Feature Article

A Microbial Biosensor for *p*-Nitrophenol Using *Arthrobacter* Sp.

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Received: July 11, 2002
Final version: August 19, 2002

Abstract
This article reports the construction, optimization of performance variables and analytical characterization of a sensitive and selective microbial amperometric biosensor for measurement of *p*-nitrophenol (PNP), a U.S. Environmental Agency priority pollutant. The biosensor consisted of PNP-degrading/oxidizing bacteria *Arthrobacter* sp. JS443 as biological sensing element and a dissolved oxygen electrode as the transducer. The best sensitivity and response time were obtained using a sensor constructed with 1.2 mg dry wt. of cells and operating in pH 7.5, 50 mM citrate-phosphate buffer. Using these conditions, the biosensor was able to measure as low as 28 ppb (0.2 μM) of PNP selectively without interference from structurally similar compounds, such as phenol, nitrophenols and chlorophenols. The service life of the microbial biosensor is around 5 days when stored in the operating buffer at 4 °C. The applicability to lake water is demonstrated.

Keywords: Biosensor, 4-Nitrophenol, *Arthrobacter* sp.

1. Introduction

p-Nitrophenol (PNP) is used in the manufacturing of one of the most popular analgesics (acetaminophen), pesticides and dyes and processing of leather. Identified as a carcinogen, mutagen and cyto- and embryotoxic to mammals, it has been found in 113 of the 1,416 National Priorities List sites identified by the U.S. Environmental Protection Agency [1]. The potential for accidental releases during these industrial processing activities requires analytical tools/devices to monitor PNP on-line.

Several chromatographic and immunoassay techniques for monitoring PNP have been reported. Although sensitive and selective, they are time-consuming, expensive and require skilled personnel and therefore are unsuitable for on-line or field monitoring [2]. The electrochemical technique of differential pulse voltammetry has been used to measure PNP in seawater and can potentially be used for on-line monitoring [3]. The method, however, requires an oxygen free sample to alleviate interference from dissolved oxygen. Additionally, other nitro aromatic compounds such as 2-nitrophenol, etc., interfere.

Microbial biosensors are cheap and easy to operate and hence are suitable for on-line process control and environmental monitoring. Several microbial biosensors, such biological oxygen demand, cyanide, etc., have been developed for these applications [4].

Microorganisms that degrade PNP have been isolated from PNP contaminated waste sites. Jain et al. described the isolation of *Arthrobacter* sp. that degrades PNP through 4-nitrocatechol, 1,2,4-benzenetriol, maleylacetate and β-ketoadipate to tricarboxylic acid intermediates while releasing nitrite and consuming oxygen (Figure 1) [5]. Thus, one can consider using the PNP degrading *A.* sp. as the biological recognition element of a biosensor. In this article, we report on the construction, characterization and evaluation of a microbial biosensor based on *A.* sp. immobilized on the surface of an oxygen electrode for the simple, sensitive, rapid and cost-effective determination of PNP.

2. Experimental

2.1. Materials

Citric acid, $MgSO_4$, KH_2PO_4, Na_2HPO_4, phenol, yeast extract and $CaCl_2$ were purchased from Fisher Scientific (Tustin, CA, USA). $FeCl_3$, $ZnSO_4$ and Na_2MoO_4 were obtained from VWR (San Diego, CA, USA). Tryptic soy broth was purchased from Becton Dickinson (Sparks, MD, USA). NH_4Cl was acquired from J. T. Baker (Phillipsburg, NJ, USA). PNP, 2-nitrophenol and 3-nitrophenol were bought from Aldrich (Milwaukee, WI, USA). 50 nm pore size Nucleopore polycarbonate membrane was acquired from Whatman (Clifton, NJ, USA). *A.* sp. JS443 was obtained from Dr. J. C. Spain, Air Force Engineering and Service Center, Tyndall Air Force Base. All solutions were prepared in distilled deionized water. The lake water was collected from Lake Elsinor, CA, USA.

2.2. Microorganism and Culture Conditions

A. sp. JS443 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

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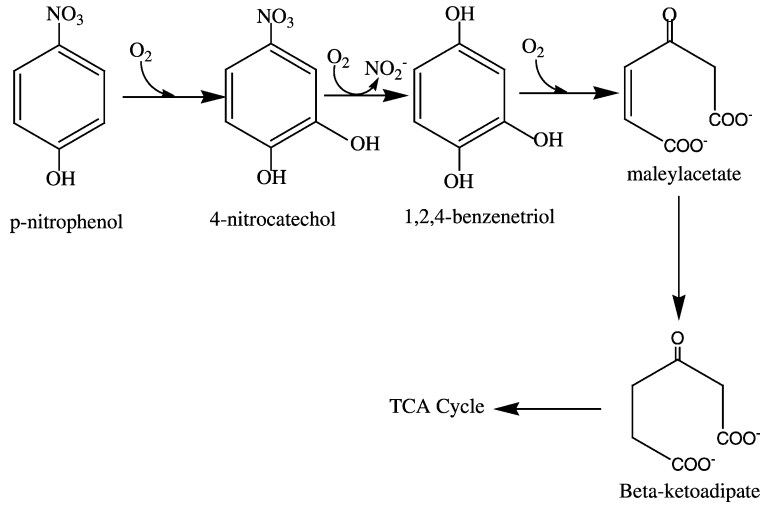

Fig. 1. Proposed pathway for PNP degradation [5].

and 300 rpm. Subsequently, these cells were inoculated ($OD_{600} = 1.0$) in Minimal Salts Medium [6] supplemented with 0.4 mM PNP and 0.1% yeast extract. The cells were incubated at 30 °C and 300 rpm until the yellow color of PNP disappeared; in approximately 3.5 – 4 hours. At this time additional PNP (0.4 mM) was added and the operation repeated three more times. The cells were then harvested by centrifuging (Model J21, Beckman Instruments, CA, USA) at 4 °C, followed by washing with buffer (50 mM citrate-phosphate pH 7.5) twice. Finally, 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) and stored at 4 °C until used.

2.3. Microbial Electrode Assembly

A predetermined amount of the cell suspension, based on the desired cell loading, was slowly dripped on a 25 mm diameter 50 nm pore size Nucleopore polycarbonate membrane 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.

2.4. Experiment Set-Up and Measurement

The experimental setup used in this research was identical to the one reported earlier [2]. All measurements were made in 4 mL of pH 7.5, 50 mM citrate-phosphate buffer, saturated with oxygen by bubbling air using a sparger, in a 10-mL jacketed glass cell, equipped with a magnetic stirrer. The cell temperature was controlled by circulating water in the cell jacket using a circulating water bath. Twenty to thirty μL of a known concentration PNP solution was added to the cell and the output of the oxygen electrode was measured using a digital biological oxygen monitor (Model YSI 5300, Yellow Springs, OH, USA) connected to a chart recorder.

3. Results and Discussion

3.1. Optimization of Operating Conditions

The response of a microbial biosensor is a function of the time of the cell harvest, the amount of cells immobilized on the transducer, operating buffer pH and temperature. Experiments were performed to investigate the effect of these variables on the oxygen consumed to 0.05 mM of PNP.

3.1.1. Effect of Cell Harvesting Time

The respiratory activity of cells to degrade a compound is a function of the activity of the enzymes involved in the metabolism. The activities of the enzymes involved are in turn a function of the incubation time of the cells. To investigate this effect, *A.* sp. JS 443 cells cultured for different time periods were harvested and the respiratory activity to metabolize PNP was measured using freely suspended cells. As shown in Figure 2, the cell density and respiratory activity of *A.* sp. JS 443 increased with the culturing time and reached a plateau after three more additions of 0.4 mM PNP to the cell culture. Cells generated after four additions of 0.4 mM PNP were used for biosensor construction.

3.1.2. Effect of Cell Loading

Figure 3 shows the effect of the cell loading on the response of the microbial biosensor. As expected, the response initially increased and reached a maximum at a 1.2 mg cell loading followed by a gradual decrease. The initial increase is attributed to an increased catalytic activity of enzyme

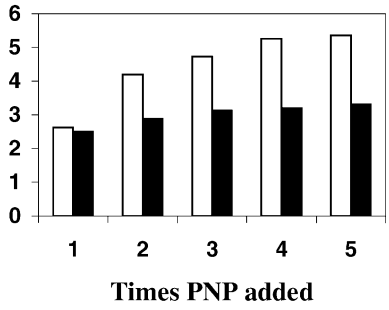

Fig. 2. Effect of culturing time on the respiratory activity of *A.* sp. JS 443. Response of 1 mg of wet cells suspended in 50 mM pH 7.5 citrate-phosphate buffer at 25 °C to 0.05 mM PNP. (□) Free cell response to 50 μM PNP in $\%O_2$ consumed/min/mg cells; (■) cell density at OD_{600}.

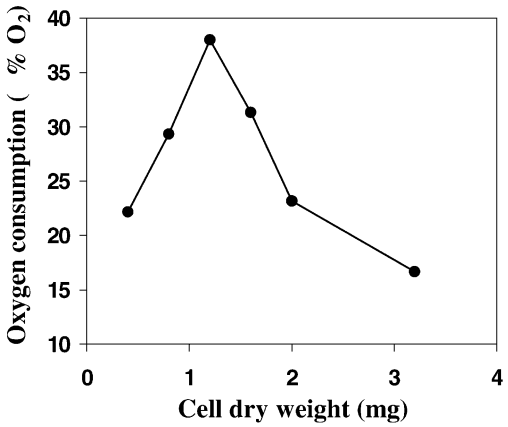

Fig. 3. Effect of cell loading on biosensor response to 0.05 mM PNP in 50 mM pH 7.5 citrate-phosphate buffer at 25 °C.

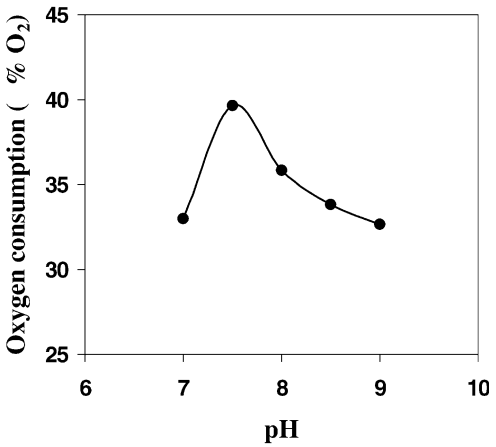

Fig. 4. Effect of pH (50 mM citrate-phosphate) on biosensor response to 0.05 mM PNP with 1.2 mg cell loading at 25 °C.

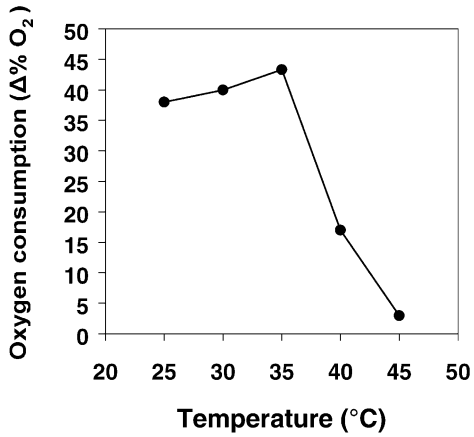

Fig. 5. Effect of temperature on biosensor response to 0.05 mM PNP in 50 mM pH 7.5 citrate-phosphate buffer with 1.2 mg cell loading.

responsible for PNP degradation, while the decrease at high cell loading is due to the transport resistance of PNP and oxygen to cells embedded deeper in the immobilized layer. A similar trend was observed with other microbial biosensors [2]. A cell loading of 1.2 mg dry weight was used in the subsequent work.

3.1.3. Effect of pH

The effect of pH on biosensor was investigated from pH 7.0 to 9.0 to establish the optimum. As shown in Figure 4 the microbial biosensor response was maximal at pH 7.5. This is in good agreement with the pH optimum of 7.5 – 8, reported by Spain and Gibson [7] for maximum activity of nitrophenol oxygenase, the first enzyme involved in PNP oxidation pathway of *A.* sp. The pH of 7.5 was used for subsequent studies.

3.1.4. Effect of Temperature

The response of the biosensor is strongly dependent on the temperature (Figure 5). As shown, the response increased with temperature up to 35 °C and then decreased with further temperature increase. The initial increase is attributed to the increase of both enzyme reaction and mass transport rates. The decrease in the response is caused by the inactivation of the microorganism by heat. Although 35 °C was determined to be the optimum temperature for the biosensor operation, subsequent experiments were still performed at 25 °C in order to overcome the problem of evaporative losses during the course of the experiment and prolong the lifetime of the biosensor [8].

3.2. Analytical Characteristics

3.2.1. Calibration

Figure 6A shows dependence of the biosensor response on the PNP concentration. As shown, the plot is linear up to 0.05 mM (6.95 ppm) with a sensitivity (slope) of 0.78% oxygen consumed per μM PNP.

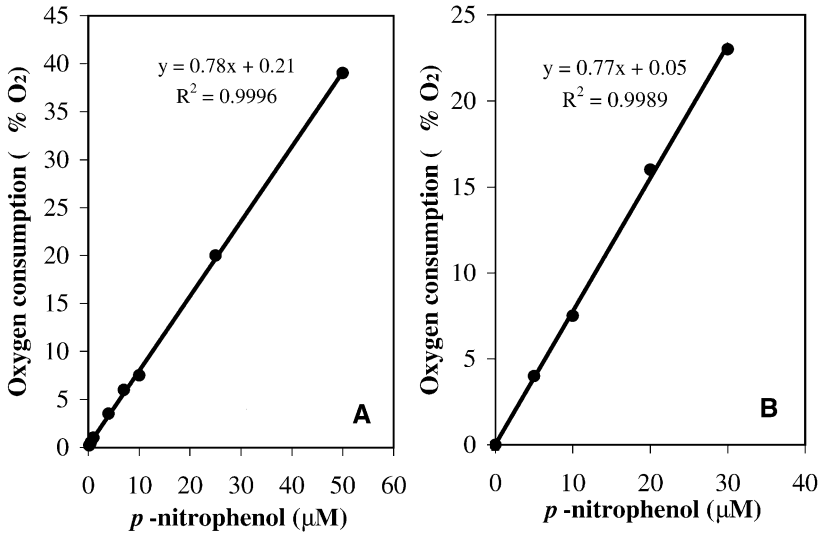

Fig. 6. Calibration plots for PNP microbial biosensor. A) In 50 mM pH 7.5 citrate-phosphate buffer and B) in lake water adjusted to pH 7.5, at 25 °C with 1.2 mg cell loading.

The lower limit of detection (LOD), determined as 3 times the standard deviation of the signal for buffer (blank), was 28 ppb (0.2 μM). The LOD is comparable to the 0.1 μM for a microbial biosensor based on another PNP degrading microorganism *Moraxella* [2]. However, it is 1–2 orders of magnitude higher than that for competitive flow-immunoassay with fluorescence detection and the maximum allowable concentration of 0.1 ppb in drinking water set by the European Union [2, 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 wastewater generated during production and consumption of the PNP and chemical or biological methods for treatment of PNP contaminated wastewaters.

In order to access the matrix effect due to naturally occurring compounds in real-world samples, the microbial biosensor response to PNP spiked in lake water from Lake Elsinor, CA, was measured. Only the pH of the water was adjusted from the original value of 9.2 to 7.5 with concentrated HCl. Despite the use of untreated water, the sensitivity of the biosensor response was similar to that observed in synthetic sample (Fig. 6B), thus demonstrating the utility and feasibility of the microbial biosensor for PNP contaminated wastewaters.

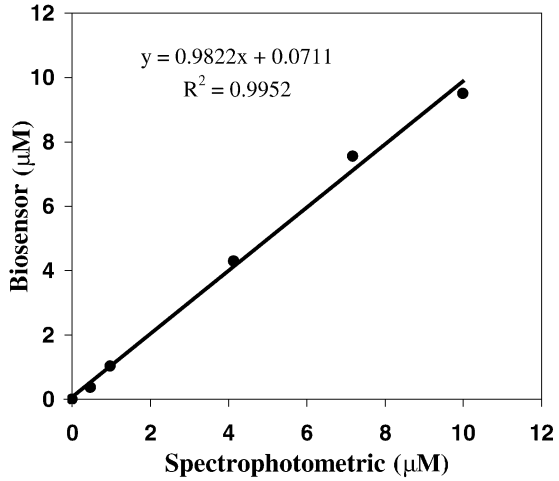

Fig. 7. Accuracy of microbial biosensor. Operating conditions: 50 mM pH 7.5 citrate-phosphate buffer at 25 °C with 1.2 mg cell loading.

Simulated samples representing PNP contaminated water were analyzed using the microbial biosensor and compared to the concentration determined by measuring absorbance at 410 nm spectrophotometrically. The good agreement between the two methods (Figure 7) reflects the high accuracy and reliability.

3.2.2. Precision and Accuracy

The low relative standard deviations of 1.94% ($n = 6$) for 50 μM PNP demonstrate the high precision of analysis. Additionally, a very low relative standard deviation of 2.56% ($n = 3$) in the response of three microbial biosensors prepared using 1.2 mg of cells to 50 μM PNP shows an excellent electrode-to-electrode reproducibility.

3.2.3. Selectivity

The selectivity of the microbial biosensor was evaluated against a range of compounds. As shown in Table 1, even molecularly similar compounds such as phenol, 2-nitrophenol, 3-nitrophenol and 4-chlorophenol, did not interfere. This high degree of selectivity is a significant advantage over other methods of determination of phenolic compounds

Table 1. Microbial biosensor selectivity.

Compounds and concentrations	*Biosensor response ($\Delta\%$ O_2)*
25 μM PNP	20
25 μM PNP + 25 μM phenol	20
25 μM PNP + 25 μM 2-nitrophenol	20.2
25 μM PNP + 25 μM 3-nitrophenol	20.6
25 μM PNP + 25 μM p-chlorophenol	20.2
25 μM PNP + 25 μM citric acid	20
25 μM PNP + 25 μM sodium acetate	20
25 μM PNP + 25 μM sodium succinate	21.5
25 μM PNP + 25 μM glucose	23
25 μM PNP + 25 μM fructose	22
25 μM PNP + 25 μM galactose	20
25 μM PNP + 25 μM sucrose	23
25 μM PNP + 25 μM lactose	20
25 μM PNP + 25 μM succinic acid	21.5

such as amperometry at ordinary electrodes [10]. However, there are small infereferences, 0 to 15% from sugars and organic acids because of non-specific cellular responses to substrate(s) and intermediates of microbial catabolism. Although the biosensor selectivity for these compounds was limited, it is expected that the biosensor will perform adequately since these compounds are not commonly present in relevant samples.

3.2.4. Response Time and Stability

The detection of PNP with the new 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 immunoassays [11].

The long-term storage stability of the microbial biosensor was investigated by evaluating the response of the same sensor to 5 μM PNP and storing it at 4 °C in pH 7.5, 50 mM citrate-phosphate buffer. The sensor response was stable for a period of 5 days and then dropped to 30% of the original after ten days. The rapid drop of the respiratory activity is hypothesized to be a result of the depletion of the NAD(P)H in the resting/non-growing cells [12].

4. Conclusions

A microbial biosensor using *Arthrobacter* sp. and a Clark dissolved oxygen electrode for the direct, rapid and selective measurement of PNP was developed. This very simple and low cost sensor had excellent precision, accuracy, short response time and selectivity for PNP over other structurally similar compounds. These features make it a potentially attractive analytical tool for on-line monitoring of effluents from the chemical processing facilities producing and using PNP.

5. Acknowledgements

This work was supported by grants from U.S. EPA (R82816001-0) and USDA (99-35102-8600). We thank Dr. J. C. Spain of the Air Force Engineering and Service Center, Tyndall Air Force Base, for providing *Arthobacter* sp. JS 443.

6. References

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