Full Paper

Arthrobacter sp. JS443-Based Whole Cell Amperometric Biosensor for p-Nitrophenol

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Abstract
An amperometric microbial biosensor for highly sensitive and selective determination of *p*-nitrophenol (PNP) is reported. The biosensor consisted of PNP-degrader *Arthrobacter* sp. JS443 immobilized by entrapment in Nafion polymer deposited on the top of the carbon paste electrode transducer. The biosensor was based on the measurement of the oxidation current of the intermediates 4-nitrocatechol and 1,2,4-benzenetriol formed by the highly selective oxidation of PNP by *Arthrobacter* sp. The sensor signal and response time were optimized with applied potential of + 0.4 V (vs. Ag/AgCl reference electrode) and 0.03 mg of cells and operating in pH 7.5, 50 mM citrate-phosphate buffer at room temperature. When operated at optimized conditions, the *Arthrobacter* sp.-based biosensor measured as low as 5 nM (0.7 ppb) of PNP. The biosensor demonstrated excellent selectivity with no interference from phenolic compounds such as 2-nitrophenol, phenol and 3-chlorophenol but was interfered by 3-nitrophenol and 3-methyl-4-nitrophenol. It had good precision and intra- and inter-day reproducibility, accuracy and was stable up to 3 days when stored in buffer at 4 °C. When applied for measurement in water from Lake Elsinore, CA, the results obtained were in excellent agreement with the amounts determined spectrophotometrically.

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

1. Introduction

Due to their extreme toxicity to mammals, humans and plants, phenol and substituted phenols have received considerable attention in waste analysis programs. *p*-Nitrophenol (PNP) is one of the substituted phenols that is listed on the U.S. Environmental Protection Agency List of Priority Pollutant [1].

Analytical techniques of chromatography with different detectors [2–9], immunoassay [10–12] and differential pulse voltammetry [13, 14] have been reported for monitoring PNP. These techniques, however, have limitations. Chromatography and immunoassay are time-consuming, expensive and require skilled personnel. Differential pulse voltammetry requires an oxygen free sample, to alleviate interference from dissolved oxygen, and is not specific for PNP as other nitroaromatic compounds such as 2-nitrophenol, etc., interfere [13, 14].

Arthrobacter sp. JS443 is a PNP degrader that was isolated by Spain and his colleagues [15]. This bacterium metabolizes PNP through 4-nitrocatechol, 1,2,4-benzenetriol, maleylacetate and β-ketoadipate to tricarboxylic acid intermediates while releasing nitrite and consuming oxygen [15]. Recently, we reported a whole cell biosensor based on a dissolved oxygen electrode modified with *Arthrobacter* sp. JS443. While simple, rapid and selective, the 28 ppb limit of detection (LOD) of the biosensor was two orders of magnitude higher than the European Commission's limit of 0.1 ppb for drinking water [16]. In this research, we investigated integrating *Arthrobacter* sp. JS443 with a carbon paste electrode (CPE) to construct a whole cell amperometric biosensor. By measuring the electrooxidation current of the intermediates 4-nitrocatechol and 1,2,4-benzenetriol, generated as a result of PNP metabolism by *Arthrobacter* sp., at the CPE we have constructed a very sensitive yet very selective and rapid biosensor for PNP. This paper reports details of biosensor operating conditions optimization, analytical characterization and the application for measurement of PNP spiked in surface water samples from Lake Elsinore, CA.

2. Experimental

2.1. Materials

Citric acid, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, $Na_2HPO_4 \cdot 7H_2O$, phenol, yeast extract, graphite powder, mineral oil and $CaCl_2$ were purchased from Fisher Scientific (Tustin, CA). $FeCl_3$, $ZnSO_4 \cdot 7H_2O$ and $Na_2MoO_4 \cdot 2H_2O$ were obtained from VWR (San Diego, CA). Tryptic soy broth was purchased from Becton Dickinson (Sparks, MD). NH_4Cl was acquired from J. T. Baker (Phillipsburg, NJ). PNP, 2-nitrophenol, 3-nitrophenol, 2,4-dinitrophenol, 3-methyl-4-nitrophenol, 4-nitrocatechol, 1,2,4-bezenetriol and Nafion solution were bought from Aldrich (Milwaukee, WI). *Arthrobacter* sp.

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JS443 was obtained from Dr. J. C. Spain (Air Force Engineering and Service Center, Tyndall Air Force Base, FL). Water sample from the Lake Elsinore, CA, was provided by Prof. Michael Anderson (University of California, Riverside). All solutions were prepared in distilled denionized water.

2.2. 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 30 °C and 300 rpm. Subsequently, these cells were inoculated (O. $D._{600} = 1.0$) in minimal salts medium [17] (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, adjusted to pH 7.0) supplemented with 0.4 mM PNP and 0.1% yeast extract. The inoculated 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 sequence repeated three more times [16]. The cells were harvested using a refrigerated centrifuge (Model J21, Beckman Instruments, CA) at 4 °C, followed by washing with buffer (50 mM citrate-phosphate pH 7.5) twice and the pellet stored in refrigerator until use.

2.3. Microbial Electrode Assembly

A 75% (w/w) graphite powder and 25% (w/w) mineral oil carbon paste was packed firmly into the electrode cavity (3 mm diameter and 1 mm deep) of a Kel-F sleeve (Bioanalytical System Inc., Lafayette, IN) and polished to a smooth shiny finish by gently rubbing over a weighing paper to make the carbon paste electrode (CPE). A 5 μL droplet containing desired amount of cells suspended in 1% Nafion was then dropped onto the polished CPE and the solvent evaporated at room temperature. The electrode was kept at 4 °C when not used.

2.4. Experimental Set-Up and Measurement

Linear scan voltammetry was performed using the VMP2 Mutichanel Potentiostat (Princeton Applied Research. NJ, USA) coupled to a computer. Amperometric measurements were performed using a Bioanalytical Systems, Inc. (BAS) voltammetric analyzer (Model LC-4C) coupled to a chart recorder (Model BD112, Kipp and Zonen, Holland). All experiments were conducted in a 10 mL 3-electrode electrochemical cell inside a Faraday cage (BAS, Model C2 cell stand), with a Ag/AgCl reference electrode (BAS, MF 2063), and a platinum wire auxiliary electrode (BAS, MF 1032). A small magnetic stirrer bar provided the convective transport.

All measurements were performed by applying appropriate potential to the working electrode vs. the Ag/AgCl reference electrode and allowing the transient current to stabilize. 20 to 30 μL of a known concentration test compound was added to 4 mL buffer or Lake Elsinore water after filtering and adjusting pH to 7.5, and the steady-state output current was recorded.

3. Results and Discussion

3.1. Optimization of Operating Conditions

The applied potential, amount of cells immobilized on the transducer and operating buffer pH, affect the amperometric microbial biosensor response. Experiments were performed to investigate the effect of these variables on the biosensor response.

3.1.1. Effect of Applied Potential

The linear scan voltammtric response to 4-nitrocatechol and 1,2,4-benzenetriol (two intermediates in the degradation pathway [15]) at CPE are shown in Figure 1. With a scan rate of 20 mV/s, oxidation peaks were observed at potentials of $+0.31$ V for 4-nitrocatechol and $+0.10$ V for 1,2,4-benzenetriol. In order to oxidize these two intermediates completely, the potential of $+0.4$ V was used in the subsequent work. This overpotential was significantly lower than the $+0.85$ V required for PNP oxidation at CPE [18] and should provide more selective analysis of PNP with an *Arthrobacter.* sp. modified-CPE.

3.1.2. Effect of Cell Loading

Figure 2 shows the effect of cell loading on the response of the amperometric microbial biosensor to PNP. In accordance with the literature [19], the response initially increased

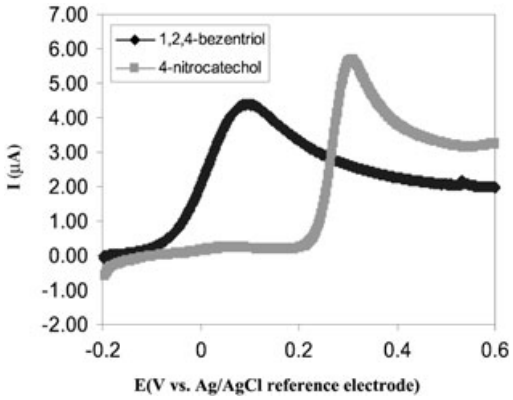

Fig. 1. Linear scan voltammograms for 100 μM 4-nitrocatechol and 100 μM 1,2,4-benzenetriol with carbon paste electrode in 50 mM pH 7.5 citrate-phosphate buffer at room temperature. Scan rate is 20 mV/s.

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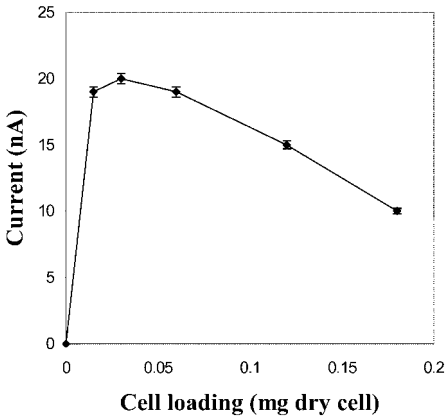

Fig. 2. Effect of cell loading on biosensor response to 5 μM PNP in 50 mM pH 7.5 citrate-phosphate buffer at room temperature. Operating potential: +0.4 V (vs. Ag/AgCl). Data are given as mean ± 1 SD for three experiments.

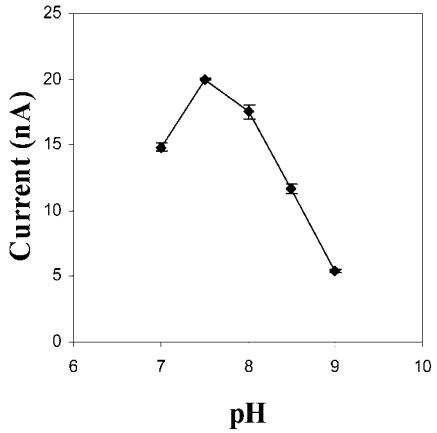

Fig. 3. Effect of pH (50 mM citrate-phosphate) on biosensor response to 5 μM PNP with 0.03 mg cell loading at room temperature. Operating potential: +0.4 V (vs. Ag/AgCl). Data are given as mean ± 1 SD for three experiments.

with cell loading reaching a maximum at 0.03 mg cell dry weight and then decreased. The observed profile can be explained by the initial increase in the catalytic activity associated with increasing cell amount that is subsequently attenuated due to the transport resistance of PNP and oxygen to cells embedded deeper in the immobilized layer. A cell loading of 0.03 mg dry weight was used in the subsequent work.

3.1.3. Effect of pH

The effect of pH on the microbial biosensor response to PNP is shown in Figure 3. The observed maximum response at pH 7.5 was in good agreement with the pH optimum of 7.5 – 8, reported by Spain and Gibson for maximum activity of nitrophenol oxygenase, the first enzyme involved in PNP oxidation pathway of *Arthrobacter* sp. [20]. The pH of 7.5 was used for subsequent studies.

3.2 Analytical Characteristics

3.2.1. Calibration

The calibration plot of the amperometric microbial biosensor for PNP is shown in Figure 4A. The response was linear up to 5 μM (0.695 ppm) with a sensitivity (slope) of 3.74 nA per μM PNP ($r^2 = 0.9989$) and limit of detection (LOD) (defined as 3 times the standard deviation of the response obtained for a blank) of 5 nM (0.7 ppb). This LOD is 1 to 2 orders of magnitude lower than that for oxygen consumption based microbial biosensors using *Moraxella* and *Arthrobacter* as biosensing elements [16, 21] and is comparable to 0.5 ppb for immunoassay [10 – 12]. This low LOD will make this biosensor suitable in meeting the health advisory level of 60 ppb in drinking water set by the US EPA without any sample pretreatment [22] and the maximum allowable concentration of 0.1 ppb in drinking water set by the European Union after a 10-fold preconcentration using standard solid phase extraction protocol [23].

In order to evaluate the effect of naturally occurring compounds (matrix) in real samples, the amperometric biosensor was applied to measure PNP spiked in surface water from Lake Elsinore, CA. Despite the use of a real sample with minimum treatment and without the addition of electrolyte, the slope of the biosensor calibration plot (Fig. 4B, 3.72 nA per μM PNP) for PNP in the Lake Elsinore water was in close agreement to that in the buffer (Fig. 4A, 3.74 nA per μM PNP) demonstrating the absence of matrix interference and therefore the potential application of the biosensor to on-line monitoring of effluents from the chemical processing facilities and environmental samples.

3.2.2. Selectivity

The microbial biosensor exhibited excellent selectivity. Even molecularly similar compounds such as phenol, 2-nitrophenol and 2,4-dinitrophenol, did not interfere (Table 1). This high degree of selectivity is a significant advantage over other PNP analysis methods such as, amperometry based on oxidation of PNP at higher potential (+0.85 V vs. Ag/AgCl reference electrode) when phenol and other substituted phenols interfere [24] or differential pulse voltammetry when oxygen, species containing nitro group and Cu(II) interfered [13, 14]. Sugars and organic acids such as glucose, sucrose and sodium succinate, that are substrate(s) and intermediates of microbial catabolism, also did not have any ineterference (data not shown). There was, however, interference from 3-nitrophenol and 3-methyl-4-nitrophenol.

3.2.3. Response Time and Stability

The detection of PNP with the new amperometric microbial biosensor is simple and rapid. The analysis time of less than 5 min is significantly shorter than the hours required for immunoassay [10] and also does not require excessive handling and multiple reaction and washing steps. Similarly,

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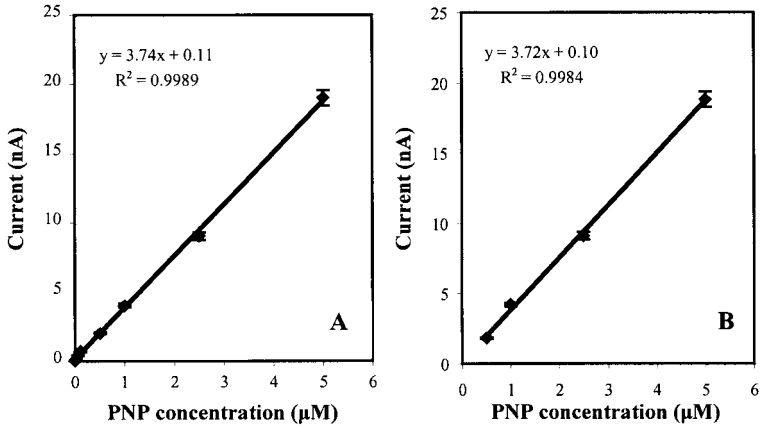

Fig. 4. Calibration plot for PNP amperometric microbial biosensor. A) in 50 mM pH 7.5 citrate-phosphate buffer and B) in Lake Elsinore water filtered and adjusted to pH 7.5, at room temperature with 0.03 mg cell loading, applying +0.4 V to working electrode vs. the Ag/AgCl reference. Data are given as mean ± 1 SD for three experiments.

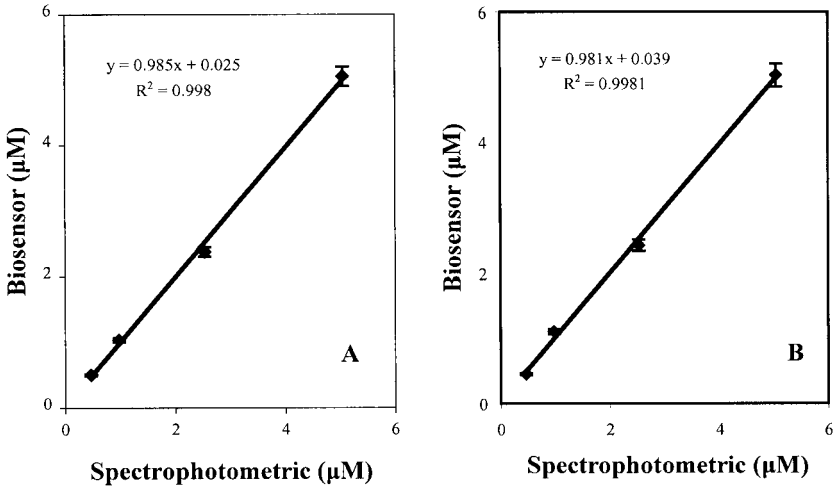

Fig. 5. Accuracy of amperometric microbial biosensor. A) in 50 mM pH 7.5 citrate-phosphate buffer and B) in Lake Elsinore water filtered and adjusted to pH 7.5, at room temperature with 0.03 mg cell loading, applying +0.4 V to working electrode vs. the Ag/AgCl reference. Data are given as mean ± 1 SD for three experiments.

Table 1. Amperometric microbial biosensor selectivity.

Compound and concentration	Biosensor response (current nA)
1 μM PNP	4
1 μM Phenol	0.6
1 μM 2-Nitrophenol	0
1 μM 3-Nitrophenol	4
1 μM 3-Chlorophenol	0.6
1 μM 2,4-Dinitrophenol	1
1 μM 3-Methyl-4-nitrophenol	4

unlike differential pulse voltammetry, deoxygenation prior to analysis to avoid oxygen interference is not necessary [14].

The long-term storage stability of the amperometric microbial biosensor was investigated by evaluating the response of the same sensor to 0.1 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 3 days and then dropped sharply over the next two days (data not shown). The quick drop in the respiratory activity is hypothesized to be a result of the depletion of the NAD(P)H in the resting/nongrowing cells [15,17,25]. Attempts to revive the cell activity by the addition of NAD(P)H were unsuccessful probably due to the inability of the cells to uptake NAD(P)H.

3.2.4. Precision and Accuracy

The response of the biosensor was highly reproducible as demonstrated by the low relative standard deviations of 2.92% ($n = 8$) for 0.1 μM 4-nitrophenol. Additionally, there was an excellent inter-day electrode-to-electrode reproducibility as characterized by a low relative standard deviation of 3.18% ($n = 6$) in the response of six microbial biosensors prepared using cells cultivated in different batches on different days.

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The excellent agreement (slopes of 0.985 and 0.981) between the PNP concentration measured by the biosensor and the independent spectrophotometric method in buffer and environmental sample (Lake Elsinore, CA) illustrates the high accuracy, reliability and applicability of the microbial biosensor to environmental monitoring of PNP (Fig. 5).

4. Conclusions

In conclusion, an amperometric microbial biosensor using *Arthrobacter* sp. JS443 for rapid, sensitive and selective measurement of PNP was developed. While the biosensor limit of detection was comparable to immunoassays, the high selectivity is its most salient feature. Additional advantages of the present amperometric microbial biosensor when compared to other techniques are the low cost (does not require expensive antibodies, trained personnel and instrumentation) and short assay time. These features should make it an ideal analytical tool for field monitoring of PNP contaminated waters and on-line monitoring of effluents from the chemical processing facilities producing and using PNP.

5. Acknowledgements

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