Detoxification of Organophosphate Nerve Agents by Immobilized Dual Functional Biocatalysts in a Cellulose Hollow Fiber Bioreactor

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Abstract: A whole-cell technology for detoxification of organophosphates based on genetically engineered *Escherichia coli* cell expressing both cellulose-binding domain (CBD) and organophosphorus hydrolase (OPH) onto cell surface was reported recently (Wang et al., 2002). This study reports the application of these biocatalysts when immobilized in a cellulose hollow fiber bioreactor (HFB) for the biodetoxification of a model organophosphate, paraoxon, in a continuous flow mode. In 24 h, 0.79 mg wet cell/cm^2 fiber surface were immobilized onto cellulose fibers specifically and strongly through the cellulose binding domain, forming a monolayer demonstrated by Scanning Electronic Micrograph, and essentially no cell was washed away by washing buffer. The immobilized biocatalyst had a high performance of detoxifying paraoxon solution of 5,220 μmol/h $\cdot$ L reactor or 990 μmol/h $\cdot$ m^2 reactor. The immobilized biocatalysts maintained a stable degradation capacity for 15 uses over a period of 48 days with only 10% decline in degradation efficiency under operating and storage conditions. In addition, the bioreactor was easily regenerated by washing with 1% sodium dodecyl sulfate (SDS), with 86.7% immobilization capacity and 93.9% degradation efficiency recovery. This is the first report using the HFB in a non-traditional way, immobilizing whole-cell biocatalysts by specific adhesion thus rendering the catalysis operation the advantages of low pressure drop, low shear force, and low energy requirement. The successful application of this genetically engineered dual functional *E. coli* strain in a model bioreactor shows its promise in large-scale detoxification of organophosphate nerve agents in bulk liquid phase.

Keywords: pesticides; degradation; OPH; CBD; hollow fiber bioreactor; immobilized cells

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INTRODUCTION

All nerve agents belong to the organophosphorus (OP) compound group. Amongst the most toxic substances known, OPs have found extensive application in agricultural and domestic pest control, and as chemical warfare agents (Yang et al., 2003). These compounds inhibit acetylcholinesterase resulting in the accumulation of acetylcholine, which interferes with muscular responses leading to failure of organs and eventually death. Because poisoning may occur through consumption of liquids or foods contaminated with nerve agents, there is a growing public concern regarding treating the wastes generated from (1) washing of storage tanks and spraying equipment, (2) excess unused pesticides and their residues, and (3) large quantity of aqueous wastes from regional/national pest control practice (Munnecke, 1980; Mulbury et al., 1996). In addition, the recently ratified Chemical Weapons Treaty requires the world stockpiles of over 200 kilotons extremely toxic organophosphate-based nerve gases such as sarin, soman, and VX to be destroyed by the year 2007 (Gill and Ballesteros, 2000; Mulchandani et al., 1998).

Biotechnological methods of detoxifying these organophosphate nerve agents based on organophosphorus hydrolase (OPH), an enzyme that hydrolyzes a wide range of organophosphates, have drawn significant attention (Benning et al., 1994; Dumas et al., 1989a; Lai et al., 1995; Rastogi et al., 1997). OPH hydrolyzes a number of OP pesticides such as paraoxon, parathion, coumaphos, diazinon, dursban, methyl parathion, etc. and chemical warfare agents, sarin, soman, and VX producing products with greatly reduced toxicity relative to the parent compounds (Dumas et al., 1989b, 1990). Although the native OPH hydrolyzes a broad range of OPs, the hydrolysis effectiveness for CWAs sarin, soman, and VX, and several pesticides and insecticides is low. However, using site-directed mutagenesis and in vitro directed evolution, OPH variants with higher activity against chemical warfare agents VX and soman, and

pesticides methyl parathion and chlorpyrifos were generated (Cho et al., 2004).

Detoxification of organophosphate nerve agents by OPH requires a stable, highly efficient, and cost-effective system. Processes based on immobilized purified enzyme have been proposed and evaluated for high throughputs of concentrated OP feeds for long-term use (Gill and Ballesteros, 2000). However, practical applications of large-scale enzymatic degradation have always been limited by the cost and stability of OPH. As a cost-effective alternative, whole cells (either growing or non-growing) rather than enzymes, can be immobilized onto the support (such as in an immobilized-cell bioreactor). However, the transport of pesticides across the cell membrane presents a major problem since the outer membrane acts as a permeability barrier and prevents the pesticides from interacting with the OPH residing within the cells. This bottleneck, however, could be eliminated if OPHs are displayed onto the cell surface. We have successfully displayed active OPH onto the cell surface of *E. coli* using either the Lpp-OmpA fusion system or the truncated ice nucleation protein (INPNC) anchor. Additionally, we combined the OPH on the cell surface with the cellulose-binding domain (CBD) protein to aid in specific and strong immobilization of cells expressing the OPH enzyme on cheap and abundantly available cellulose support for sustained long-term operation and stable high efficiency degradation (Wang et al., 2002).

Discovery and development of novel biological treatment systems must be merged with the design of practical treatment processes in order to realize the full potential for industrial applications. From a practical standpoint, any detoxification process based on these novel biocatalysts must be more efficient and economical than the existing technology of organophosphate detoxification. To be efficient, the process will require a high activity of the enzyme catalyzing the hydrolysis of organophosphates (OPH) with low mass transport resistance, that is, the effectiveness factor (the ratio of the actual reaction rate to the reaction rate when there is no diffusion and mass transport resistances) is close to unity. For the process to be economical, cells with surface-expressed OPH should be reuseable for many times, and the reactor setup should be easy and not labor intensive. Immobilizing the cells with the biocatalyst on the cell surface in traditional immobilizing matrix, such as beads, particles, will lose the benefit of surface expression.

Hollow fiber bioreactor (HFB) system was originally developed to simulate an in vivo capillary system for in vitro growth of cells, mainly to commercially produce monoclonal antibody from hybridoma cells in place of mice (Lipman and Jackson, 1998). Recently, it has found many novel applications ranging from glycerol-3-phosphate synthesis (Li et al., 2001) to heavy metal removal (Chen et al., 1998). As a basic pattern, the hollow fibers are packed as a bundle and are potted at both ends within a cartridge. The fiber walls are constructed of a material, which acts as a semi-permeable ultrafiltration membrane. The size of the membrane's pores, characterized by its molecular weight cut-off, can be small enough to retain cells and large enzymes while permitting small molecules move freely down their concentration gradients (Lipman and Jackson, 1998). It can accommodate two independent medium streams (either one or both can be continuous) in a cross-flow mode thus providing a maximal contact of the two phases, with the hollow fiber wall as the interface or support for the immobilization of cells or enzymes (Deng and Wilson, 2001; Lloyd et al., 1997; Sousa et al., 2001).

This study reports the application and evaluation of a cellulose HFB for detoxification of organophosphate nerve agents using genetically engineered *E. coli* cells expressing OPH and CBD on cell surface constructed in our earlier study (Wang et al., 2002). A model organophosphate, paraoxon, was used as the substrate for this study. Traditionally, cell immobilization in a hollow fiber reactor is achieved through confinement of the cells behind the hollow fiber membrane. Cells are circulated through the lumen or extra-capillary space (ECS) at high flow rate to provide high tangential velocity to prevent fouling/cake deposition on the filtration surface (Chen et al., 1998). Because of the high shear rate, the cells in such a system are prone to damage. Since the newly constructed *E. coli* cells with surface-expressed OPH and CBD were shown to be immobilized specifically and tightly through CBD to cellulose fibers, forming essentially a monolayer on the fiber surface (Wang et al., 2002), it was expected that immobilization of these cells in the HFB would take advantage of its high surface area while alleviating the disadvantages of high pressure drop, high shear and high energy input necessary in the traditional immobilization based on non-specific confinement.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Culture Conditions, and Buffer System

Escherichia coli strain 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^qZΔM15, Tn10 (Tet^r)]) was used in this study. Plasmid pUCBD (Wang et al., 2001), a pUC18 derivative, was used for expression of Lpp-OmpA-CBD on the cell surface. Plasmid pPNCO33 (Wang et al., 2002) was used to express INP-OPH onto the cell surface.

E. coli cells carrying both pUCBD and pPNCO33 plasmids were cultivated in buffered LB media (10 g/L Difco tryptone, 5 g/L Difco yeast extract, 10 g/L NaCl, 1 g/L KH_2PO_4, 3 g/L K_2HPO_4, pH 7.0) and supplemented with ampicillin (100 μg/mL) and kanamycin (20 μg/mL). Induction of the expression of Lpp-OmpA-CBD and INP-OPH was achieved by adding 0.05 mM IPTG as an inducer (Wang et al., 2002) at an OD_{600} of 0.5. One millimole $CoCl_2$ was added 24 h after induction of OPH expression (OPH contains a metal center and cobalt is the preferred metal for optimal activity). Cells were harvested 48 h after induction.

The harvested cells were resuspended in 150 mM carbonate-bicarbonate buffer, pH 9.5 (30% v/v 150 mM

sodium carbonate and 70% v/v 150 mM sodium bicarbonate) supplemented with 0.05 mM $CoCl_2$ (henceforth designated as buffer A).

Hollow Fiber Reactor

The hollow fiber reactor CELLMAX® Artificial Capillary Cartridge (Cellulosic 400–022) was obtained from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). It contains ca. 500 fibers running the length of the cartridge of 7.7 cm with a pair of shell-side (ECS) ports and another pair of lumen-side ports. The shell-side ports (III and IV) connect to the outside surface of the hollow fibers and the lumen-side ports (I and II) directly connect to the inside of the hollow fibers (Fig. 1). The outer wall is made of a transparent material, which facilitates observation on the hollow fibers. As per manufacturer specifications the cartridge has a capillary fiber surface area of 250 cm^2 and ECS volume of 2.2 mL with a 95% molecular weight cut-off at 150 kDa or 50% molecular weight cut-off at 30 kDa.

Reactor System Set-Up

Figure 1 shows the experimental set-up used in the study. The system is consisted of the hollow fiber microfiltration catridge, a multi-channel peristaltic pump (PumpPro® MPL, Watson Marlow, Inc., Wilmington, MA), a cell suspension feed reservoir, a substrate (paraoxon) feed reservoir, and a waste collection reservoir connected by tubing and valves.

Cell Amount Determination

Because of the need to measure very low amounts of cells, wet weight of cells was determined by measuring the cell protein. For this purpose, the wet cell weight was correlated to the cell protein concentration. Protein content of the cells was determined by centrifuging 5 mL of cell suspension at 16,000*g* and resuspending in 50 μL of buffer. Four hundred twenty-five microliters of 0.1 M NaOH was then added, and the sample was heated at 80°C for 10 min. Subsequently, 425 μL of 0.1 M HCl was added to neutralize the sample and then 100 μL of 0.5 M citrate phosphate pH 8 buffer added to give a final volume of 1 mL. Two hundred microliters of the above solution was then mixed with 800 μL of concentrated dye reagent (BioRad, Hercules, CA) and incubated for 5 min. Optical density at 595 nm was recorded and converted to protein concentration following the Bradford method.

The cell wet weight was determined by measuring the weight of the cell pellet recovered from 5 mL of cell suspension.

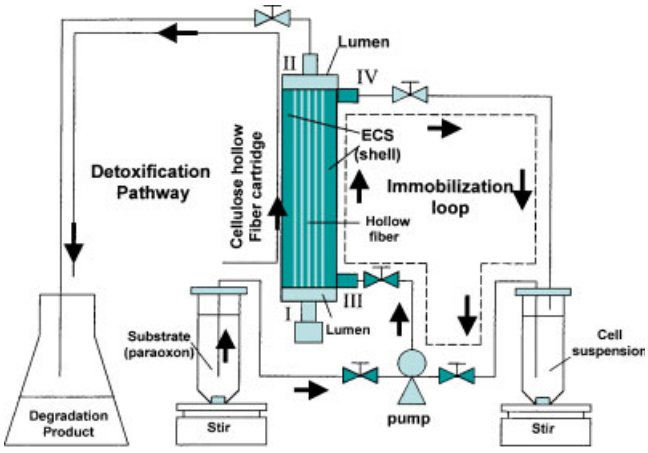

Figure 1. Schematic diagram of the hollow fiber bioreactor system. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Measurement of Void Volume

Both compartments of the cartridge—lumen and ECS—and all connecting tubing were first primed with buffer A by circulating the buffer overnight. Twenty milliliters of the buffer A containing 50 μM of *p*-nitrophenol (Aldrich, Milwaukee, WI) was then introduced into the system and circulated for 4.5 h at a flow rate of 0.53 mL/min. Concentration of the *p*-nitrophenol in the feed reservoir was measured at different time intervals until it reached steady-state. The total volume of the bioreactor system (lumen plus ECS) was calculated from the difference between the initial and final *p*-nitrophenol concentrations, minus the volume of the tubing measured beforehand (1.6 mL).

Measurement of Pressure Drop

For measuring pressure drop during immobilization, washing, and regeneration, a manometer made of a U-shape glass tube filled with mercury is connected between the lumen and ECS ports (either I and IV, or III and II in Fig. 1) so as to measure the pressure difference across the hollow fiber wall.

Organophosphorus Hydrolase Assay

Cells were centrifuged and resuspended in 150 mM carbonate-bicarbonate buffer, pH 9.5 (30% (v/v) 150 mM sodium carbonate and 70% (v/v) 150 mM sodium bicarbonate). Activity assays were conducted in 1.5 mL disposable methacrylate cuvettes (Fisher). For each assay, 900 μL of cells ($OD_{600} = 0.5$) were combined with 100 μL of 20 mM paraoxon (Sigma, St. Louis, MO) dissolved in methanol. Cells with no OPH activity were used as control. Change in absorbance (412 nm, $\varepsilon_{410} = 16{,}500/M \cdot cm$ for *p*-nitrophenol) was measured with a Beckman DU-60 spectrophotometer for 2 min at 37°C. Whole cell activities were expressed as U (micromoles of paraoxon hydrolyzed per minute) per mg protein.

Whole Cell Immobilization on Hollow Fibers

Cells were harvested and resuspended in buffer A to cell density (OD_{600nm}) of ca. 2.0. After measuring the OPH activity by measuring *p*-nitrophenol formation kinetics using paraoxon as substrate (Wang et al., 2002), the cell suspension

(stirred constantly to keep cells in suspension) was circulated through the ECS (immobilization loop) for 24 h at 25°C (Fig. 1, dashed lines). The OD_{600nm} of the cell suspension before and after the circulation was measured to determine the amount of cells immobilized on the fiber. The immobilized cells were then washed by pumping 1,000 mL of buffer A from the lumen side to shell side (ports I to IV) to remove cells bound non-specifically to the hollow fiber. The washing buffer was collected and concentrated to determine the amount of proteins and thereby cells washed out. In the event a large amount of cells were found washing away, the immobilization procedure was repeated a second time to ensure good cell immobilization.

The cell coverage of the fiber surface was determined by pressure drop measurement. At a given pump speed, buffer A was pumped from ECS to lumen side (ports III to II) through the hollow fiber wall. By measuring the pressure-drop across the hollow fiber wall before and after the immobilization, the pressure-drop increase would indicate the cell coverage on the fiber outside surface.

Biodetoxification of Paraoxon by Immobilized Whole-Cells

A known concentration of paraoxon solution in buffer A was pumped at desired flow rate though the ''biodetoxification loop'' (Fig. 1, solid line). The solution entered the immobilized cell cartridge from the shell side (port III) and exited from the lumen side (port II) (transverse or cross-flow mode). The degree of paraoxon hydrolysis was determined continuously as a function of time by measuring the concentration of the degradation product *p*-nitrophenol formed by monitoring its absorbance at 412 nm using a UV-vis spectrophotometer (Beckman DU-60) in flow through mode. The experiment was stopped when *p*-nitrophenol concentration was constant. The system was then washed thoroughly with buffer A, until no *p*-nitrophenol was detected in the effluent by the spectrophotometer, and was either used for next experiment or stored at room temperature in buffer A.

Regeneration of the Bioreactor

The immobilized whole-cell biocatalyst reactor was washed with a 1% (w/v) solution of sodium dodecyl sulfate (SDS) in water to release the cells from the surface of the hollow fibers. The pressure drop across the fiber membrane was monitored during the process. When the pressure drop decreased to a constant, the reactor was washed with water to remove any residual SDS. The bioreactor was then used to immobilize the cells again and the immobilized cells evaluated for detoxification of paraoxon. The immobilization and degradation efficiencies of the regenerated bioreactor were compared with that of last batch to show the regeneration efficiency. This process was repeated to determine the number of times the cellulose fibers can be successfully reused as support in immobilization and detoxification.

Scanning Electron Microscopy

The plastic wall of the bioreactor cartridge was cross-cut open to remove a bundle of cellulose fibers with immobilized *E. coli* cells. The fibers were cut into 1 cm–long fragments, washed once with 150 mM carbonate-bicarbonate buffer (pH 9.5), and then soaked in the same buffer containing 2% glutaraldehyde overnight to fix the cells. The fiber was washed five times with 150 mM carbonate-bicarbonate buffer (pH 9.5), followed by secondary fixation with 1% w/v osmium tetroxide for 1 h at 4°C and consecutive washing with carbonate-bicarbonate 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 to ensure complete dehydration of the sample. The ethanol was removed by a critical point dryer (Balzers CPD0202). The sample was then placed onto a SEM sample holder and coated with gold using a sputter coater (Emscope ES500). The gold-coated samples were viewed in an electron microscope (PHILIPS XL30-FEG).

RESULTS AND DISCUSSIONS

Measurement of Void Volume

The evaluation of the residence time requires reliable and accurate information of the actual void volume of the specific cartridge used in the work. The void volume of 4.76 ± 0.03 mL ($n = 3$) determined by our method was in close agreement with the 4.9 mL reported by the manufacturer.

Immobilization of Whole-Cell Biocatalyst Onto Cellulose Hollow Fiber

The pH of 9.5 determined as optimum for the immobilization of XL1-Blue through the CBD expressed on the cell surface (Wang et al., 2001) and the OPH activity (Richins et al., 2000) was used in the present study for cell immobilization and detoxification. Additionally, the buffer was supplemented with 0.05 mM $CoCl_2$ to maintain OPH activity in a continuous flow bioreactor (Mulchandani et al., 1998).

The immobilization of cells was carried out as described in Materials and Methods. By measuring the cell density of the suspension before and after the immobilization, approximately 197 ± 14 mg (wet weight) of cells ($n = 2$) were shown to be immobilized on the surface of the cellulose fiber after 24 h circulation of the cell suspension. This corresponded to 4.14 g dry weight of cells per liter of total reactor volume or 0.79 mg wet weight cell/cm^2 fiber surface. This cell loading is comparable to the 4.47 g dry weight of cells per liter of reactor volume reported by Lloyd et al. (1997). In that study, cells were immobilized by growing them in situ into the outer spongy matrix layer of the fibers for 24 h. The cells in this case were immobilized primarily by entrapment (86%), in the fiber pores and confinement (14%) in the ECS. In comparison, in this study, cells were immobilized as a monolayer on the fiber surface by contacting resting cells

(circumventing the need for aseptic conditions) with smooth surface of the cellulose hollow fiber, and there were no loose cells in the ECS even after long backwash indicating the strong and specific attachment of cells through CBD. Additional benefits of immobilizing the cells on the membrane surface through affinity interaction as opposed to in the membrane pores will include: (1) no substrate and product mass transfer limitation into and out of the pores; (2) low transmembrane pressure drop and therefore pumping energy; and (3) regeneration of the bioreactor when the cells become inactive.

The strength and specificity of cell binding through CBD to cellulose support were evaluated by back-flushing/washing the immobilized cells from the direction opposite to that used for cell immobilization. Such a black-flush is traditionally used to clean the cake deposited on filtration membrane/medium. Back-flushing with 1,000 mL of buffer A at significantly high permeate rate released negligible amount [$(2.6 \pm 1.5) \times 10^{-4}$ mg wet weight] of cells from the support, indicating very strong and specific binding of cells to cellulose hollow fiber support matrix through CBD (Wang et al., 2001, 2002).

The pressure-drop profile monitored during immobilization and washing, at a fixed pumping speed of 30 mL/h, showed a gradual increase in pressure-drop from the initial value of 100 mm Hg to a final stable value of 250 mm Hg after 60 min, implying the saturation of the fiber surface after a gradual covering with cells. In washing stage, the pressure-drop did not change at all. Combining this and the result that essentially no cell was washed out by the washing buffer after immobilization, according to our previous observation (Wang et al., 2002), it was probable that the outside surface of the hollow fiber was covered with a monolayer of the dual functional whole-cell biocatalyst. This monolayer coverage of cells on fiber surface was further confirmed by direct observation of the fiber using Scanning Electron Microscope (Fig. 2).

Traditionally, to prevent fouling on the filtration surface in hollow fiber reactor, cells are circulated through the lumen or ECS space at high flow rate. This requires high energy input for pumping and causes shear force on biocatalysts, which is detrimental for their activity and stability. In this work, by the CBD-based specific adhesion, immobilization of the dual functional biocatalysts was not only granted the advantage of high surface area of hollow fibers, but also provided operation with low pressure drop, low shear force, and low energy requirement.

Determination of Degradation Capacity of Immobilized-Cell Reactor

The dynamics and steady-state biodetoxification of model organophosphate, paraoxon, was investigated as a function of the residence time (flow rate) and substrate concentration (Figs. 3 and 4). A careful analysis of the dynamic profile of the concentration of hydrolysis product for the same flow rate at different concentrations showed that the steady-state in all the cases were achieved after same time period, which was approximately three times the residence time (Fig. 3). The long time required for the product concentration to reach the steady-state after the introduction of the substrate suggests the flow pattern in the reactor was close to a mixed-type reactor. The mixed-type characteristic was confirmed by the good linear fit to the plot of inverse of the rate of OP degradation against the inverse of the stead-state OP

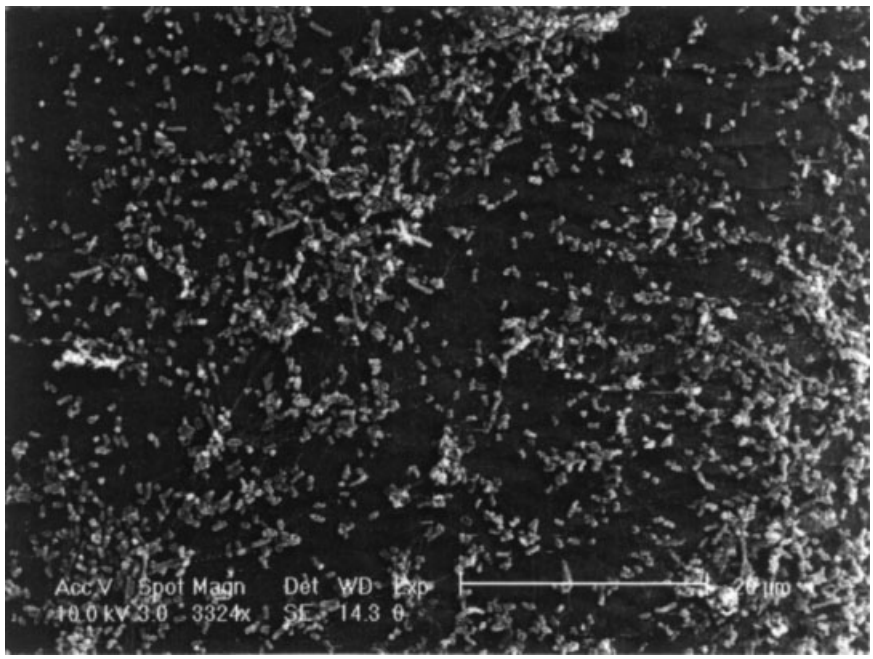

Figure 2. Scanning electron micrograph (SEM) of hollow fiber surface with immobilized dual-functional *E. coli* cells on the outside surface.

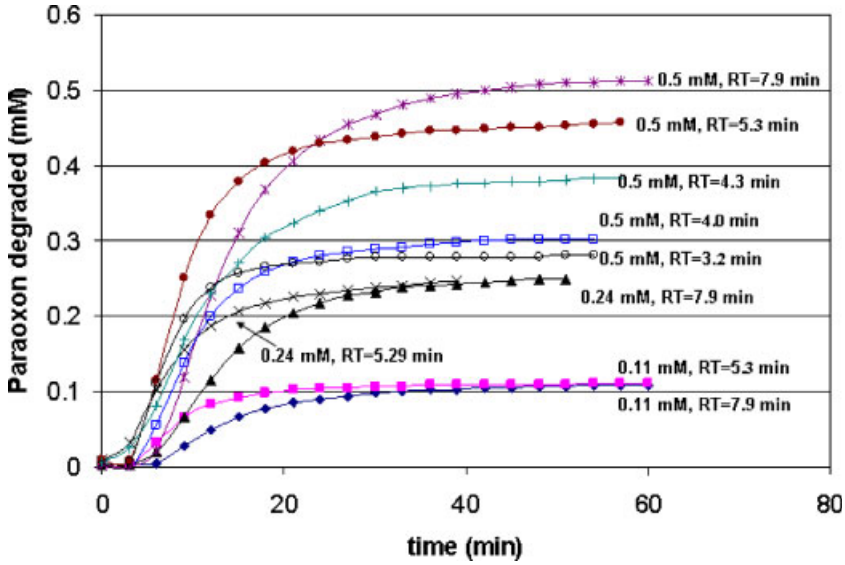

Figure 3. Dynamics of paraoxon degradation at various substrate concentrations and residence time (RT). [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

concentration in the effluent (Fig. 5) in Equation (1)

$$(1/r_s) = 1/V_m + (K_M/V_m)(1/S_o) \quad (1)$$

derived from the following balance equation for an ideal mixed-type reactor,

$$r_s = (F/V_R)(S_i - S_o) = V_m S_o/(S_o + K_M) \quad (2)$$

where, r_s is the rate of substrate consumption, F is flow rate in L/min, V_R is reactor volume in L, S_i and S_o are inlet and outlet paraoxon concentrations, respectively, in mmol/L, V_m is the maximum rate of OPH catalyzed paraoxon hydrolysis in mmol/L min and K_M is Michaelis–Menten constant in mmol/L.

The V_m and K_M estimated from the intercept and slope of the line fitted to the plot in Figure 5 were 88 μmol/L · min and 2 μmol/L, respectively.

With increasing paraoxon concentration and increasing flow rate, the degradation efficiency was maintained at 100% until the inlet flux reached 0.3 μmol/min (Fig. 4). The corresponding maximum degradation capacity of this immobilized-cell reactor for paraoxon was 5,220 μmol/h · L reactor or 990 μmol/h · m^2 reactor. At higher flux, the degradation efficiency decreased.

Stability of Immobilized Biocatalyst

The operational and storage stability of the immobilized cell HFB was evaluated by evaluating repeated hydrolysis of paraoxon over a period of 7 weeks. The hydrolysis performance of the bioreactor was extremely stable with essentially no decrease of degradation capacity for the first 2 weeks and 15 repeated uses, and a small gradual decrease to 90% of the original at the end of 7 weeks (data not shown). This is in line with our previous observation of less than 10% decline in the paraoxon hydrolysis efficiency by the same

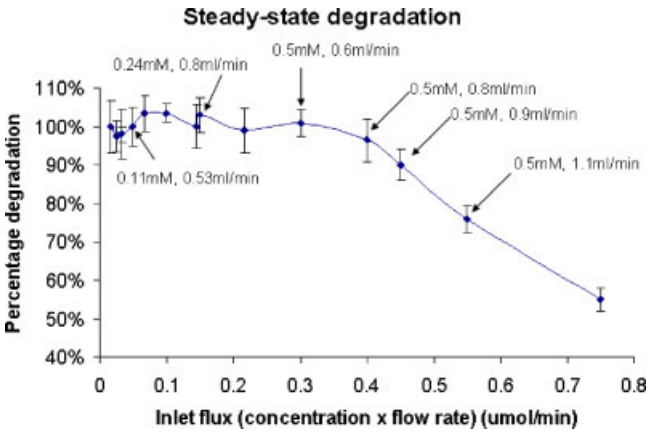

Figure 4. Steady-state paraoxon degradation as a function of paraoxon concentration and flow rate. Data points represent average of three measurements ± standard deviation. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

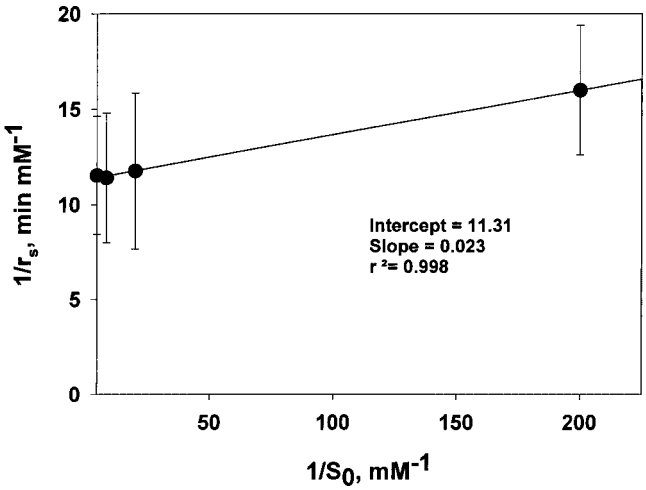

Figure 5. Lineweaver-Burk plot for paraoxon hydrolysis in immobilized cells hollow fiber bioreactor operating in mixed-type reactor mode. S_0 is the outlet paraoxon concentration in mM and r_s is the rate of paraoxon hydrolysis in mM/min.

dual functional whole-cell biocatalyst immobilized on a non-woven cellulose fiber support over a 45-day period (Wang et al., 2002).

While the high stability of degradation efficiency under storage condition was mostly from the enhanced stability of the OPH enzyme from surface expression (Chen and Mulchandani, 1998) and upon cell immobilization (Wang et al., 2002), the detoxification stability under repeated operations and washing conditions was obviously contributed by CBD-based specific adhesion, which rendered the whole-cell biocatalyst tight binding on the surface of cellulose hollow fiber to sustain the extensive degradation and washing of large volume buffer.

Regeneration of Bioreactor

An advantage of immobilizing biomolecule through affinity interaction is the potential to regenerate the immobilization matrix once the biomolecule is not functional. According to Ong et al. (1989), affinity interaction between CBD and microcrystalline surface is reversible and can be easily disrupted by washing with low pH buffer, guanidine hydrochloride, or distilled water. Since washing with water is the simplest and cheapest method of regeneration, if successful, it was used to investigate the regeneration of hollow fiber reactor. An observation of the cellulose hollow fiber surface after washing the ECS of the reactor (where the cells were immobilized on fiber surface) with 1,000 mL distilled water overnight revealed the presence of yellowish biofilm on the fiber, indicating that distilled water was not able to release the cells. This is in agreement with the observations of Tomme et al. (1998), who reported the binding between CBD_{cex} (used in this study) and crystalline and regenerated cellulose (used in the present investigation) was irreversible. Regeneration by guanidine hydrochloride washing was not investigated because it was expensive in a large-scale process.

Since the purpose of regeneration is to recover the support surface when the immobilized biocatalysts lose their degradation activity, harsher conditions may be applied to denature the CBD and thus release the cells from the immobilization support. However, these conditions may change the characteristics of the support surface thus affecting the re-immobilization efficiency. SDS solution is commonly used in protein gel electrophoresis to denature protein samples with no known detrimental effect on cellulose materials. A 1% SDS solution in distilled water was therefore used to wash the ECS where the cells were immobilized onto cellulose hollow fiber, until the pressure drop across the fiber membrane decreased to a constant, which indicated no more cells were being removed. After washing thoroughly with 2 L of water to ensure that there was no residual SDS, the ECS fiber surface was reused for immobilizing cells.

Approximately 171 mg (wet weight) of cells were immobilized after regeneration which was 86.7% of the amount immobilized in the first time. The steady-state paraoxon hydrolysis with the cells immobilized the second time at 0.3 μmol/min inlet flux was determined as 84.5%, which corresponds to 94% of the hydrolysis achieved the first time. This decrease can be attributed to the reduction in the OPH activity as a result of the reduced amount of immobilized cells in the bioreactor.

CONCLUSIONS

A genetically engineered *E. coli* cell line expressing both OPH and CBD onto the cell surface was successfully applied in a HFB. With the CBD-based specific adhesion, the biocatalyst was tightly immobilized onto cellulose hollow fiber surface and degraded paraoxon efficiently, affording low pressure drop, low shear on cells, and low energy cost. Its degradation activity was stable for 48 days under either operation or storage conditions. This immobilized bioreactor was also easily regenerated with a recovery efficiency of 90% in terms of both immobilization efficiency and degradation capacity. The high degradation capacity, low cell plugging on the immobilization support/filtration membrane, high stability, and easy regeneration adequately showed a promise of this dual functional biocatalyst upon immobilization in large scale biodegradation of organophosphate nerve agents.

NOMENCLATURE

OP	organophosphate
OPH	organophosphorus hydrolase
CBD	cellulose-binding domain

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