

Biosensor for Direct Determination of Organophosphate Nerve Agents Using Recombinant *Escherichia coli* with Surface-Expressed Organophosphorus Hydrolase.

1. Potentiometric Microbial Electrode

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A potentiometric microbial biosensor for the direct measurement of organophosphate (OP) nerve agents was developed by modifying a pH electrode with an immobilized layer of *Escherichia coli* cells expressing organophosphorus hydrolase (OPH) on the cell surface. OPH catalyzes the hydrolysis of organophosphorus pesticides to release protons, the concentration of which is proportional to the amount of hydrolyzed substrate. The sensor signal and response time were optimized with respect to the buffer pH, ionic concentration of buffer, temperature, and weight of cells immobilized using paraoxon as substrate. The best sensitivity and response time were obtained using a sensor constructed with 2.5 mg of cells and operating in pH 8.5, 1 mM HEPES buffer. Using these conditions, the biosensor was used to measure as low as 2 μ M of paraoxon, methyl parathion, and diazinon. The biosensor had very good storage and multiple use stability. The use of cells with the metabolic enzyme expressed on cell surface as a biological transducer provides advantages of no resistances to mass transport of the analyte and product across the cell membrane and low cost due to elimination of enzyme purification, over the conventional microbial biosensors based on cells expressing enzyme intracellularly and enzyme-based sensors, respectively.

Synthetic organophosphorus (OP) compounds, among the most toxic substances known,¹ are used as pesticides, insecticides, and chemical warfare agents.^{2–5} The growing public concern about their safety and the widespread use of the acutely toxic compounds in modern agriculture has stimulated the development

of technologies to effectively treat effluents generated at both the producer and consumer levels.^{6–12} Additionally, the recently ratified Chemical Weapons Treaty requires the United States to destroy all of its chemical weapons arsenal, including the organophosphorus-based nerve gases, within 10 years.^{13,14} The successful use of any laboratory-developed technology for detoxification of the organophosphate neurotoxins will require analytical tools to monitor and control the process.

Gas, liquid, and thin-layer chromatographies coupled with different detectors and different types of spectroscopy are the most commonly used analytical methods for OP determination.^{15,16} However, these techniques are time-consuming, are expensive, and require highly trained personnel, and therefore not suitable for on-line monitoring of detoxification processes.¹⁷

Biological methods, such as immunoassays and inhibition of cholinesterase activity, for OP determination have also been reported.¹⁵ Despite the promise of immunoassay techniques, these methods require long analysis time (1–2 h) and extensive sample handling (large number of washing steps) and are therefore unsuitable for on-line monitoring of detoxification process.

Biosensing analytical devices, based on an acetylcholine esterase (AChE) inhibition test, using AChE-modified amperometric,^{18–26} potentiometric,^{27–32} or fiber optic^{33–38} transducers

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have been reported. Although sensitive and ideal as single-use disposable sensors for environmental monitoring, biosensors based on AChE inhibition have limitations for application in on-line monitoring of detoxification process. These biosensors (1) have a long and tedious protocol that requires long incubation with inhibitors prior to analysis for good sensitivity and treatment with pyridine-2-aldoxime after analysis to partially regenerate/recover the enzyme activity which is inhibited irreversibly by OP^{18,21,31} and (2) are not selective since AChE is inhibited by not only OP pesticides but also by carbamate pesticides and many other compounds.

Organophosphorus hydrolase (OPH), a biological catalyst, has been shown to effectively hydrolyze a range of organophosphate esters, pesticides such as parathion, coumaphos, and acephate, and chemical warfare agents such as soman, sarin, VX, and tabun.^{1,39–44} The catalytic hydrolysis of each molecule of these compounds releases two protons. The measurement and correlation of the released protons to the OP concentration forms the basis of a potentiometric enzyme electrode. The detection scheme based on the monitoring of OPH-catalyzed hydrolysis products of OPs offers advantages over AChE-based biosensors. Unlike the AChE-based system, which responds to all types of neurotoxins and involves multiple step indirect measurement, the OPH-based system is simple, direct, and quick, measuring only the organophosphorus class of nerve agents. Additionally, the

OPH-based biosensor can potentially be used to quantify individual organophosphate pesticides when used as a detector in conjunction with an HPLC for chromatographic separation.

Recently, an OPH-based potentiometric enzyme electrode for rapid and direct determination of parathion, paraoxon, methyl parathion, and diazinon was developed.⁴⁵ It consisted of a hydrogen ion sensitive pH electrode modified by a layer of OPH immobilized by cross-linking with glutaraldehyde. Although elegant, a drawback of the enzyme electrode is the time, effort, and cost to isolate and purify the OPH.

Immobilized cells have been shown as an alternative biological transducer to enzymes. Many examples of microbial-based biosensors for a variety of applications have been reported.⁴⁶ One such recently reported microbial biosensor system for OPs was based on recombinant *Escherichia coli* cells expressing OPH intracellularly.¹⁷ It consisted of cells cryoimmobilized by entrapment in poly(vinyl alcohol) gel and packed in a column reactor upstream of a pH sensor. A limitation of the reported biosensor system was the slow response, 20 min, compared to 2 min in kinetic mode and 10 min in steady-state mode for the potentiometric OPH-based enzyme electrode. The slow response can be attributed to the various mass-transfer barriers present in the system. The most significant mass transport resistance in the above system, and for that matter in many microbial systems with intracellular enzymes,⁴⁷ is the transport of the OPs and products across the cell membrane to the cytoplasm where the expressed OPH resides. We recently showed that the uptake of parathion and paraoxon is indeed the rate-limiting step in the degradation of these pesticides by recombinant *E. coli* cells expressing OPH intracellularly.⁴⁸ Moreover, other recent reports have also shown that transport of OP nerve agents is restrictive.^{49,50} 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.⁴⁸ 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.¹¹

This paper describes the construction, characterization, and evaluation of a potentiometric microbial biosensor based on the recombinant *E. coli* cells expressing OPH on the cell surface immobilized on the surface of a pH electrode for the direct, sensitive, and rapid determination of organophosphate nerve agents.

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MATERIALS AND METHODS

Reagents. Luria-Bertani (LB) media, HEPES, potassium monobasic phosphate, potassium dibasic phosphate, and cobalt chloride were purchased from Fisher Scientific (Tustin, CA). Paraoxon, methyl parathion, diazinon, sevin, sutan, atrazine, and simazine were acquired from Supelco Inc. (Bellefonte, PA). 0.05- μm pore size Nucleopore polycarbonate membrane was purchased from Corning Costar Corp. (Cambridge, MA). All the solutions were made in distilled deionized water.

Bacterial Strains and Plasmids. *E. coli* strains XL1-Blue (*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* (r_k^- , m_k^+), *supE44*, *relA1*, *lac[F' proAB, lac^qZDM15, Tn10 (Tet^r)]*, and JM105 (*endA1*, *thi*, *rpsL*, *sbcb15*, *hsdR4*, *D(lac-proAB)*, [*F'*, *traD36*, *proAB*, *lac^qZDM15*]) were used in this study. Plasmid pOPK132 was used for expressing Lpp-OmpA-OPH on the cell surface.⁴⁸ Expression of OPH is tightly regulated by a *tac* promoter due to the presence of the *lacI^q* gene on the plasmid. Plasmid pJK33, which contains the *opd* gene from *Flavobacterium sp.* was used as a control for the production of native OPH in the cytoplasm.⁵¹

Growth Conditions. Strain bearing plasmid pOPK132 was grown in 50 mL of LB media buffered to pH 7.0 with 0.017 M KH_2PO_4 and 0.072 M K_2HPO_4 , supplemented with 100 $\mu\text{g}/\text{mL}$ ampicillin in a 250-mL Erlenmeyer flask at 37 °C and 300 rpm on an orbital incubator–shaker (Innova 4000, New Brunswick Scientific, Edison, NJ). 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. CoCl_2 (1 mM) was added to the culture 24 h after induction. After 48 h of growth, cells were harvested by centrifugation at 5000g for 10 min, washed with pH 8.5, 1 mM HEPES/150 mM NaCl/0.05 mM CoCl_2 buffer (henceforth designated as buffer A) twice, resuspended in 2 mL of buffer A, and stored at 4 °C until use.

Strain bearing plasmid pJK33 was grown as reported earlier.⁴⁵

Assembly of Microbial Biosensor. Microbial biosensor was constructed by immobilizing *E. coli* XL-1 Blue cells expressing OPH on the cell surface directly on the hydrogen ion sensing glass membrane of the pH electrode (Accumet, model 13-620-289, Fisher Scientific, Tustin, CA). A predetermined volume of the cell suspension, based on the desired cell loading, was slowly dropped at the center of the 0.05- μm polycarbonate membrane with slight suction. The cell retaining membrane was then attached to the hydrogen ion sensing glass surface of the pH electrode and held in place by an O-ring (Figure 1).

Experimental Setup and Measurement. The experimental setup is schematically depicted in Figure 1. All measurements were made in 5 mL of an appropriate buffer, maintained at the desired temperature, in a 10-mL working volume jacketed glass cell, equipped with a magnetic stirrer. The temperature of the liquid in the cell was controlled by circulating water in the cell jacket using a circulating water bath (model 1160, VWR Scientific, San Francisco, CA). OP nerve agent (5–10 μL), dissolved in pure methanol, was added to the cell and the change in potential, i.e., pH, was recorded with a pH/ion analyzer (model 255, Corning Science Products, Corning, NY) connected to a flat bed chart recorder (model BD112, Kipp and Zonen, Holland).

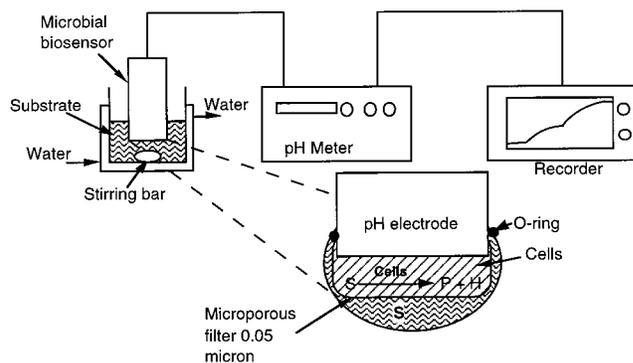


Figure 1. Schematic drawing of the potentiometric microbial biosensor.

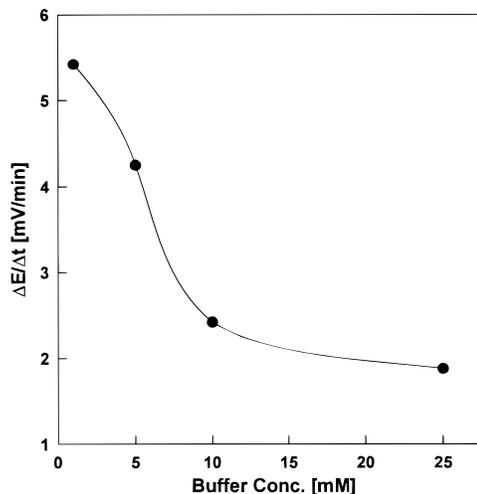


Figure 2. Effect of buffer concentration on the response of the microbial biosensor to 0.1 mM paraoxon in pH 8.5 HEPES buffer with 0.05 mM CoCl_2 at 20 °C. Cell loading, 4.5 mg.

RESULTS AND DISCUSSION

Effect of Various Parameters on the Response of the Microbial Electrode. The sensitivity, change of potential per unit change in analyte concentration, and response time of a potentiometric microbial electrode are functions of the buffer concentration, starting pH of buffer, temperature, and cell weight immobilized on the surface of the electrode. Experiments were performed to investigate the effect of these variables on the rate of change of the initial response (determined by drawing a tangent to the response curve) of the electrode to 100 μM injections of paraoxon.

Effect of Buffer Concentration. The rate of potential change is a strong inverse function of the buffer concentration (Figure 2). This inverse relationship is due to the fact that a higher concentration buffer counteracts the pH change resulting from protons released during the OPH-catalyzed hydrolysis of organophosphate nerve agents better than a lower concentration buffer. Although the magnitude, the lower detection limit, and the response time of the electrode were better in the weak buffer, the linear dynamic concentration range was better in the stronger buffer (data not shown). These results are in agreement with the observations for other potentiometric biosensors⁵² and OPH-based microbial and enzyme biosensors for OPs.^{17,45} (A total salt

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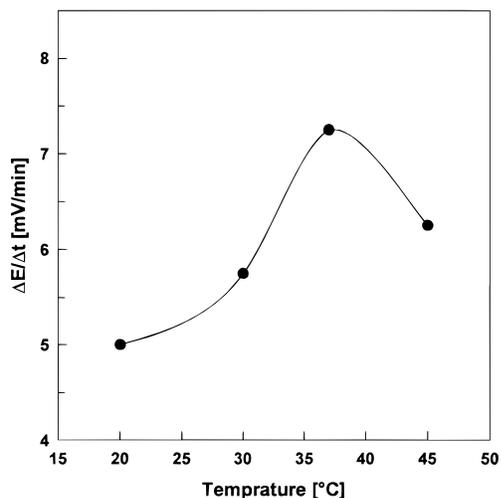


Figure 3. Effect of temperature on the response of the microbial biosensor to 0.1 mM paraoxon in pH 8.5, 1 mM HEPES/150 mM NaCl/0.05 mM CoCl₂. Cell loading, 4.5 mg.

concentration of 150 mM was maintained in this study by addition of an appropriate amount of sodium chloride to the buffer in order to prevent any adverse osmotic shock, which could rupture the cell membrane. Besides balancing the osmotic pressure, the neutral salt also aided in stabilization of weak buffers.) Since an objective of this work was to develop a rapid and sensitive biosensor for organophosphate nerve agents, 1 mM HEPES/150 mM NaCl/0.05 mM CoCl₂ buffer was selected for subsequent investigations.

Effect of Temperature. Figure 3 shows the effect of temperature on the response of the potentiometric microbial biosensor. As shown, response increased with temperature up to 37 °C and then decreased with further temperature increase. The initial increase in the rate is attributed to the increase of both the enzyme reaction and mass transport rates. The decrease in the rate at higher temperatures is due to enzyme denaturation. The optimum temperature of 37 °C for the microbial biosensor is a few degrees lower than the optimum of 45 °C for the enzyme electrode.⁴⁵ This small shift can be due to the modification/alteration in the OPH when fused to the linker Lpp-OmpA. Although 37 °C was determined to be the optimum temperature for the biosensor operation, subsequent experiments were still performed at room temperature, 20 °C. This was done in order to overcome the problem of evaporative losses during the course of the experiment and ease of operations.

Effect of Cell Loading. As can be seen in Figure 4, the rate of potential change initially increased and reached a maximum at a 2.5-mg cell loading followed by a steep decrease. This trend is similar to that reported for other microbial biosensors.^{53–55} The initial increase is attributed to an increased catalytic activity of the OPH enzyme responsible for OP hydrolysis. However, as the cell loading on the pH electrode is increased and the cells get packed more densely, forming a thicker layer, the resistance to the transport of OPs to cells embedded deeper in the immobilized layer and for the protons to reach the transducer is increased. A cell loading of 2.5-mg dry weight was used subsequently.

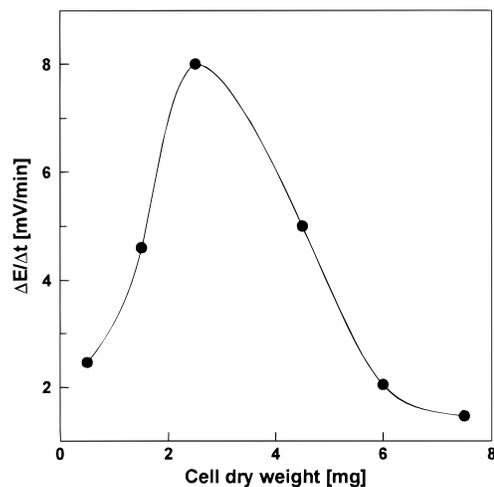


Figure 4. Effect of cell loading on the response of the microbial biosensor to 0.1 mM paraoxon in pH 8.5, 1 mM HEPES/150 mM NaCl/0.05 mM CoCl₂ at 20 °C.

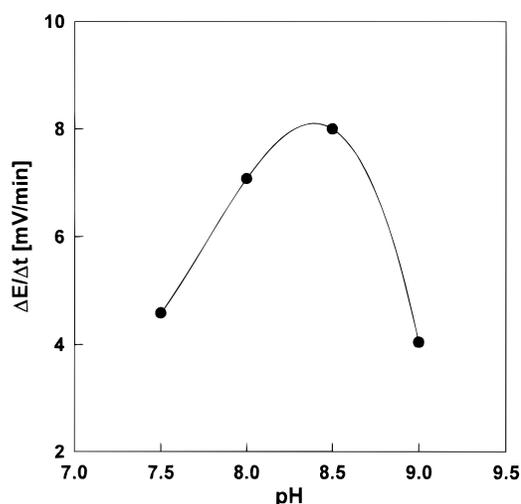


Figure 5. Effect of buffer starting pH on the response of the microbial biosensor to 0.1 mM paraoxon in 1 mM HEPES/150 mM NaCl/0.05 mM CoCl₂ buffer at 20 °C. Cell loading, 2.5 mg.

Effect of Starting pH. Figure 5 shows the pH profile for the potentiometric microbial biosensor. This profile is similar to the OPH-based potentiometric enzyme electrode and the free enzyme.⁴⁵ This suggests that the observed pH dependence of the sensor response is due to the pH dependence of the OPH activity present on the cell surface. The optimum pH for both the potentiometric enzyme electrode and potentiometric microbial electrode is 0.5 unit lower than for the free enzyme. Such a shift in optimum pH for immobilized enzymes is not uncharacteristic and can be attributed to the alteration of the physicochemical characteristics of the enzyme when immobilized.⁵⁶ pH 8.5, which gave the maximum sensitivity, lowest response time, and largest dynamic range, was selected.

Analytical Characteristics of the Microbial Electrode. *Calibration Plots for Organophosphates.* Figure 6 shows the calibration plots for paraoxon, methyl parathion, and diazinon using the potentiometric microbial biosensor (these plots were

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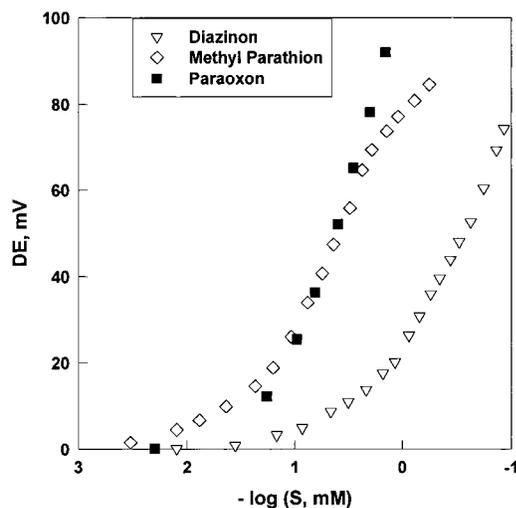


Figure 6. Calibration plots for organophosphates: Conditions: 1 mM HEPES/150 mM NaCl/0.05 mM CoCl₂, pH 8.5, 20 °C; 2.5 mg of cells.

Table 1. Analytical Characteristics of Potentiometric Microbial Biosensor

compound	max sensitivity, mV/decade concn	linear range corresponding to max sensitivity, mM	detection limit, μ M
paraoxon	90.62	0.055–1.8	2
methyl parathion	53.24	0.06–0.91	2
diazinon	45.1	0.46–8.56	5

prepared from the sensor steady-state response data). The calibration graphs are not linear. This is stated to be a general property of potentiometric biosensors.^{57,58} The nonlinearity of calibration graphs can be easily taken into consideration with computer support. The sensor operating range for these analytes spans 2 orders of magnitude. The maximum sensitivity of the microbial electrode was in the order paraoxon > methyl parathion > diazinon. Table 1 summarizes the important characteristics—maximum sensitivity, linear dynamic range corresponding to the maximum sensitivity, and lower detection limit—for the different analytes measured using the sensor.

The 2 μ M lower detection limit (3 times the standard deviation of the response obtained for a blank) of the present microbial electrode for paraoxon is comparable to the OPH-based enzyme and microbial biosensors.^{17,45} This detection limit, however, is 1–3 orders of magnitude higher than for AChE-based biosensors.^{19–38} This will therefore limit the applicability of the present sensor for environmental monitoring. For any such application of the present biosensor, off-line sample preparation involving solvent extraction and sample concentration will be necessary. The present microbial electrode, however, will be ideal for selective (see Selectivity) on-line monitoring of detoxification or disposal processes for wastewater generated during production and consumption of the organophosphate-based pesticides and insecticides and organophosphate-based nerve gases.

Selectivity. The microbial biosensor was highly selective for organophosphates. There was no interference from the other

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Table 2. Response of the Microbial Electrode to Sugars and Other Pesticides

substrate	concn, mM	ΔE , mV ^a	substrate	concn, mM	ΔE , mV ^a
paraoxon	0.04	10.16 \pm 0.24	lactose	5	0.10
glucose	5	0	atrazine	0.04	0.17 \pm 0.01
fructose	5	0.1	sevin	0.04	0.25
galactose	5	0	sutan	0.04	0.1
sucrose	5	0	simazine	0.04	0.32 \pm 0.02

^a Results are average of three individual measurements.

commonly used pesticides, atrazine, sutan, sevin, and simazine. The output voltage for 40 μ M (20-fold higher concentration than the paraoxon lower detection limit) injections of these pesticides was less than the output of 0.5 mV corresponding to the paraoxon lower detection limit (Table 2). This is a significant benefit over AChE-based biosensors, which are unable to differentiate between organophosphates and other neurotoxic compounds, especially for on-line monitoring of detoxification processes.^{19–38}

Nonspecific cellular responses generally limit the selectivity of microbial biosensors. *E. coli* can metabolize a variety of sugars to produce acidic products that can cause the pH drop. As illustrated in Table 2, sugars such as glucose, sucrose, lactose, fructose, and galactose at 125-fold higher concentrations do not interfere with the OPH-specific response of the cells.

Precision and Accuracy of the Microbial Electrode. The low relative standard deviations of 4.83 ($n = 6$) and 4.84% ($n = 6$), respectively, for 10 μ M paraoxon and methyl parathion, demonstrate the high precision of analysis. Additionally, a very low relative standard deviation of 4.8% ($n = 3$) in the response of four microbial electrodes prepared using 2.5 mg of cells to 0.1 mM paraoxon demonstrates an excellent electrode-to-electrode reproducibility.

Simulated samples representing a feed to biological (enzymatic/microbial) detoxification process^{11,12} were analyzed using the microbial electrode and conventional enzymatic assay (based on the concentration of *p*-nitrophenol formed by enzymatic hydrolysis of the three tested pesticides). A good agreement (Figure 7) between the two methods indicates the sensor is very accurate and reliable.

Stability and Response Time of the Microbial Electrode. The long-term storage and multiple-use stability of the microbial biosensor was investigated by evaluating the response of the same sensor to paraoxon and storing it at 4 °C in pH 8.5, 1 mM HEPES/150 mM sodium chloride/0.05 mM CoCl₂ buffer. As the results show (Figure 8), the biosensor was stable for over 2 months of investigation. During this period, the same sensor was used for a total of 20 times. This stability is far superior than that for the microbial biosensor prepared using *E. coli* cells carrying plasmid pJK33 that expressed native OPH intracellularly. On the basis of the comparison of stability of the two microbial sensors and the fact that purified OPH is very stable,⁴⁵ we hypothesize that expression of OPH on the cell surface (1) protects the enzyme from intracellular proteases and (2) prevents it from being washed away from the pH transducer surface in the event the cell lysis, since the cell wall debris to which the enzyme is attached is retained by the 0.05- μ m pore size membrane. In comparison, AChE-based biosensors lost as much as 40% of the original

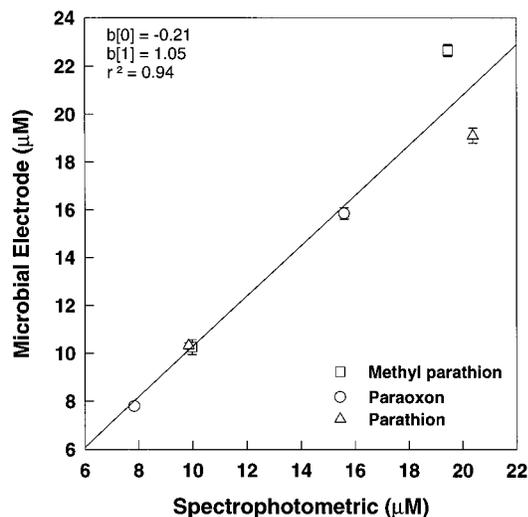


Figure 7. Comparison of the organophosphate measurement by the microbial electrode with enzyme assay technique. Results are average of three measurements.

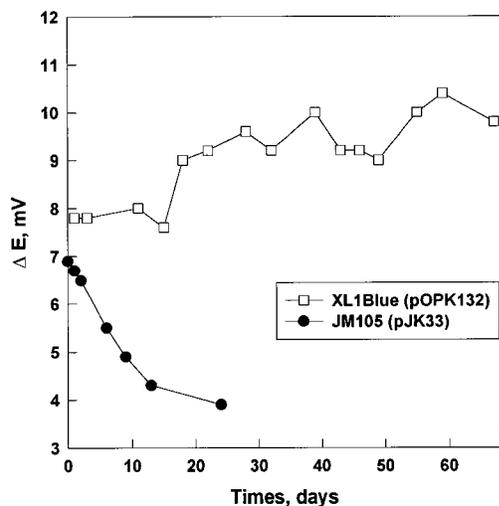


Figure 8. Stability of the potentiometric microbial biosensor. Response to 0.1 mM paraoxon in 1 mM HEPES/150 mM NaCl/0.05 mM CoCl_2 , pH 8.5 at 20 °C. Cell loading, 2.5 mg.

response after as few as three repeated uses.^{18,21} The low stability of AChE-based biosensors can be attributed to the inactivation of AChE every time the biosensor is exposed to neurotoxins.^{18,21,30}

The detection of OPs by the new microbial electrode is a simple, single-step, and direct measurement. The electrode can be operated either in steady-state or kinetic response mode, and the analysis involves a simple, single direct measurement step. The analysis times for each sample in the two modes were 10 (determined from the time required to achieve 90% of maximum response) and 2 min, respectively (data not shown). These times are comparable to the OPH-based enzyme and microbial biosensors.^{17,45} In contrast, AChE-based biosensors involve multiple steps (measurement of the initial AChE activity, incubation with the neurotoxin, determination of the inhibited enzyme activity, and regeneration of enzyme activity) and analysis times range

anywhere from 15 min for the disposable type (where the enzyme reactivation step is excluded and hence unsuitable for multiple use on-line process monitoring) to 5 h for reusable types.^{18,26,29,32,36,37}

CONCLUSIONS

A potentiometric microbial biosensor using recombinant *E. coli* cells expressing OPH on the cell surface and a hydrogen ion sensitive pH glass electrode for the direct, rapid, and selective measurement of organophosphate nerve agents was developed. The expression of the enzyme on the surface of cell membrane alleviates (1) problems of mass transport across the cell membrane encountered with microbial biosensors constructed with cells expressing enzyme intracellularly and (2) need for isolation and purification of enzyme as is the case with enzyme-based sensors and thereby reducing the overall cost. The sensor had excellent stability, short response time, reproducibility, and accuracy. These features will make it an ideal analytical tool for long-term on-line monitoring of chemical or biological detoxification processes. The applicability of the present sensor for environmental monitoring, however, will be limited to off-line analysis. This is because the concentration of OPs in environmental samples is usually below the 2 μM detection limit of the present sensor. For any such application, off-line sample preparation involving solvent extraction and concentration will be necessary. Additionally, the microbial biosensor can be used as a detector downstream of any chromatographic separation to determine concentrations of individual organophosphate nerve agents.

Unlike other microbial biosensors where either membrane transport may influence the overall response or other enzyme(s) may participate in the cascade of enzymes responsible for the final response, for the biosensor developed in this study, the response is only due to the OPH on the cell surface. Hence, for all purposes this microbial biosensor is similar to any enzyme biosensor with the cell wall basically acting as an immobilization support for the enzyme. Therefore, theoretical models for enzyme-based electrodes can be applied to predict the direction of future research to improve the sensitivity and detection limit of this microbial biosensor. Theoretical models for an enzyme-based biosensor with nonreactive transducer predict that enzyme electrode sensitivity and detection limit can be improved by either lowering the enzyme K_M or increasing the bimolecular rate constant.^{58,59} The advancements in enzyme engineering has made these goals potentially achievable. One such example is the site-directed mutagenesis of OPH in order to improve the rate of hydrolysis of the chemical warfare agent soman, recently reported.⁶⁰ Thus, it is foreseeable that a microbial biosensor of higher sensitivity and lower detection limit will be realizable by expressing an OPH variant that has a lower K_M and/or higher bimolecular rate constant for the nerve agents to be analyzed.

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