosphate Buffer, and for pHs 6-10, beads were kept in Tripartate Buffer. The buffer had no effect on the beads. Free enzyme was assayed over the same range after being stored at 4°C at the varying pHs (Courtesy of K. Lai).

Temperature effect and thermal stability were examined from 22°C to 60°C, Fig. 3. The measured activity is relative to beads stored at 4°C, which maintained stable activity throughout the investigation. All of the beads experienced a slight loss in activity within 10 min; 22°C decreased to 87%, where activities were stable for 2 hr. Retentive activity for beads at 60°C was 15% after 10 min and 4% after 30 min. After 24 hr, 22°C beads retained 66% of activity, but all other conditions lost over 85% of activity after 24 hr.

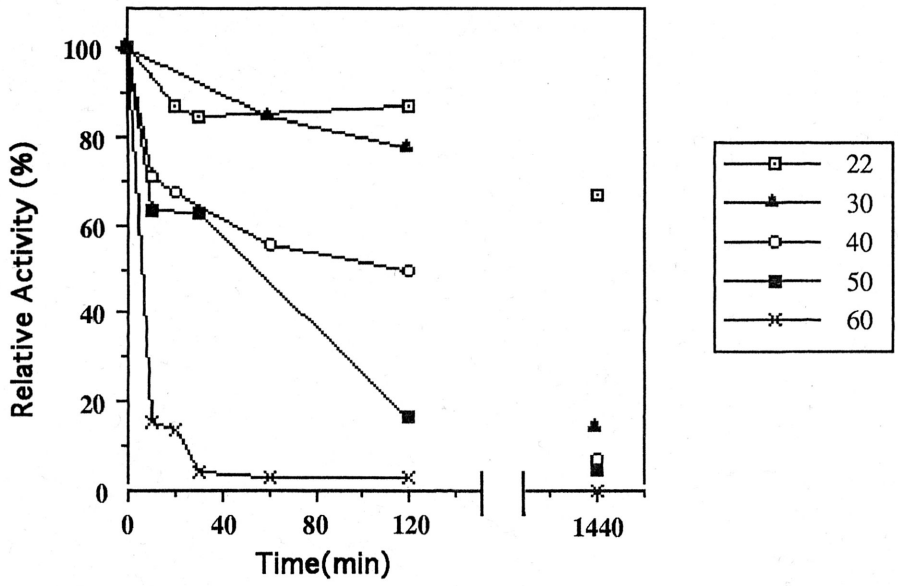


Figure 3. Beads were stored varying temperatures from 22°C to 60°C for 24 hr. Enzymatic assays were then determined relative to beads stored at 4°C.

In order to assess the capacity of the beads to hydrolyze substrate in a scaled down bioreactor simulation, beads were packed in a straw column and a reaction mixture with 1mM paraoxon was pumped through at varying flow rates at room temperature (Fig. 4). The absorbances (400 nm) of the collected fractions were monitored until they had demonstrated a steady state of substrate hydrolysis. This data was presented as the stable level of paraoxon degraded at a given flow rate. The same column, with washing, could be reused with no decrease in activity. Rates to 1.2 ml/min could degrade 100% of the substrate. At 3.0 ml/min 90% of the paraoxon was hydrolyzed, but by 5 ml/min only 37% was hydrolyzed. Rates greater than 10.0 ml/min hydrolyzed less than 5% of the paraoxon. There was no damage to the structure of the beads nor any decrease in its ability to degrade substrate due to compaction or structure breakdown at any flow rate.

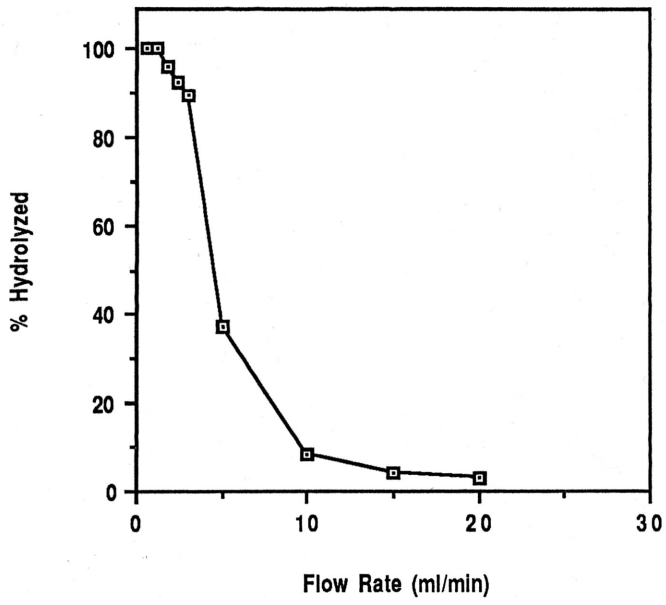


Figure 4. One gram of beads were placed in a 7.0 mm in diameter column that was directly attached to a pump and sample collector. Assay buffer was then pumped through the column at varying rates and collected in 5 ml fractions. 10-12 samples were collected at each flow rate for absorbance measurements on the spectrophotometer.

The physical integrity of cryoimmobilized cells and the composition of the matrix was examined by electron microscopy . While scanning the surface of the beads, no cells were observed on the edge of the beads, suggesting that the beads did not lose cells while being used nor would they be released them into the environment . Cross-sectional evaluation of cryoimmobilized gels demonstrate that the cells were well distributed within the beads(Fig. 5 10,000X magnification). Fig. 6 shows the structure of immobilized cells at 54,000X magnification with a transmission

electron microscope. The micrograph showed little deterioration in cell morphology when cryoimmobilized, demonstrating the gentle nature of the encapsulation process. Maintaining the integrity of the cellular structure benefits the system by lessening the chance of losing the plasmid, evidenced by the continued activity of the beads; similar results have been found with this technique in other systems (Varfolomeyev et al 1992).

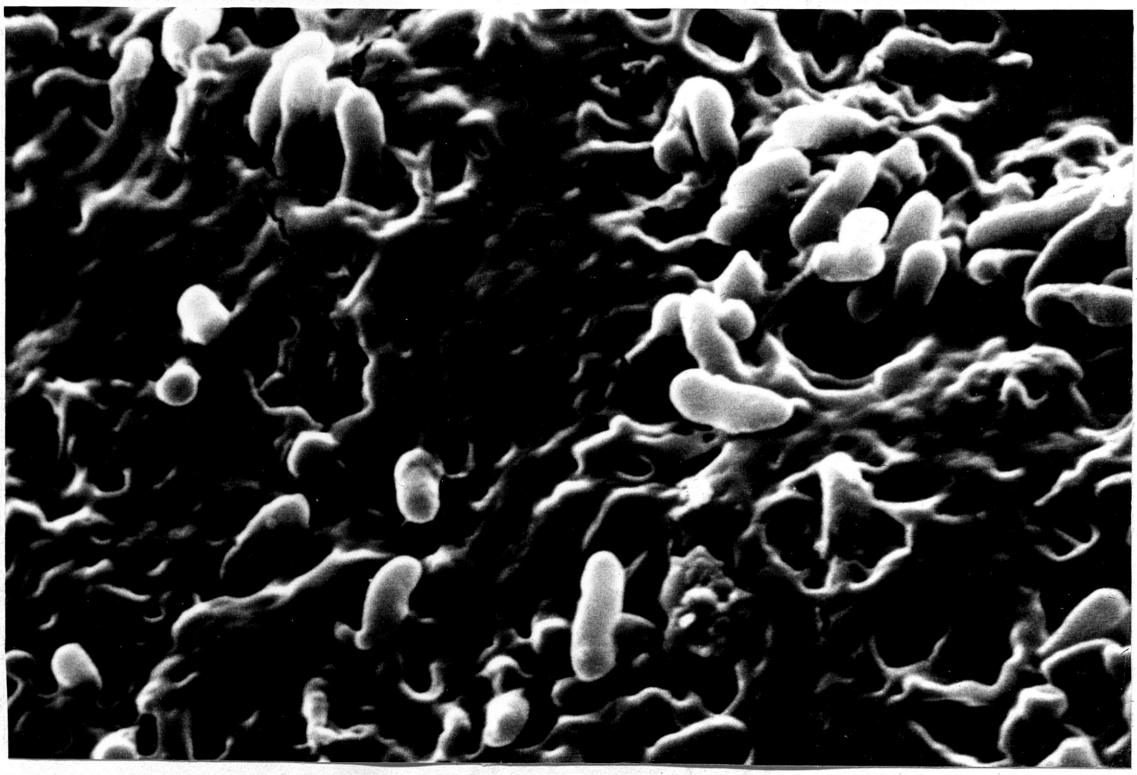


Figure 5. A scanning electron microscope view at 10,000X magnification showing the *E. coli* well distributed throughout the bead.

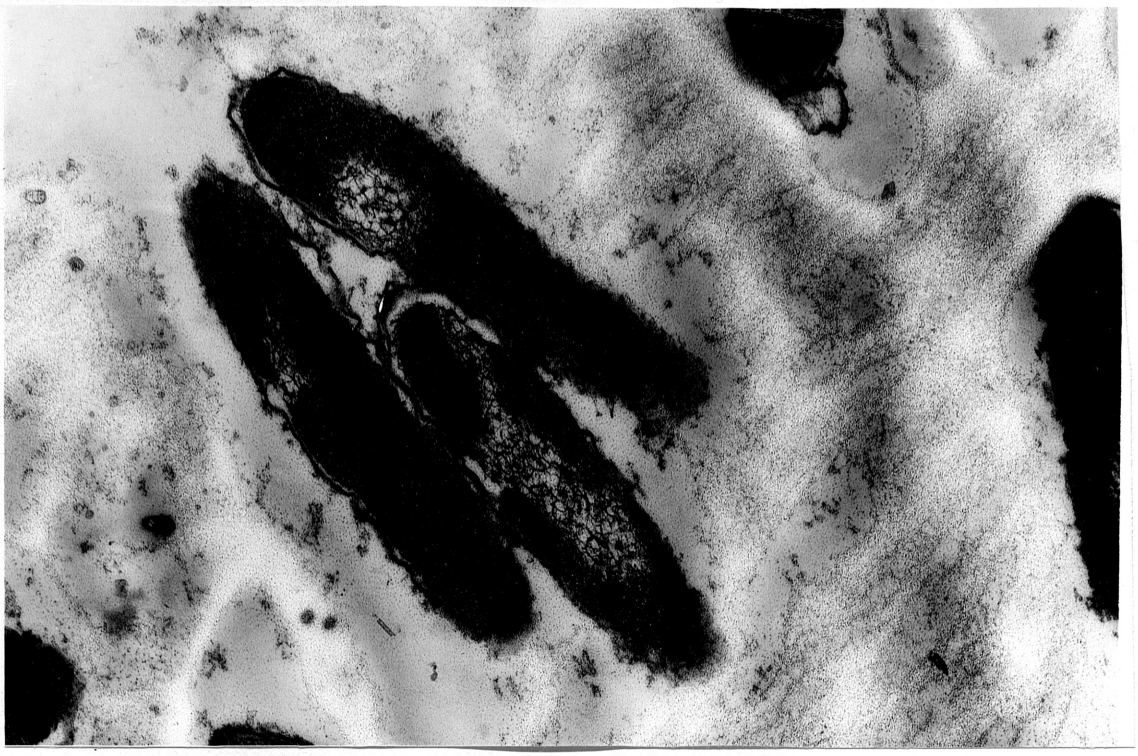


Figure 6. A transmission electron microscope micrograph at 54,000X magnification shows the intact morphology of the encapsulated cells after the process of cryoimmobilization.

The encapsulation of bacterial cells with cryoimmobilization has excellent potential for bioremediation. It has the advantages of tolerance to varying pHs and temperatures, as well as, its ability to withstand a constant flow rate without altering its capacity to degrade substrate. This is also an economical and environmentally responsible way of disposing of many neurotoxic organophosphates.

ACKNOWLEDGMENTS

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