

# Whole Cell–Enzyme Hybrid Amperometric Biosensor for Direct Determination of Organophosphorous Nerve Agents With *p*-Nitrophenyl Substituent

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**Abstract:** In this paper, we reported the construction of a hybrid biosensor for direct, highly selective, sensitive, and rapid quantitative determination of organophosphate pesticides with *p*-nitrophenyl substituent using purified organophosphorus hydrolase (OPH) for the initial hydrolysis and *Arthrobacter* sp. JS443 for subsequent *p*-nitrophenol oxidation. The biocatalytic layer was prepared by co-immobilizing *Arthrobacter* sp. JS443 and OPH on a carbon paste electrode. OPH catalyzed the hydrolysis of organophosphorus pesticides with *p*-nitrophenyl substituent such as paraoxon and methyl parathion to release *p*-nitrophenol that was oxidized by the enzymatic machinery of *Arthrobacter* sp. JS443 to carbon dioxide through electroactive intermediates 4-nitrocatechol and 1,2,4-benzenetriol. The oxidation current of the intermediates was measured and correlated to the concentration of organophosphates. The best sensitivity and response time were obtained using a sensor constructed with 0.06 mg dry weight of cell and 965 IU of OPH operating at 400 mV applied potential (vs. Ag/AgCl reference) in 50 mM citrate-phosphate pH 7.5 buffer at room temperature. Using these conditions, the biosensor measured as low as 2.8 ppb (10 nM) of paraoxon and 5.3 ppb (20 nM) of methyl parathion without interference from phenolic compounds, carbamate pesticides, triazine herbicides, and organophosphate pesticides that do not have the *p*-nitrophenyl substituent. The biosensor had excellent operational lifetime stability with no decrease in response for more than 40 repeated uses over a 12-h period when stored at room temperature, while its storage life was approximately 2 days when stored in the operating buffer at 4°C. © 2004 Wiley Periodicals, Inc.

**Keywords:** OPH; amperometric; biosensor; organophosphate; pesticides; nerve agents; paraoxon; methyl parathion; *Arthrobacter* sp.

## INTRODUCTION

Organophosphorus compounds (OPs) are widely used as pesticides, insecticides and chemical warfare agents (Compton, 1988; FAO, 1989; USDA, 1992). Due to their extreme toxicity (Chapalamadugu and Chaudhry, 1992), there are urgent needs for innovative analytical tools/devices to monitor OPs at both the producer and the consumer levels effectively (LeJeune et al., 1997; Mulbury et al., 1996). Several techniques, such as chromatography, immunoassay, and enzyme biosensors based on inhibition of cholinesterase activity, for monitoring OPs have been reported. Although sensitive, these methods are time-consuming, expensive, and require skilled personnel and therefore unsuitable for on-line or field monitoring (Sherma, 1993).

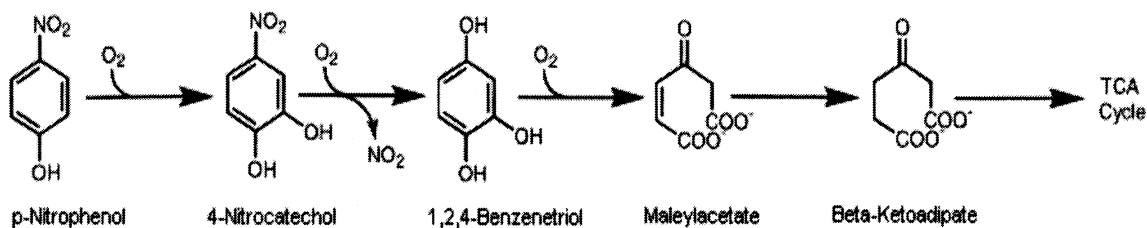
Organophosphorus hydrolase (OPH) catalyzes hydrolysis of a wide range of OP nerve agents (Donarski et al., 1989; Dumas et al., 1989a,b). Because of the superiority offered by biosensor based on catalytic reaction (direct monitoring) over the inhibition of enzyme activity (indirect monitoring), the use of OPH for developing potentiometric, optical, and amperometric biosensor based on purified enzyme or whole cells has been exploited (Mulchandani et al., 1998a,b, 1999a,b,c, 2001a,b,c; Roger et al., 1999; Wang et al., 1999). Recently, we reported OPH-based amperometric enzyme biosensors based on the electrochemical oxidation of *p*-nitrophenol (PNP), a product of OPH-catalyzed hydrolysis of *p*-nitrophenyl substituent OPs (paraoxon, methyl parathion, parathion, EPN, and fenitrothion) (Mulchandani et al., 1999c, 2001a,b). While these biosensors had improved sensitivity, their selectivity was compromised in presence of other phenols (Mulchandani et al., 2001a) and hence not suitable for most practical environmental applications.

*Arthrobacter* sp., isolated from PNP contaminated waste sites, degrades PNP through 4-nitrocatechol, 1,2,4-benzenetriol, maleylacetate, and  $\beta$ -keto adipate to tricarboxylic acid intermediates while releasing nitrite and consuming

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**Figure 1.** Proposed pathway for PNP degradation by *Arthrobacter* sp. (Jain et al., 1994).

oxygen (Fig. 1) (Jain et al., 1994). 4-Nitrocatechol and 1,2,4-benzenetriol can be electrochemically oxidized, and the oxidation current can be correlated to the concentration of PNP resulting from the OPH-catalyzed hydrolysis of OPs.

Hybrid biosensors, incorporating a tandem of biological recognition elements such as enzyme–microorganisms and enzyme–tissues, have been constructed to detect disaccharides sucrose (Barlikova et al., 1991) and lactose (Svorc et al., 1990), inorganic phosphate in drug formulations and biological fluids (Campenella et al., 1990), and improve selectivity for sucrose in samples containing glucose (Rotariu et al., 2002). In this article, the enzyme–microorganism, organophosphorus hydrolase-*Arthrobacter* sp., hybrid biorecognition element was combined with an amperometric detector to improve the selectivity and sensitivity for the detection of organophosphate nerve agents with a *p*-nitrophenyl substituent, such as paraoxon, methyl parathion, parathion, fenitrothion, and EPN, over the previously reported biosensors for these targets (Mulchandani et al., 1999c, 2001a,b). Details of construction, optimization, characterization, and application of the new hybrid biosensor are reported.

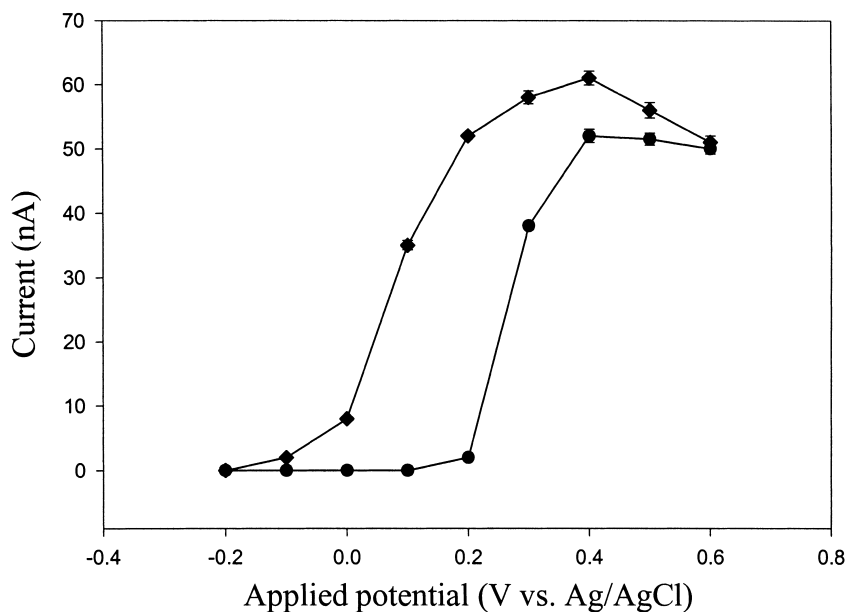
## EXPERIMENTAL

### Materials

Citric acid,  $MgSO_4 \cdot 7H_2O$ ,  $KH_2PO_4$ ,  $Na_2HPO_4 \cdot 7H_2O$ , yeast extract, graphite powder, mineral oil, phenol, and  $CaCl_2$  were purchased from Fisher Scientific (Tustin, CA). PNP, 2,4-dinitrophenol, 2-chlorophenol, 2-nitrophenol, and Nafion solution were bought from Aldrich (Milwaukee, WI).  $FeCl_3$ ,  $ZnSO_4 \cdot 7H_2O$ , and  $Na_2MoO_4 \cdot 2H_2O$  were obtained from VWR (San Diego, CA). Coumaphos, diazinon, sevin, sutan, atrazine, paraoxon, and methyl parathion were acquired from Supelco Inc. (Bellefonte, PA). Tryptic soy broth was purchased from Becton Dickinson (Sparks, MD).  $NH_4Cl$  was acquired from J.T. Baker (Phillipsburg, NJ). All solutions were prepared in distilled deionized water.

### Microorganism and Culture Conditions

*Arthrobacter* sp. JS443 was inoculated into tryptic soy broth and incubated overnight on a gyratory incubator shaker (Innova 4000, New Brunswick Scientific, Edison, NJ) at



**Figure 2.** Hydrodynamic voltammograms for 1  $\mu M$  4-nitrocatechol (◆) and 1  $\mu M$  1,2,4-benzenetriol (●) with carbon paste electrode in 50 mM pH 7.5 citrate–phosphate buffer at room temperature. Data are given as mean  $\pm$  1 SD for three experiments.

30°C and 300 rpm. Subsequently, cells were inoculated ( $OD_{600} = 1.0$ ) in minimal salts medium (Spain and Nishino, 1987) (composition per liter minimal salts medium: 112 mg of  $MgSO_4 \cdot 7H_2O$ , 5 mg of  $ZnSO_4 \cdot 7H_2O$ , 2.5 mg of  $Na_2MoO_4 \cdot 2H_2O$ , 340 mg of  $KH_2PO_4$ , 670 mg of  $Na_2HPO_4 \cdot 7H_2O$ , 14 mg  $CaCl_2$ , 0.13 mg of  $FeCl_3$ , and 0.5 g of  $NH_4Cl$ , pH adjusted to 7.0) supplemented with 0.4 mM PNP and 0.1% yeast extract and incubated at 30°C and 300 rpm for approximately 3.5–4 h until the yellow color of PNP disappeared. At this time, additional PNP (0.4 mM) was added and the sequence repeated for three more times. The cells were harvested using a refrigerated centrifuge (Model J21, Beckman Instruments, Fullerton, CA) at 4°C, followed by washing with buffer (50 mM citrate-phosphate pH 7.5) twice. The pellet was stored in refrigerator until use.

OPH was purified from recombinant *E. coli* carrying plasmid pJK 33 according to the reported protocol (Mulchandani et al., 1999a; Omburo et al., 1992) and stored at -70°C till use.

### Microbial Electrode Assembly

The carbon paste working electrode (CPE) was prepared by firmly packing a 75% (w/w) graphite powder and 25% (w/w) mineral oil carbon paste into the 3-mm diameter and 1-mm deep cavity of a Kel-F sleeve (Bioanalytical System Inc., Lafayette, IN) and polished to a smooth shiny finish by gently rubbing over a weighing paper.

The biocatalytic layer containing the immobilized cells and OPH was prepared by casting a 5- $\mu$ L droplet of 0.5% (wt.%) Nafion containing a known amount of the cells and OPH onto the carbon paste electrode tip and allowing the

solvent to evaporate. The electrode was subsequently dried completely at room temperature and kept in 4°C till use.

### Experimental Setup and Measurement

Amperometric measurements were performed using a Bioanalytical Systems (BAS) voltammetric analyzer (Model LC-4C) coupled to a chart recorder (Model BD112, Kipp and Zonen, Delft, Holland). All experiments were conducted using a three-electrode electrochemical cell (10-mL volume with a working volume of 4 mL) inside a Faraday cage (BAS, Model C2 cell stand), with an Ag/AgCl reference electrode (BAS, MF 2063), and a platinum wire auxiliary electrode (BAS, MF 1032) in appropriate electrolyte.

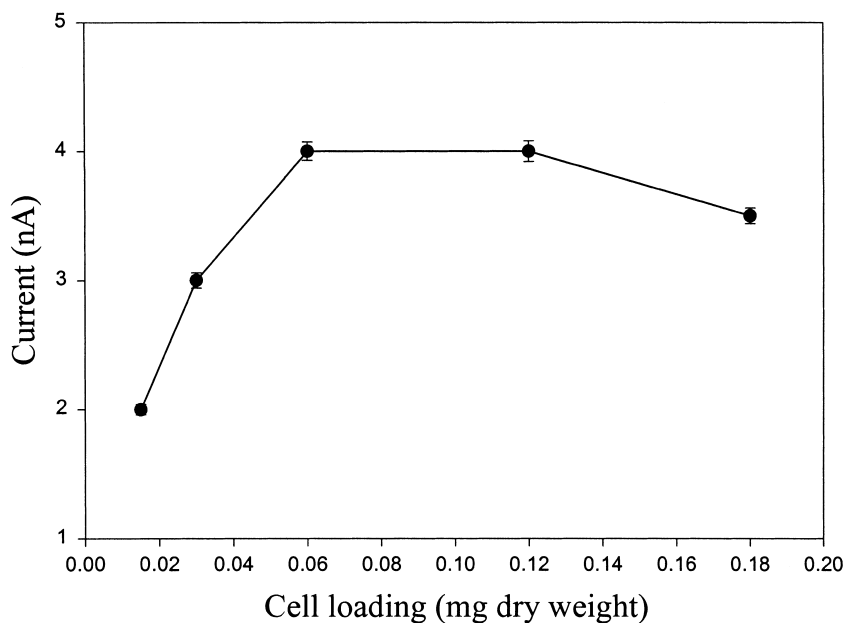
All measurements, unless stated otherwise, were performed by applying a potential of +0.4 V (vs. the Ag/AgCl reference) to the working electrode and allowing the transient background current to decay to a steady-state value, prior to the addition of 10–20  $\mu$ L of OP dissolved in pure methanol. A stirred solution was employed to provide convective transport.

## RESULTS AND DISCUSSION

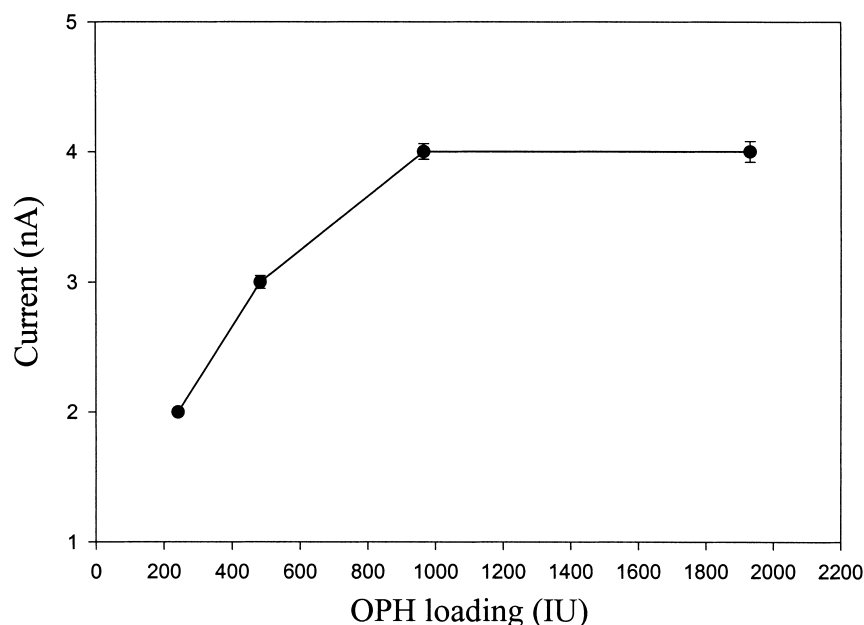
### Optimization of Operating Conditions

#### Effect of Applied Potential

Figure 2 shows hydrodynamic voltammograms for 1  $\mu$ M 4-nitrocatechol and 1  $\mu$ M 1,2,4-benzenetriol over the -0.2 to



**Figure 3.** Effect of cell loading on biosensor response to 1  $\mu$ M paraoxon with 965 IU OPH. Electrolyte, 50 mM pH 7.5 citrate-phosphate buffer; temperature, 20°C; operating potential, +0.4 V versus Ag/AgCl reference. Data are given as mean  $\pm$  1 SD for three experiments.



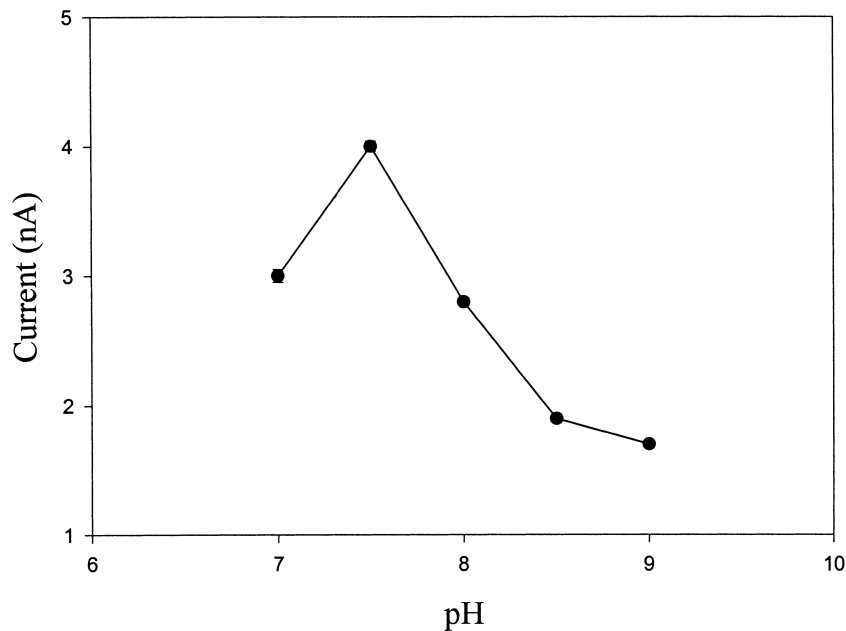
**Figure 4.** Effect of OPH loading on biosensor response to  $1 \mu\text{M}$  paraoxon with 0.06 mg dry weight of cells. Electrolyte, 50 mM pH 7.5 citrate–phosphate buffer; temperature,  $20^\circ\text{C}$ ; operating potential, +0.4 V versus the Ag/AgCl reference. Data are given as mean  $\pm$  1 SD for three experiments.

+0.6 V potential range in 50 mM pH 7.5 citrate–phosphate buffer at room temperature. The oxidation current for both 4-nitrocatechol and 1,2,4-benzenetriol increased sharply up to +0.4 V and leveled off thereafter. An applied potential of +0.4 V (vs. Ag/AgCl reference electrode) corresponding to the maximum oxidation current was used in the subsequent work. The lower operating potential of the electrode should make this amperometric biosensor less prone to interference than the earlier OPH-based amperometric biosensors operating at +0.85 V at which phenol, pentachlorophenol,

and other electroactive pollutants interfere (Mulchandani et al., 1999c, 2001a).

#### *Effect of Cell Loading*

Figure 3 shows the effect of cell loading on the response of the hybrid amperometric biosensor. The output response increased with increasing cell loading up to 0.06 mg dry weight and reached a plateau. The initial increase can be attributed to an increased catalytic activity of enzymes in



**Figure 5.** Effect of pH (50 mM citrate–phosphate) on biosensor response to  $1 \mu\text{M}$  paraoxon. Operating conditions: 0.06 mg dry weight cell loading and 965 IU OPH; temperature,  $20^\circ\text{C}$ ; operating potential, +0.4 V versus Ag/AgCl reference. Data are given as mean  $\pm$  1 SD for three experiments.

bacteria which are responsible for PNP degradation, while attainment of plateau at higher cell loading is due to the transport resistance of OP compounds and oxygen to enzyme and cells embedded deeper in the immobilized layer (Han et al., 2001). A cell loading of 0.06 mg dry weight corresponding to the maximum current output was used in the subsequent work.

### Effect of OPH Loading

The effect of the amount of OPH immobilized in the Nafion membrane on the hybrid biosensor response is shown in Figure 4. While the output response initially increased with increasing OPH loading, it reached a plateau above 965 IU. The observed response profile can be attributed to the OPH being the limiting factor initially whereas the cells being the limiting factor when the response plateau. Since OPH loading of greater than 965 IU was not beneficial this OPH amount was used for subsequent experiments.

### Effect of pH

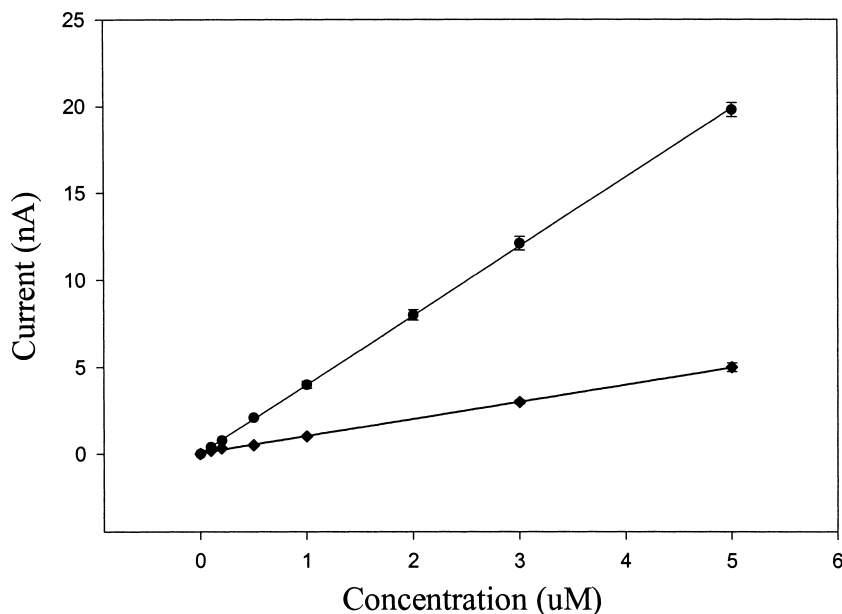
The catalytic activities of OPH and the enzymes involved in PNP catabolism are a function of the pH. As shown in Figure 5, the hybrid biosensor response was maximal at pH 7.5. The observed profile mirror the pH profile for the *Arthobacter* sp.-based amperometric biosensor for PNP. Furthermore, literature reports have shown that the maximal activities of OPH and nitrophenol oxygenase (the first enzyme involved in PNP oxidation of *Arthrobacter* sp.) are

achieved at pH of 8.5 (Mulchandani et al., 2001b) and 7.5–8 (Spain and Gibson, 1991), respectively. These suggest that the PNP oxidation enzyme is the rate-limiting biosensing molecule in the series reaction of OPH-catalyzed hydrolysis of OP and the subsequent oxidation of the resulting PNP. It should be noted that, while the activity of OPH is maximal at pH 8.5, the enzyme retains 60–80% of its maximum activity between pH 7.5 and 8. In comparison, the PNP oxygenase has only 45% of its maximum activity at the OPH optimal pH of 8.5. A pH of 7.5 was used for subsequent studies.

## Analytical Characteristics

### Calibration

Figure 6 shows dependence of the hybrid biosensor response on paraoxon and methyl parathion concentrations. As shown, the plots were linear up to 5  $\mu\text{M}$  with sensitivities (slope) of 3.979 nA per  $\mu\text{M}$  ( $R^2 = 0.9999$ ) and 0.989 nA per  $\mu\text{M}$  ( $R^2 = 0.9995$ ) for paraoxon and methyl parathion, respectively. The lower detection limits (defined as 3 times the standard deviation of the response obtained for a blank) of the present hybrid electrode for paraoxon and methyl parathion were 10 nM (2.8 ppb) and 20 nM (5.3 ppb), respectively. These are 9- and 3.5-fold, respectively, lower than the OPH-based amperometric biosensor based on measurement of PNP electrooxidation (Mulchandani et al., 1999c, 2001a,b) and are comparable to the lower detection limit for the AChE-based biosensor.



**Figure 6.** Calibration plots for paraoxon (●) and methyl parathion (◆). Electrolyte, 50 mM pH 7.5 citrate–phosphate buffer; temperature, 20°C; biocatalytic layer, 0.06 mg dry weight of cells and 965 IU OPH; operating potential, +0.4 V versus Ag/AgCl reference. Data are given as mean  $\pm$  1 SD for three experiments.

**Table I.** Hybrid amperometric biosensor selectivity.

Compound	Concentration ( $\mu\text{M}$ )	Current (nA)
Paraoxon	1	4
Methyl parathion	1	1
Coumaphos	1	0
Diazinon	1	0
Sutan	1	0
Sevin	1	0
Atrazine	1	0
Phenol	1	0.6
2-nitrophenol	1	0
<i>p</i> -chlorophenol	1	0.6
2,4-dinitrophenol	1	1

### Selectivity

The hybrid biosensor selectivity was evaluated against a range of compounds. As shown in Table I, there is no interference from different phenolic compounds and other commonly used pesticides, such as atrazine, sutan, sevin, coumaphos, and diazinon, which belong to three different groups of pesticides, i.e., triazine (atrazine), carbamates (sutan and sevin), and OP group without *p*-nitrophenol substituent (coumaphos and diazinon). This is a marked improvement over AChE-based biosensors, which are unable to differentiate between organophosphates and other neurotoxic compounds such as carbamate pesticides, triazine herbicides, heavy metals, etc., the potentiometric and optical OPH-based biosensors that are unable to differentiate between subclasses of OPs (Mulchandani et al., 1998a,b, 1999a,b) and OPH-based amperometric biosensors that are interfered by phenolic compounds. Additionally, as demon-

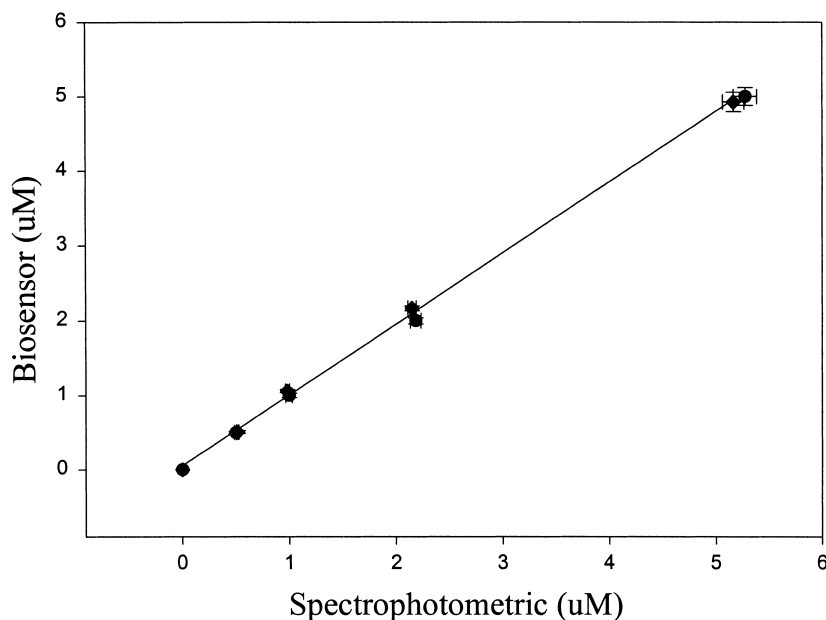
strated previously, combining the OPH-based amperometric detector with OPH-based potentiometric biosensor will enhance the information content allowing discrimination between the two subclasses of OPs (Wang et al., 2002). The ability to discriminate between the OPs with and without a PNP substituent is potentially helpful as the chemicals with a PNP substituent are more toxic than the ones without.

Because nonspecific cellular responses to substrate(s) and intermediates of microbial catabolism can limit the selectivity of microbial-based biosensors, it is necessary to evaluate interference from sugars and organic acids. No interference from sugars such as glucose and sucrose and organic acid, sodium succinate, at 25-fold higher concentration was observed (data not shown).

### Precision and Accuracy

The response of the hybrid biosensor is very reproducible as demonstrated by the low relative standard deviations of 1.39% ( $n = 6$ ) for 1  $\mu\text{M}$  paraoxon and 2.63% ( $n = 6$ ) for 1  $\mu\text{M}$  methyl parathion. Additionally, there is an excellent electrode-to-electrode reproducibility as characterized by the very low relative standard deviations of 3.56% ( $n = 6$ ) and 3.89% ( $n = 6$ ) in the response of six microbial biosensors prepared at different times using different batches of cells to 1  $\mu\text{M}$  paraoxon and methyl parathion, respectively.

The accuracy of the newly developed hybrid biosensor was determined by comparing concentrations of paraoxon and methyl parathion determined by the biosensor (using the calibration curves in Fig. 6) with conventional enzymatic assay (based on the measurement of PNP (at 410 nm) formed by the OPH catalyzed hydrolysis). As



**Figure 7.** Accuracy of amperometric hybrid biosensor for paraoxon (●) and methyl parathion (◆). Slope = 0.946,  $R^2 = 0.9989$ . Electrolyte, 50 mM pH 7.5 citrate–phosphate buffer; temperature, 20°C; biocatalytic layer, 0.06 mg dry weight of cells and 965 IU OPH; operating potential, +0.4 V versus Ag/AgCl reference. Data are given as mean  $\pm$  1 SD for three experiments.

shown in Figure 7, there is an excellent agreement between the two methods indicating a high degree of accuracy and reliability of the biosensor.

### Response Time and Stability

The detection of paraoxon and methyl parathion with the new hybrid amperometric biosensor is simple and rapid. Even though the detection involves a cascade of enzyme-catalyzed reactions the analysis time for each sample was less than 5 min, which is significantly shorter than the long incubation period required for AChE-based biosensor (Paddle, 1996; Palchetti et al., 1997). The rapid response is attributed to the single-step, and direct and reversible measurement of the OPs from the biocatalytic action of OPH on OPs compared to the multisteps (measurement of initial AChE activity, washing, incubation with inhibitor, washing, measurement of the AChE residual activity, incubation with pyridine-2-aldoxime to reactivate the enzyme and washing) and indirect (the response is not a result of direct biocatalytic action of the enzyme, as with OPH) measurement in the AChE-based biosensors.

The new hybrid biosensor had excellent operational stability as evident from the low RSD of 4.9% of current output for 42 repeated analyses of 0.1  $\mu\text{M}$  paraoxon over a 12-h period; the biosensor was stored in the operating buffer at room temperature in between analyses. The repeated use capability of the hybrid biosensor is a significant advantage over the AChE-based biosensors that are either single use or have limited reuse because of incomplete regeneration of the AChE activity even after treatment with reactivating agent 2-pyridine aldoxime (La Rossa et al., 1994; Skladal, 1991) an attribute necessary for on-line monitoring application.

To determine the long-term storage stability the biosensor response was measured once every 24 h, with hour zero as the initial response, and stored at 4°C in the operating buffer (pH 7.5, 50 mM citrate-phosphate buffer). The sensor response was stable for a period of 48 h (2 days) and then dropped sharply (data not shown). Based on the literature information of the excellent stability, more than a month, for OPH-based biosensors (Mulchandani et al., 1998a,b, 1999a,b, 2001a,b) the short life-span of the hybrid biosensor may be attributed to the drop of *Arthrobacter* sp. respiratory activity probably from the depletion of the NAD(P)H pool in the resting/non-growing cells (Han et al., 2001; Jain et al., 1994). Addition of NAD(P)H to the storage buffer, however, did not reactivate the biosensor response, probably because the whole cell could not intake NADPH.

### CONCLUSIONS

In conclusion, a whole cell-enzyme hybrid amperometric biosensor combining *Arthrobacter* sp. and OPH for the direct measurement of paraoxon and methyl parathion was developed. This very simple and low-cost sensor had excellent sensitivity, precision, accuracy, response time, and selectivity for paraoxon and methyl parathion over the other

phenolic compounds, organophosphates without nitrophenyl substituent, carbamates, and triazines. While the amperometric concept has been illustrated in connection with the biosensing of paraoxon and methyl parathion, it can also be applied to other nitrophenyl-substituted OP pesticides, such as parathion, fenitrothion, and ethyl *p*-nitrophenol thiobenzenephosphonate (EPN). Further, work done in our laboratories has shown that the matrix of river (Rio Grande, Las Cruces, NM) and lake (Lake Elsinore, CA) water did not have any effect on the response of the OPH- and *Arthrobacter*-based biosensors for OPs and PNP, respectively (Lei et al., 2003; Mulchandani et al., 1999c). These features make it a potentially ideal analytical tool for on-line monitoring of effluents from the chemical processing facilities producing and using OP pesticides with *p*-nitrophenol substituent and environmental samples after preconcentration with commonly used solid phase extraction. Additionally, as demonstrated earlier (Wang et al., 2002), combining this amperometric biosensor with OPH-based potentiometric biosensor will greatly enhance the information content giving information on the total OPs and the fraction of PNP containing OPs.

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