Biosensor for Direct Determination of Fenitrothion and EPN Using Recombinant *Pseudomonas putida* JS444 with Surface Expressed Organophosphorus Hydrolase. 1. Modified Clark Oxygen Electrode

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**Abstract:** This paper reports a first microbial biosensor for rapid and cost-effective determination of organophosphorus pesticides fenitrothion and EPN. The biosensor consisted of recombinant PNP-degrading/oxidizing bacteria *Pseudomonas putida* JS444 anchoring and displaying organophosphorus hydrolase (OPH) on its cell surface as biological sensing element and a dissolved oxygen electrode as the transducer. Surface-expressed OPH catalyzed the hydrolysis of fenitrothion and EPN to release 3-methyl-4-nitrophenol and *p*-nitrophenol, respectively, which were oxidized by the enzymatic machinery of *Pseudomonas putida* JS444 to carbon dioxide while consuming oxygen, which was measured and correlated to the concentration of organophosphates. Under the optimum operating conditions, the biosensor was able to measure as low as 277 ppb of fenitrothion and 1.6 ppm of EPN without interference from phenolic compounds and other commonly used pesticides such as carbamate pesticides, triazine herbicides and organophosphate pesticides without nitrophenyl substituent. The applicability of the biosensor to lake water was also demonstrated.

**Keywords:** Organophosphorus, fenitrothion, EPN, biosensor, *Pseudomonas putida.*
Introduction

Organophosphate pesticides fenitrothion and EPN are widely used for agricultural purpose in many countries, such as China, Brazil, Japan and Australia, due to their low cost and broad spectrum activity. Like other organophosphates, they are neurotoxic, inhibiting the activity of cholinesterase and over-stimulating the nervous system causing nausea at lower level exposure, and even death at higher and prolonged exposure [1,2]. Because of increasing concerns over agricultural worker health and its potential environmental impacts, there is a growing interest to develop novel analytical methods capable of performing rapid detection of these compounds in the field.

Many analytical methods, including gas and liquid chromatographic technologies [3-7], immunoassays [8-11], and biosensors based on cholinesterase or alkaline phosphatase inhibition [12-15], have been reported for the determination of organophosphorus pesticides. Although very sensitive, these methods are not satisfactory for rapid and on-line and in-field monitoring.

With the discovery of organophosphorus hydrolase (OPH), an enzyme that can hydrolyze a wide range of organophosphate pesticides releasing detectable products [16,17], several enzyme and microbial biosensors based on OPH for rapid, simple, selective monitoring of these neurotoxic pesticides with the potential for in the field analysis have been reported [18-29].

Recently, OPH was functionally expressed onto the surface of a natural p-nitrophenol (PNP) degrader, Pseudomonas putida JS444, using an ice nucleation protein (INP) anchor, creating a single microorganism with the capability to rapidly degrade organophosphate pesticides and PNP simultaneously while consuming oxygen [30]. Using this genetically engineered Pseudomonas putida we modified the Clark dissolved oxygen electrode, to construct a simple microbial biosensor for rapid, cost-effective, selective, precise and accurate determination of organophosphates with PNP substituent and demonstrated the application to paraoxon, parathion and methyl parathion monitoring [31]. In this communication, we extend the application of this biosensor to two other OPs with nitrophenyl substituent, fenitrothion and EPN under the optimum operating conditions reported before and demonstrate the applicability of the microbial biosensor for the rapid, cost-effective, selective, precise and accurate detection of fenitrothion and EPN.

Materials and methods

Materials, bacterial strains and growth conditions

All chemical reagents and the details of the recombinant PNP-degrader Pseudomonas putida JS444 anchoring and displaying OPH on the cell surface and its growth conditions used in this study have been described elsewhere [30].

Microbial electrode assembly

A predetermined amount of the cell suspension, based on the desired cell loading, was slowly dropped on a 25 mm diameter 0.4 µm pore size Nucleopore polycarbonate membrane (Whatman, NJ, USA) with slight suction. The cell retaining membrane was then placed on the top of the Teflon
membrane of the oxygen electrode (Model YSI 5331, Yellow Springs, OH, USA) and fixed in place by a rubber O-ring. The cells were thus immobilized (entrapped) between the two membranes.

Experiment set-up and measurement

The experimental set-up used in this research was identical to the one reported earlier [32]. All measurements were made at our reported optimized conditions with 0.086 mg dry weight of cell loading, operating in 50 mM pH 7.5 citrate-phosphate buffer with 50 µM CoCl₂ at room temperature [31]. The steady-state output of the oxygen electrode (after 5 minutes) was measured using a digital biological oxygen monitor (model YSI 5300, Yellow springs, Ohio, USA) connected to a chart recorder.

Results and discussion

Analytical characteristics

Figure 1A shows the dependence of the biosensor response on fenitrothion and EPN concentrations in operating buffer. As shown, the biosensor had a wide dynamic range with the oxygen consumption and a linear function of the pesticide concentrations up to 0.05 mM. The sensitivity (slope) of the microbial biosensor was 0.20% oxygen consumed per µM and 0.10% oxygen consumed per µM for fenitrothion and EPN, respectively.

\[
\begin{align*}
\text{Fenitrothion:} & \quad y = 0.20x + 0.19 \\
\text{EPN:} & \quad y = 0.10x + 0.03
\end{align*}
\]

\[
\begin{align*}
\text{R}^2 & = 0.9983 \\
\text{R}^2 & = 0.9985
\end{align*}
\]

Figure 1. Calibration plots for fenitrothion and EPN. A) in 50 mM pH 7.5 citrate-phosphate buffer with 50 µM CoCl₂ and B) in Lake Elsinore water filtered and adjusted to pH 7.5 and 50 µM CoCl₂, at room temperature, with 0.086 mg cell loading. Data are given as mean ± 1 standard deviation for three experiments.
The lower limit of detection (LOD), determined as 3 times the standard deviation of the signal for buffer (blank), were 277 ppb of fenitrothion and 1.6 ppm of EPN, respectively. While comparable to the lower detection limit for biosensors based on cholinesterase and alkaline phosphatase inhibition [12-15], they were 2~3 orders of magnitude higher than those for immunoassays [8-11] and gas and liquid chromatography methods [3-7]. 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 solid-phase extraction and sample concentration will be necessary. The present microbial electrode, however, will be ideal for selective on-line monitoring wastewater generated during production and consumption of the organophosphate pesticides (OP) and chemical or biological methods for treatment of OP-contaminated wastewaters.

In order to evaluate the matrix effect of naturally occurring compounds in real samples, the microbial biosensor was applied to measure target compounds spiked in lake water from Lake Elsinore, CA, after the lake water filtered through a 0.22 µm membrane and adjusted to pH 7.5 and 50 µM CoCl₂. The sensitivity of the biosensor response was similar to that observed in synthetic samples (Fig. 1B), thus demonstrating there was no interference from the components such as chlorophyll, phosphorus, nitrogen and several metal ions in the water from Lake Elsinore, CA, and the applicability of the microbial biosensor for organophosphate pesticides contaminated wastewaters. Additionally, as reported previously, the biosensor response was not interfered by 20-fold higher concentrations of sugars such as sucrose, fructose and galactose and other organic compounds such as glycerol and sodium acetate. However, high concentrations of glucose and lactic acid interfered. This interference is, however, not a concern as these compounds are not expected to be present in samples of interest [31].

Reproducibility is an important parameter for the sensor performance. The microbial biosensor demonstrated a very good reproducibility as evidenced by the low relative standard deviations (n=6) of 4.4% and 4.8% for 50 µM fenitrothion and EPN, respectively. Additionally, there was an excellent electrode-to-electrode reproducibility as characterized by the low relative standard deviations of 5.0% and 5.4% in the response of six microbial biosensors prepared at different times using different batches of cells to 50 µM fenitrothion and EPN, respectively.

In order to validate the microbial sensor, simulated samples (prepared by addition of OP compounds into buffer) representing OP-contaminated water were analyzed using the microbial biosensor and compared to a spectrophotometric assay based on the measurement of PNP (at 412 nm) formed by the OPH-catalyzed hydrolysis of OPs. As shown in Figure 2, there was an excellent agreement between the two methods confirming the accuracy and reliability of the developed microbial biosensor.

The detection of fenitrothion and EPN with the novel microbial biosensor is simple, direct, single step and rapid. The analysis time for each sample was less than 5 min, which is significantly shorter than the hours required for gas and liquid chromatography and immunoassays. The biosensor also shows excellent precision and selectivity described in our previous report [31]. When stored at operating buffer at 4 °C, the sensor response was stable for a period of 5 days. The rapid drop of the respiratory activity after 5 days is hypothesized to be a result of the depletion of the NAD(P)H in the resting/non-growing cells [33,34].
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Figure 2. Accuracy of microbial biosensor. Fenitrothion and EPN as target compounds and operating in 50 mM pH 7.5 citrate-phosphate buffer with 50 μM CoCl₂ at room temperature, with 0.086 mg cell loading. Data are given as mean ± 1 standard deviation for three experiments.

Conclusions

In conclusion, a novel microbial biosensor using recombinant Pseudomonas putida JS444 and a Clark dissolved oxygen electrode for the direct, rapid and selective measurement of fenitrothion and EPN was reported. As a first reported microbial biosensor for fenitrothion and EPN, this very simple and low cost sensor showed excellent precision, accuracy, short response time and selectivity. These features make it a potentially attractive analytical tool for on-line monitoring of fenitrothion and EPN.

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