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Short communication

Amperometric microbial biosensor for *p*-nitrophenol using *Moraxella* sp.-modified carbon paste electrode

Priti Mulchandani, Carlos M. Hangarter, Yu Lei, Wilfred Chen, Ashok Mulchandani*

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

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Abstract

An amperometric microbial biosensor for highly specific, sensitive and rapid quantitative determination of *p*-nitrophenol was developed. The biosensor takes advantage of the ability of *Moraxella* sp. to specifically degrade *p*-nitrophenol to hydroquinone, a more electroactive compound than *p*-nitrophenol. The electrochemical oxidation current of hydroquinone formed in biodegradation of *p*-nitrophenol was measured at *Moraxella* sp.-modified carbon paste electrode and correlated to *p*-phenol concentrations. The optimum response was realized by electrode constructed using 15 mg of dry cell weight per 1 g of carbon paste and operating at 0.3 V (versus Ag/AgCl reference) in pH 7.5, 20 mM sodium phosphate buffer. Operating at these optimum conditions the biosensor had excellent selectivity against phenol derivatives and was able to measure as low as 20 nM (2.78 ppb) *p*-nitrophenol with very good accuracy and reproducibility. The biosensor was stable for approximately 3 weeks when stored at 4 °C. The applicability of the biosensor to measure *p*-nitrophenol in lake water was demonstrated.

Keywords: Amperometric; Microbial biosensor; *p*-Nitrophenol; *Moraxella* sp.

1. Introduction

Aromatic nitrocompounds such as nitrobenzene, nitrotoluenes and nitrophenols are found widely as environmental contaminants in freshwater, in marine environments and in the atmosphere. They have been used commonly in the manufacture of explosives, pesticides, dyes, plasticizers and pharmaceuticals (Hallas and Alexander, 1983; Hanne et al., 1993; Munnecke, 1976; Spain and Gibson, 1991). *p*-Nitrophenol, because of its toxicity is listed as U.S. Environmental Protection Agency priority pollutant. Acute (short-term) inhalation or ingestion of *p*-nitrophenol in humans causes headaches, drowsiness, nausea, and cyanosis (blue color in lips, ears, and fingernails). The detection of this compound is therefore a matter of concern for environmental control.

Chromatography (gas, liquid and capillary electrophoresis), enzyme-linked immunosorbent assay (ELISA) and electroanalytical techniques (polarography, cyclic voltammetry, adsorptive stripping and differential pulse voltammetry) are among the many techniques reported for *p*-nitrophenol determination (Clement et al., 1995; Sherma, 1993; Zietek, 1975; Barek et al., 1994; Hernandez et al., 1993; Rodriguez et al., 1997a,b,c). Although sensitive, these techniques have limitations that limit their applicability to on-line or field monitoring (Nistor et al., 2001).

Recently, we reported microbial biosensors incorporating *p*-nitrophenol degraders *Moraxella* sp. and *Arthrobacter* sp. in conjunction with a Clark oxygen electrode correlating the respiratory activities of these microorganisms to the *p*-nitrophenol concentration (Mulchandani et al., 2002; Lei et al., 2003). Both these microorganisms oxidize *p*-nitrophenol specifically using a cascade of enzymes through a series of intermediates consuming oxygen while releasing nitrite (Jain et al., 1994; Spain and Gibson, 1991; Spain

* 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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et al., 1979; Mulchandani et al., 2002). While extremely selective, the biosensors could only detect concentrations higher than 100 nM (14 ppb). In this paper we report a biosensor for *p*-nitrophenol based on the amperometric determination of the metabolic intermediate hydroquinone during the specific oxidation of *p*-nitrophenol by 4-nitrophenol monooxygenase of *Moraxella* sp. (Spain and Gibson, 1991; Spain et al., 1979; Mulchandani et al., 2002) that can be electrooxidized at a relatively low potential of 300 mV (versus Ag/AgCl reference). The amperometric measurement at this low potential resulted in a lower detection limit comparable to that obtained during detection based on electrooxidation of *p*-nitrophenol at the significantly higher applied potential of 900 mV (versus Ag/AgCl reference) while retaining the selectivity obtained in the Clark oxygen electrode modified *Moraxella* sp. case. The details of biosensor operating conditions optimization, analytical characterization and the application for measurement of *p*-nitrophenol spiked in surface water samples from Lake Elsinore, CA, are reported.

2. Materials and methods

2.1. Materials

Yeast extract, phenol, K_2HPO_4, KH_2PO_4, $MgSO_4$, $FeSO_4$, Na_2HPO_4, NaH_2PO_4, graphite powder and mineral oil (white, light) were purchased from Fisher Scientific (Tustin, CA, USA). NH_4Cl was bought from J.T. Baker (Phillipsburg, NJ, USA). Tryptic soy broth was purchased from Becton Dickinson (Sparks, MD, USA). *p*-Nitrophenol, Chlorophenol, 3-nitrophenol, 2-nitrophenol, 2,4-dinitrophenol, and 3-methyl-4-nitrophenol were obtained from Aldrich (Milwaukee, WI, USA). All the solutions were made in distilled deionized water.

2.2. Microorganism and culture conditions

Moraxella sp. isolated from activated sludge was received from Dr. Jim C. Spain (US Airforce Research Laboratory, Tyndall Airforce Base, FL). This *Moraxella* sp. is reported to degrade *p*-nitrophenol using the following enzymes: *p*-nitrophenol monooxygenase, benzoquinine reductase, hydroquinone dioxygenase, hydroxymuconic semialdehyde dehydrogenase and maleylacetate reductase (Spain and Gibson, 1991). *Moraxella* sp. stock culture maintained on tryptic soy broth was inoculated into tryptic soy broth and incubated overnight on a gyratory incubator shaker (Innova 4000, New Brunswick Scientific, Edison, NJ, USA) at 30 °C and 300 rpm. Subsequently, these cells were inoculated ($OD_{600} = 0.1$) in 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 $MgSO_4 \cdot 7H_2O$ and 0.02 mM $FeSO_4 \cdot 7H_2O$) supplemented with 0.4 mM *p*-nitrophenol and 0.2% yeast extract. The cells were incubated at 30 °C and 300 rpm until the yellow color of *p*-nitrophenol disappeared in approximately 5 h. At this time, additional PNP (0.4 mM) was added and the sequence repeated for three more times over 1.25 h. The cells were harvested using a refrigerated centrifuge (Model J21, Beckman Instruments, CA, USA) at 4 °C, followed by washing with buffer (20 mM sodium phosphate, pH 7.5) twice, resuspended in 20 mM sodium phosphate buffer, pH 7.5, and stored at 4 °C. Appropriate amount of cell suspension (corresponding to the desired cell weight) was then dried at 25 °C under vacuum and mixed in carbon paste.

2.3. Electrode fabrication

The working carbon paste electrode was prepared by mixing appropriate weight of cells with 1 g of carbon paste containing 75% (w/w) graphite powder and 25% (w/w) mineral oil. The whole cell modified carbon paste was subsequently packed firmly into the electrode cavity (3 mm diameter × 1 mm deep) of a Kel-F sleeve (Bioanalytical System Inc., Lafayette, IN) and polished to a smooth shiny finish by gently rubbing over an ordinary weighing paper. The electrode was stored at 4 °C until use.

2.4. Experimental set-up and measurement

Amperometric measurements were done using a Bioanalytical Systems (BAS) voltammetric analyzer (Model LC-4C) coupled to a chart recorder (Model BD 112, Kipp and Zonen, Holland). All experiments were conducted using a three-electrode electrochemical cell (10 ml volume with a working volume of 3 ml), with an Ag/AgCl reference electrode (BAS, MF 2063), and a platinum wire auxiliary electrode (BAS, MF 1032). A small magnetic stirrer and bar provided the convective transport, 0.3 mM of NADPH and 0.03 mM of FAD was added to the cell. When the baseline was established then 5–10 μl of *p*-nitrophenol was added to the cell and the steady-state current was recorded.

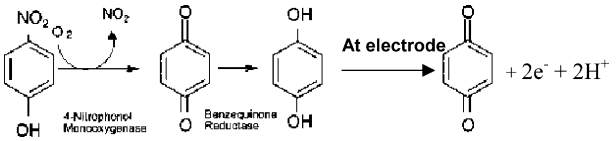
3. Results and discussion

3.1. Optimization of operational conditions

The parameters of applied potential, amount of biocatalyst, and operating pH, affecting the response of microbial biosensor were optimized using 0.05 mM *p*-nitrophenol.

3.1.1. Hydrodynamic voltammogram for hydroquinone

A review of the *p*-nitrophenol degradation/oxidation pathway in *Moraxella* sp. indicates the presence of intermediates benzoquinone and hydroquinone, compounds that are known to be electroactive (Spain et al., 1979; Spain and Gibson, 1991; Mulchandani et al., 2002). The former is an oxidized compound and can be electroreduced, while the later is a reduced compound that can be electrooxidized (Eq. (1)).



(1)

As shown in Fig. 1, the electrooxidation current response of the intermediate hydroquinone increased as a function of applied potential to reach a maximum at 300 mV (versus Ag/AgCl) and plateaued. The potential of 0.3 V was used in the subsequent studies.

3.1.2. Effect of cell loading

The effect of cell loading on the response of the amperometric microbial biosensor is shown in Fig. 2. The biosensor response increased with increased cell loading. This trend differed from the normally observed profile of the current response passing through a maximum for microbial biosensors (Lei et al., 2003; Mulchandani et al., 1998, 2002). Increasing the cell loading beyond 15 mg, however, increased the background current and also the signal was very noisy. Therefore, 15 mg of dry cell weight per 1 g of carbon paste was selected for subsequent experiments.

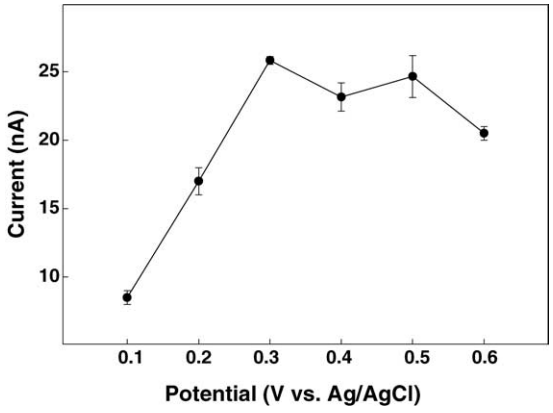


Fig. 1. Hydrodynamic voltammogram for 1 μM hydroquinone at the carbon paste electrode in 50 mM, pH 7.0, citrate–phosphate buffer at 20 °C. Data are given as mean ± 1S.D. for three measurements.

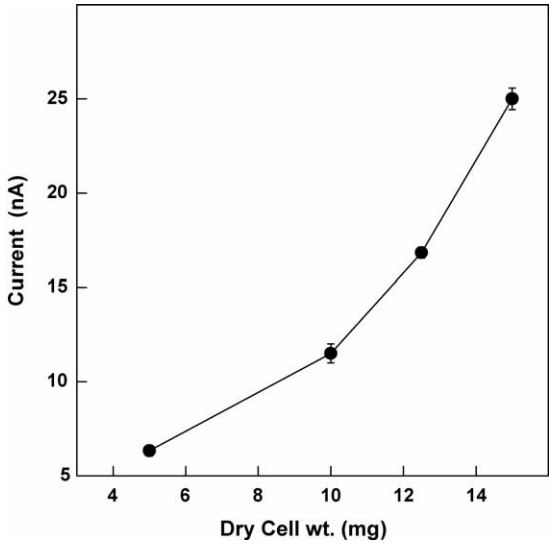


Fig. 2. Effect of cell loading on biosensor response to 0.05 mM *p*-nitrophenol in 20 mM sodium phosphate buffer, pH 7.0, at 20 °C. Data are given as mean ± 1S.D. for three measurements.

3.1.3. Effect of pH

The catalytic activities of the enzymes involved in *p*-nitrophenol catabolism are a function of the pH. Therefore, it is necessary to determine the optimum pH that will result in the maximum sensitivity of the amperometric microbial biosensor. To establish the optimum conditions, the effect of pH on biosensor was investigated from pH 6.5 to 8.5 and the highest response was achieved at pH 7.5 (data not shown). The pH profile and optimum were in accordance with that reported by Spain and Gibson (1991) for nitrophenol oxygenase, the first enzyme involved in *p*-nitrophenol oxidation pathway of *Moraxella* sp. The pH of 7.5 was therefore used for subsequent studies.

3.2. Analytical characteristics of the microbial electrode

3.2.1. Calibration

The calibration plot generated using the optimum conditions determined above (pH 7.5, 20 mM sodium phosphate buffer, 15 mg cell loading per 1 g of carbon paste and working electrode poised at 0.3 V) is shown in Fig. 3 (the plot was prepared from the sensor steady-state response data). The biosensor response was linear up to 20 μM (2.78 ppm) with a sensitivity (slope) of 1.93 nA per μM *p*-nitrophenol (r^2 = 0.994). The lower detection limit (defined as three times the standard deviation of the response obtained for a blank)

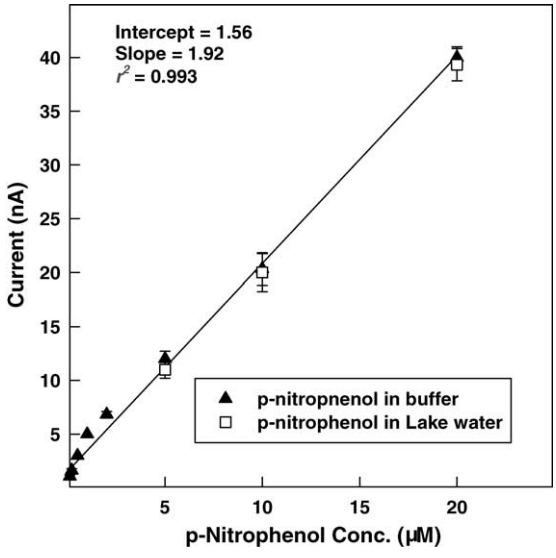


Fig. 3. Calibration plot for *p*-nitrophenol microbial biosensor: in 20 mM, pH 7.5, sodium phosphate buffer (▲) and filtered and pH adjusted Lake Elsinore water (□) at 20 °C with 15 mg cell loading. Data are given as mean ± 1S.D. for three measurements.

Table 1
Microbial biosensor selectivity

Compound	Concentration (mM)	Biosensor response (current, nA) $n = 3$
p-Nitrophenol	0.05	40 ± 1.4
Chlorophenol	0.05	1.0 ± 0
3-Nitrophenol	0.05	1.0 ± 0.04
2-Nitrophenol	0.05	1.0 ± 0
Phenol	0.05	1.0 ± 0
2,4-Dinitrophenol	0.05	0 ± 0
3-Methyl-4-nitrophenol	0.05	40 ± 1.5

was 20 nM (2.78 ppb). The LOD was 5- and 10-fold lower than that for oxygen consumption based microbial biosensors using *Moraxella* and *Arthrobacter*, respectively, as biosensing elements (Mulchandani et al., 2002; Lei et al., 2003). However, it is 1–2 orders of magnitude higher than that for competitive flow-immunoassay with fluorescence detection (Nistor et al., 2001). This detection limit should make the biosensor suitable for selective on-line monitoring wastewater generated during production and consumption of the *p*-nitrophenol and chemical or biological methods for treatment of *p*-nitrophenol contaminated wastewaters. For environmental monitoring application, however, sample pretreatment involving extraction and sample concentration will be necessary.

3.2.2. Selectivity

Selectivity is an important criterion for any analytical tool. As shown in Table 1, the microbial electrode was highly specific for *p*-nitrophenol. Even molecularly similar compounds, chlorophenol, 3-nitrophenol, 2-nitrophenol, phenol and 2,4-dinitrophenol, did not interfere. This high degree of selectivity is a significant advantage over other methods of determination such as, amperometry based on oxidation of *p*-nitrophenol that is performed at a much higher potential of 0.9 V (versus Ag/AgCl reference) when phenol and other substituted phenols interfere (Mulchandani et al., 2001a,b) or differential pulse voltammetry when oxygen, species containing nitro group and Cu(II) interfered (Hu et al., 2001; Cordero-Rando et al., 1999). As expected, the biosensor responded to 3-methyl-4-nitrophenol, a *p*-nitrophenol derivative.

The microbial biosensor was also evaluated for matrix effect of naturally occurring compounds in real samples. *p*-Nitrophenol spiked in lake water from Lake Elsinore, CA (filtered and pH adjusted from original 9.2 to 7.5) was measured. As shown in Fig. 3, the sensitivity (slope of the line) of the biosensor response in the lake water was similar to that in the buffer; validating the potential utility of the present biosensor for PNP contaminated wastewaters.

3.2.3. Response time and stability

For field monitoring, a biosensor should be simple to operate, have short response time, have excellent multiple use capability and have good long-term storage stability. The short analysis time of less than 5 min per sample in conjunction with simple protocol for the present biosensor are significant advantages of the reported biosensor over the more tedious and time consuming (hours) immunoassay technique (Oubiña et al., 1999).

The newly developed biosensor had excellent long-term storage stability and multiple use capability. The microbial electrode response was stable for approximately 3 weeks period during which it was used a total of 20 times (data not shown). This stability is far superior than that for oxygen consumption based microbial biosensors using *Arthrobacter* and *Moraxella* (Lei et al., 2003; Mulchandani et al., 2002).

3.2.4. Precision and accuracy

The biosensor had very good precision as evidenced by the low relative standard deviation of 3.77% ($n = 8$) of the biosensor response to eight repeated measurements of 0.05 mM *p*-nitrophenol. Additionally, there was a very good electrode-to-electrode reproducibility as evidenced by the low standard deviation of 4.49% ($n = 5$) in the response of five microbial biosensors prepared at different times using different batches of cells to 0.05 mM *p*-nitrophenol.

The microbial biosensor accuracy was evaluated by comparing the concentration determined to the independent absorbance spectroscopy method (measuring absorbance at 412 nm). A slope of 1.013 with regression coefficient of the correlation of 0.999 between the two methods demonstrated the excellent accuracy and reliability of the biosensor (figure not shown).

4. Conclusions

In conclusion, we have taken the advantage of the specific degradation of *p*-nitrophenol by *Moraxella* sp. to hydroquinone to develop an amperometric microbial biosensor using for sensitive, selective and rapid determination of *p*-nitrophenol. The biosensor exhibited excellent stability, precision, accuracy, short response time and selectivity for *p*-nitrophenol over other structurally similar compounds. This very simple and low cost sensor does not require trained personnel and expensive instrument. These features make it a potentially attractive analytical tool for on-line monitoring of effluents from the chemical processing facilities producing and using *p*-nitrophenol and field-monitoring of environmental samples after conventionally employed simple solid phase extraction pretreatment protocol.

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