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Microbial biosensor for direct determination of nitrophenyl-substituted organophosphate nerve agents using genetically engineered *Moraxella* sp.

Priti Mulchandani, Wilfred Chen, Ashok Mulchandani *

Department of Chemical and Environmental Engineering, University of California, Riverside, CA 92521, USA

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Abstract

A microbial biosensor consisting of a dissolved oxygen electrode modified with the genetically engineered PNP-degrader *Moraxella* sp. displaying organophosphorus hydrolase (OPH) on the cell surface for sensitive, selective, rapid and direct determination of *p*-nitrophenyl (PNP)-substituted organophosphates (OPs) is reported. Surface-expressed OPH works in tandem with the PNP oxidation machinery of the *Moraxella* sp. to degrade PNP-substituted OPs and PNP simultaneously while consuming oxygen, that is proportional to the analyte concentration. The optimum performance was obtained by electrodes constructed using 0.35 mg dry weight of cell and operating at pH 7.5. Operating at optimum conditions the biosensor was able to measure as low as 0.1 μM (27.5 ppb) of paraoxon and had excellent selectivity against triazines, carbamates and OPs without PNP substitutent. The biosensor was stable for a week when stored at 4 °C. The applicability of the biosensor to measure OPs in lake water was demonstrated.
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Keywords: Nerve agents; Pesticides; Organophosphorus hydrolase; Microbial biosensor; *Moraxella* sp.

1. Introduction

Because of their high efficiency, low bioaccumulation and relatively short half-life in the environment parathions, ethyl and methyl, fenitrothion and ethyl *p*-nitrophenol thiobenzenephosphonate (EPN) are used worldwide as agriculture pesticides and insecticides [1]. The large volumes of high concentration (1–10,000 ppm) wastes produced from the extensive use of these pesticides are environmental and health concern [2–4]. Decisions to dispose, reuse or recycle as make-up water of these wastes have to be made at the site of use. This requires simple, rapid, cost-effective and reliable analytical device for their determination in the field.

Analytical methods such as gas, liquid and thin-layer chromatography and immunoassays are widely used for the determination of organophosphorus compounds. While very sensitive and selective these techniques are not amenable for on-field analysis, as they are either time consuming, require expensive instrumentation or require highly trained technicians [4–6].

Biosensors are well-suited to meet the demands of on-field monitoring. Several biosensors based on cholinesterase and organophosphorus hydrolase have been reported. The former, based on the inhibition of cholinesterase activity, while extremely sensitive suffers from poor selectivity, irreversible response or a multiple step protocol (involving activity measurement, incubation with sample, washing, measurement of residual activity and regeneration of activity) [7–9]. The class of biosensors based on OPs as substrates of organophosphorus hydrolase (OPH) as opposed to inhibitors is single step, simple, rapid, reversible and selective for OPs [10]. However, the lower sensitivity of potentiometric and optical biosensors and poor selectivity of amperometric biosensors over phenolic compounds limit their applications in environmental monitoring.

Several microorganisms capable of degrading *p*-nitrophenol (PNP) while releasing nitrite and consuming oxygen have been isolated from PNP contaminated environments [11–14]. Thus, combining PNP degrader and OPH integrated with an appropriate transducer can provide the basis of an elegant biosensor for PNP-substituted OPs. Recently, a hybrid microbial-enzyme biosensor combining purified OPH with PNP-degrader *Arthobacter* sp. was reported [15]. While elegant, a better alternative to the hybrid biorecognition element

* Corresponding author. Tel.: +1 951 827 6419; fax: +1 951 827 5696.
E-mail address: adani@engr.ucr.edu (A. Mulchandani).

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will be a single biological recognition element with dual functionality.

Moraxella sp. is an efficient PNP degrader that metabolizes PNP specifically. It was utilized to construct an amperometric microbial biosensor for selective detection of PNP [16], The bacterium has been genetically engineered to express functional OPH onto its cell surface using an ice nucleation protein (INP) anchor resulting in a single microorganism capable of degrading OPs with a PNP moiety and PNP simultaneously [17]. This new capability provides a unique biological recognition molecule that can be integrated with an appropriate transducer for selective, simple and rapid detection of PNP-substituted OPs.

In this paper we report the development, characterization and application of a microbial biosensor for PNP-substituted OPs such as ethyl parathion, methyl parathion, fenitrothion and EPN based on genetically engineered *Moraxella* sp. expressing OPH on the cell surface that is able to simultaneously degrade both organophosphate pesticide and PNP integrated to a Clark dissolved oxygen electrode. The use of this recombinant microorganism as the biorecognition element provided a simpler sensor construction protocol and also a more cost-effective analytical device because of the elimination of purified OPH which is labor intensive and time consuming. Paraoxon was used as a model for PNP-substituted OPs used as pesticides and insecticides, to demonstrate the proof-of-concept of the microbial biosensor.

2. Materials and methods

2.1. Materials

Yeast extract, phenol, K_2HPO_4, KH_2PO_4, $MgSO_4$, $FeSO_4$, Na_2HPO_4 and NaH_2PO_4 were purchased from Fisher Scientific (Tustin, CA, USA). NH_4CI was bought from J.T. Baker (Phillipsburg, NJ, USA). Tryptic soy broth was purchased from Becton Dickinson (Sparks, MD, USA). PNP and paraoxon were obtained from Aldrich (Milwaukee, WI, USA). Coumaphos, diazinon, sutan and atrazine were acquired from Supelco Inc. (Bellefonte). Polycarbonate membrane (Nucleopore, 50 nm pore size, 25 mm diameter disc) was obtained from Whatman (Clifton, NJ, USA). All the solutions were made in distilled deionized water.

2.2. Microorganism and culture conditions

Moraxella sp. isolated from activated sludge by selective enrichment with PNP [11] harboring the plasmid pPNCO33 for expressing OPH on the bacterium surface using an INPNC anchor was used in this study [17]. This resulted in a single microorganism capable of degrading organophosphate pesticides and PNP simultaneously.

Strain bearing plasmid pPNCO33 was grown in tryptic soy broth supplemented with 50 μg of kanamycin overnight in a 250-ml Erlenmeyer flask at 30 °C and 300 rpm on an orbital incubator-shaker (Innova 4000, New Brunswick Scientific, Edison, NJ). Subsequently, the cells were used to inoculate (OD_{600} = 0.05) pH 7.2 minimal salt medium (3.73 mM K_2HPO_4, 1.25 mM KH_2PO_4, 1.4 mM NH_4Cl, 0.4 mM $MgSO_4·7H_2O$ and 0.02 mM $FeSO_4·7H_2O$) supplemented with 0.4 mM PNP and 0.2% yeast extract. The cells were incubated at 30 °C and 300 rpm. Once the OD_{600} of cell suspension reached 0.2, the culture was induced for the expression of OPH on the cell surface with 1 mM IPTG. When the yellow color of PNP disappeared in approximately 9 h, 0.4 mM PNP was added three more times. The cells were harvested using a refrigerated centrifuge (Model J21, Beckman Instruments, CA, USA) at 4 °C, followed by washing with buffer (50 mM citrate phosphate pH 7.5) twice. The pellet was resuspended in appropriate volume of buffer to obtain a suspension of known cell concentration (determined using dry weight and optical density calibration plot generated for this organism). Cells were stored in refrigerator until use.

2.3. Microbial electrode assembly

A known and desired weight of cell suspension in buffer was dripped onto a 25 mm diameter 0.05 μm pore size polycarbonate membrane with slight suction. The cells retaining membrane was then attached to the surface of the Teflon membrane of the dissolved oxygen electrode (Model 5332, Yellow Springs Instrument, Yellow Springs, OH, USA) and held in-place by a rubber O-ring. The cells were thus immobilized (entrapped) between the two membranes.

2.4. Experimental set-up and measurement

The experimental set-up used in the study consisted of a 10 ml detection cell in which the microbial electrode described earlier (Section 2.3) was inserted through a hole in the rubber stopper, a magnetic stirrer, an oxygen monitor (Model 5300, Yellow Springs Instruments) and a chart recorder.

The measurements were made in 3 ml of pH 7.5, 50 mM citrate phosphate buffer that was saturated with oxygen by bubbling air using sparger until an equilibrium/steady state response of 100% dissolved oxygen was established. At this time, 20–30 μl of a known concentration of paraoxon solution was added and the changes in dissolved oxygen were recorded.

3. Results and discussion

3.1. Optimization of operating conditions

The response of the microbial biosensor was optimized as a function of the amount of cells immobilized on the transducer and operating buffer pH by measuring the amount of oxygen consumed in response to 0.05 mM of paraoxon at room temperature.

As shown in Fig. 1 the biosensor response increased initially with the increase of cell loading, reaching a maximum at 0.35 mg of cell dry weight loading followed by a gradual decrease. The trend was similar to reported for other microbial biosensors [18,19]. The initial increase of biosensor response is attributed to an increased catalytic activity with increasing cell amount, while the transport resistance of substrate and oxygen to cells embedded deeper in the immobilized layer is responsible

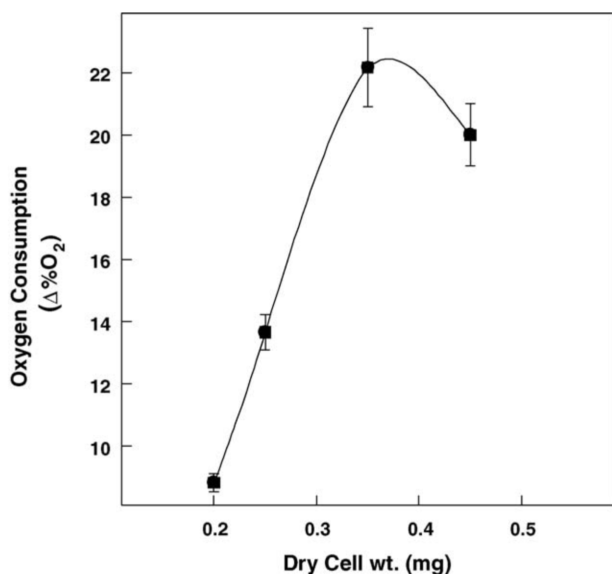

Fig. 1. Effect of cell loading on biosensor response to 0.05 mM paraoxon in 50 mM pH 8.0 citrate-phosphate buffer at 20 °C. Each point represents an average of three measurements and the error bars represent ±1S.D.

for the decrease at higher cell loading. A cell loading of 0.35 mg dry weight was used for the subsequent studies.

Fig. 2 shows the effect of operating pH on the microbial biosensor response. As shown, the biosensor response was maximum at pH 7.5. Studies have shown that the OPH has the maximum activity at pH 8.5 [10,20,21] and nitrophenol monooxygenase, the first enzyme involved in PNP oxidation pathway of *Moraxella* sp., has the maximum activity at pH 7.5 [12,18]. The pH optimum of 7.5 for detecting paraoxon suggests that the PNP oxidation enzyme is the rate-limiting biosensing molecule in this biosensor. The pH of 7.5 was used in the further studies.

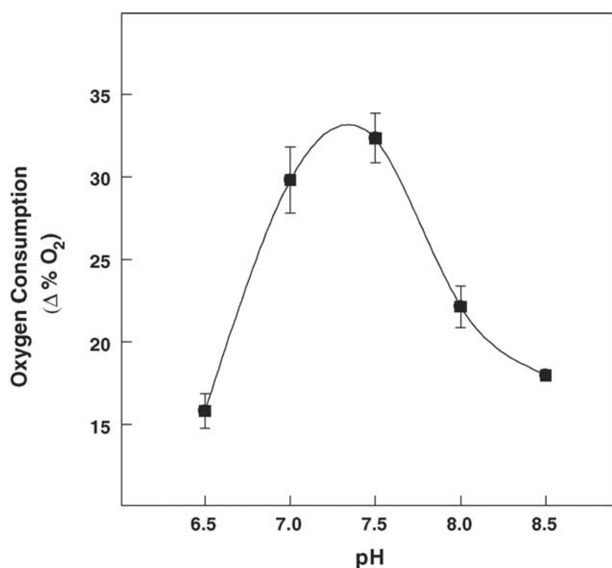

Fig. 2. Effect of pH (50 mM citrate-phosphate) on biosensor response to 0.05 mM paraoxon with 0.35 mg cell loading at 20 °C. Each point represents an average of three measurements and the error bars represent ±1S.D.

3.2. Analytical characteristics

3.2.1. Calibration

When operated at the optimum conditions determined above (pH 7.5, 50 mM citrate phosphate buffer and 0.35 mg cell loading), the response of the microbial biosensor (Δ% O_2 consumed) was a linear function of the paraoxon concentration up to 0.05 mM (13.75 ppm) with a corresponding sensitivity (calibration plot slope) of 0.61 Δ% O_2 consumed/μM paraoxon ($r^2 = 0.999$). The lower detection limit (defined as three times the standard deviation of the response obtained for a blank) of the microbial electrode for paraoxon was 0.1 μM (27.5 ppb). The LOD was 2-fold lower than for oxygen consumption-based microbial biosensor using *Pseudomonas putida* and amperometric microbial biosensor using the genetically engineered *Moraxella* sp. (used in this study) detecting the PNP released from OPH catalyzed hydrolysis of paraoxon as biosensing element and 20-fold lower than OPH enzyme-based potentiometric biosensors [20,22–24]. This detection limit, however, was 1–2 orders of magnitude higher than that for acetylcholinesterase (AChE) inhibition-based biosensor, immunoassay, and gas, liquid and thin-layer chromatography [5,9]. 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 extraction and sample concentration will be necessary. The present microbial electrode, however, will be ideal for selective on-line monitoring of detoxification or disposal process for wastewater generated during production and consumption of the organophosphate-based pesticides and insecticides.

To evaluate the matrix effect of naturally occurring compounds in real samples the response of the microbial biosensor to paraoxon spiked in Lake water from Lake Elsinore, CA (filtered and pH adjusted to 7.5 from original 9.2) was measured. As shown in Table 1, the sensitivity of the biosensor response was similar to that observed in synthetic sample. No interference from the components of Lake Elsinore, CA such as phosphorus, nitrogen, metal ions and chlorophyll [25], demonstrated the applicability of the microbial biosensor for organophosphate pesticide contaminated wastewaters.

3.2.2. Selectivity

The biosensor selectivity was evaluated against a range of compounds. As illustrated in Table 2, there was no interference from the widely used pesticides such as atrazine, sutan and even other organophosphate insecticides such as coumaphos and diazinon, that do not produce PNP upon hydrolysis. This is a benefit of the present biosensor over the AChE-based biosensors,

Table 1
Effect of Lake Elsinore water components on microbial biosensor response

Paraoxon added (μM)	Buffer (Δ% O_2)	Lake water (Δ% O_2)
5	3.50	3.48
10	6.25	6.18
20	12.00	11.67

Table 2
Microbial biosensor selectivity

Compound	Concentration (mM)	Biosensor response (Δ% O_2)
Paraoxon	0.05	32
Coumaphos	0.05	0
Diazinon	0.05	0
Sutan	0.05	0
Atrazine	0.05	0

which are unable to differentiate between organophosphates and other neurotoxic compounds [5,9].

Non-specific cellular responses to substrate(s) and intermediates of microbial catabolism can limit the selectivity of microbial biosensors. As reported earlier [20] no interference from sugars such as sucrose, fructose and galactose and salts of acetic and citric acids at 100-fold higher concentrations was observed (data not shown).

3.2.3. Response time and stability

The newly constructed microbial biosensor is simple to use and has a short response time. The analysis time for each sample was less than 5 min. This is an advantage when compared to hours required for immunoassays and AChE inhibition methods [4,5]. The rapid response is attributed to the close proximity of the surface-expressed OPH and the membrane associated nitrophenol monooxygenase in *Moraxella* sp.

To investigate the long-term storage stability and multiple uses capability, the microbial biosensor was used once every 24 h to measure the response to 0.05 mM paraoxon in triplicate and then stored at 4 °C in 50 mM citrate phosphate buffer, pH 7.5. As demonstarted in Fig. 3, there was a less than 10% loss of its original response over the one week period in which the biosensor was used a total of 20 times. This stability is superior to the *Arthobacter* sp. and *P. putida*-based microbial sensors for OPs [15,22].

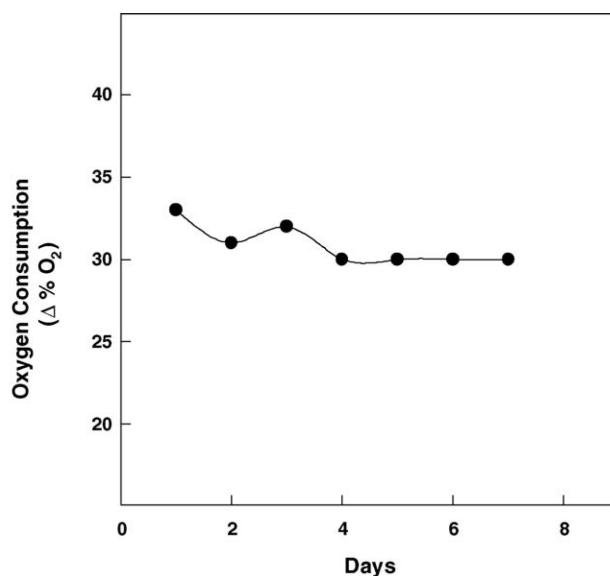

Fig. 3. Stability of microbial biosensor; response to 0.05 mM paraoxon in 50 mM pH 7.5 citrate-phosphate buffer at 20 °C with 0.35 mg cell loading.

3.2.4. Precision and accuracy

The microbial biosensor showed excellent precision as demonstrated by the low relative standard deviation of 3.5% ($n = 10$) for 0.05 mM paraoxon. Additionally, the biosensor had very good electrode to electrode reproducibility as demonstrated by the low standard deviation of 4.71% in the response of three microbial electrodes prepared using 0.35 mg of the cells at different times using different lots of cells to 0.05 mM paraoxon.

To confirm the biosensor accuracy, four different paraoxon concentrations were analyzed by the microbial biosensor (concentration of paraoxon was determined using the calibration relationship of Δ% O_2 consumed = 0.61 × paraoxon concentration in μM, established above) and compared to conventional spectrophotometric assay based on the measurement of PNP formed by the enzymatic hydrolysis (concentration of PNP was determined using the extinction coefficient of 17,000 at λ_{max} of 412 nm). A slope of 1.001 with regression coefficient of the correlation of 0.999 (data not shown) between the two methods demonstrated the excellent accuracy and reliability of the biosensor.

4. Conclusions

In conclusion, a microbial biosensor based on Clark dissolved oxygen electrode modified with genetically engineered PNP-degrader *Moraxella* sp. endowed with OPH activity was developed for the direct determination of PNP-substituted OPs. This newly developed biosensor had excellent sensitivity, precision, accuracy and selectivity for paraoxon over other organophosphates without nitrophenyl substituent, carbamates and triazines. Further, the matrix of real water sample (water from Lake Elsinore) did not have any effect on biosensor response for OPs. The above features make this microbial biosensor an ideal analytical tool for field monitoring of organophosphate contaminated waters and on-line monitoring of effluents from facilities producing and consuming these pesticides.

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