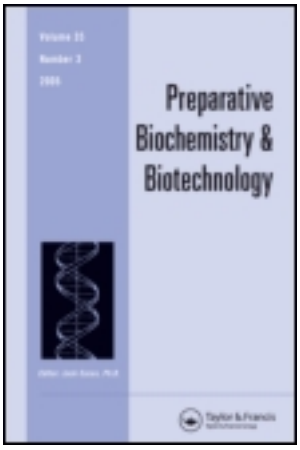

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Pseudomonas putida Based Amperometric Biosensors for 2,4-D Detection

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Abstract: Amperometric biosensors using *Pseudomonas putida* cells as a bioelement were developed for 2,4-dichloro phenoxy acetic acid (2,4-D). After the adaptation process of *Pseudomonas putida* to 2,4-D, cells were immobilized onto the screen printed graphite electrodes (SPG) as well as Clark oxygen probe by gelatin and glutaraldehyde. Optimum pH, temperature, and stability of the biosensor were investigated. Substrate specificities for various phenolic compounds were also searched. In repeatability studies, variation coefficients and standard deviations for both type of systems were calculated; SPG and Clark electrodes were calculated and results are given as a comparison of two systems. Finally, the biosensors were applied to 2,4-D determination in a real herbicide sample.

Keywords: 2,4-D, Herbicides, Microbial biosensors, *Pseudomonas putida*, Screen printed electrodes

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INTRODUCTION

2,4-D (2,4-dichloro phenoxy acetic acid) is one of the most widely used herbicides for control of broad leaf weeds in agriculture, forestry, and right-of-way applications.[1,2] It has been associated with the occurrence of cancer in humans, endocrine-disrupting activities, and acute congestion and degenerative changes in the central nervous system. The maximum admissible concentration for pesticides in drinking water is restricted, by the European Drinking Water Act (EDWA), to 0.1 mg/mL for an individual pesticide and to 0.5 ng/mL for the combined total of pesticides.[3,4]

Biosensors are considered to provide efficient and superior solutions relative to conventional laboratory-based chromatographic and mass spectroscopic techniques for analyzing trace amounts of various substances in complex matrices.

Microorganisms have a number of advantages as biological sensing materials in the fabrication of biosensors. They are present ubiquitously and are able to metabolize a wide range of chemical compounds. Over 90% of the enzymes known to date are intracellular. In this respect, the utilization of whole cells as a source of intracellular enzymes has been shown to be a better alternative to purified enzymes in various industrial processes.[5,6] Whole cells also provide a multipurpose catalyst, especially when the process requires the participation of a number of enzymes in sequence.

Selection of an appropriate culture is essential, as the specific microbial species used in biosensors have characteristic substrate spectra which may or may not correspond well with the spectrum of compounds present in the sample. Adaptation of a microorganism for induction of desirable metabolic pathways and uptake systems by cultivation in a medium containing appropriate substrates may often be desirable.

2,4-D, present in the environment, agriculture products, and physiological solutions, is typically monitored using HPLC and GC/MS methods, which require extensive sample pretreatments and preparation, including derivatization, and highly trained technical personnel. Due to its widespread use, many methods have been suggested for the determination of 2,4-D and its metabolites in matrices such as natural waters, soils, and agricultural products. A few examples of these methods include gas chromatography, high-performance liquid chromatography (HPLC), capillary HPLC, HPLC mass spectrometry,[7–17] fluorescence,[18] and electrochemical methods.[19]

In this contribution, we focus on the development of two different biosensors for detection of 2,4-D. Adapted *Pseudomonas putida* cells were used as a bioactive material for these biosensors. *Pseudomonas putida* is a non-pathogenic, gram negative, rod-shaped, non-spore forming bacteria.

They have an aerobic metabolism and are able to grow on a wide variety of organic substrates.

EXPERIMENTAL

Chemicals

Chemicals were obtained from either E. Merck (Germany) or Sigma-Aldrich Chemical Co. (USA) and were analytical grade and used as received.

Apparatus

WTW Inolab Oxi Level 2 model oxygen meter, based on an amperometric mode, was used for the experiments. All signals were recorded as dissolved oxygen level (mg/L). Chronoamperometric measurements were performed using a Palmsens electrochemical measurement system from Palm Inst., B.V. (Netherlands). A water bath was used for preparation of bioactive material (Stuart Sci.Linear Shaker bath SBS 35) (UK). All the measurements were carried out at constant temperature using a thermostat (Nuve, BM302) (Turkey). A magnetic stirrer (IKA-Combimag, RCO) and pH-meter with electrode (WTW pH 538, Germany) for preparing buffer solutions were used. The temperature was maintained constant in the reaction cell by circulating water around the cell during the experiment.

Inks for printing working electrodes were prepared by using a commercially available carbon ink (Du Pont 7101). Printed electrodes were fabricated by depositing several layers of inks on a PVC substrate. The conducting paths and pads were deposited directly onto the PVC sheets using Ag/Pd ink (Du Pont 5025). Ag/AgCl ink was deposited to obtain the reference electrode. Carbon ink was printed to obtain the working electrodes. Finally, an insulator layer was placed over the conducting paths. After each printing step, the paths were treated at 60°C for 60 min.

Procedure

Biological Material

Pseudomonas putida DSM 50026 was sub-cultured on nutrient agar. In order to obtain non-adapted, control cells which use glucose as a carbon source, the microorganism was inoculated into MSM (mineral salt

medium) containing glucose and incubated at 28°C. After 20 h, the biomass was harvested by centrifugation. The supernatant was removed and cellular paste was used for making biosensors. Adaptation of cells to 2,4-D was performed with gradually increasing 2,4-D and decreasing glucose concentration by daily inoculations.

Preparation of the Biosensors

Both dissolved oxygen probe (Type I) and SPGE (Type II) surfaces were used for immobilization of *Pseudomonas putida*. In the step of preparation of the bioactive layer material, *P. putida* (100 μL) and 10 mg gelatin were added to a test tube containing 150 μL of phosphate buffer (pH 7.5, 50 mM). This mixture was incubated at 38°C for 5–10 minutes to dissolve the gelatin completely. For construction of the biosensor based dissolved oxygen probe, 250 μL of the *P. putida*-gelatin mixture was dispersed over the dissolved oxygen probe membrane surface and allowed to dry at 4°C for an hour. Then, for the crosslinking by glutaraldehyde, the probe carrying bioactive layer was immersed into 2.5% (v/v) glutaraldehyde solution (in phosphate buffer, pH 7.5, 50 mM) for 5 minutes. Finally, the electrode was washed with distilled water. Construction of the biosensor based SPGE was the same as for the dissolved oxygen probe, except 2.5 μL of *P. putida*-gelatin mixture was placed on a graphite working electrode. After use, the biosensors were stored at 4°C in a flask which contained distilled water. Daily prepared biosensors including fresh cells in the logarithmic phase were used in all experimental steps.

Measurement Procedure

In order to determine the concentration of 2,4-D, oxygen consumption due to the metabolic activity of the bacteria was followed. Chronoamperometric measurements were performed by using SPG electrodes at −0.7 V. When a Clark electrode was used for the biosensors, the signals were obtained with a dissolved oxygen-meter as mg/L. All the measurements were done at optimum temperature and pH mentioned below in steady-state conditions.

RESULTS AND DISCUSSIONS

Optimization Studies of the Biosensors

In these studies, the effects of pH and temperature were investigated for the biosensor. In order to determine the temperature effect on the biosensor response, varying assay temperatures were examined. Figure 1 shows

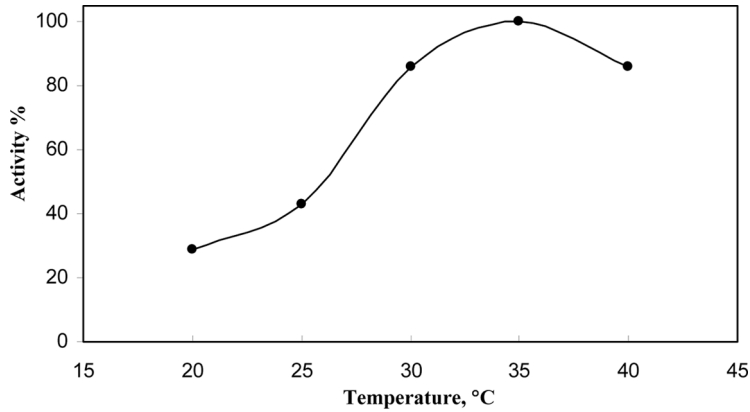

Figure 1. Optimum temperature of the biosensor. (Working conditions: 2,4-D concentration used was 80 μM, amounts of *Pseudomonas putida* cells-gelatin mixture and percentage of glutaraldehyde were kept constant as 250 μL and 2.5%, respectively. pH 7.0, 0.05 M phosphate buffer was used as working buffer solution).

the temperature effect on the biosensor. As can be seen from the figure, the best results were obtained at 35°C. However, in order to prolong the life of the microbial cells in the bioactive of the biosensor, the working temperature was maintained at 30°C in all experiments.

As a rule, the maximum of the enzyme activity is evaluated as most appropriate. Maximum biosensor response for 2,4-D was obtained at pH 7.0. The response decreased at higher pH values. Besides, at more acidic pH values, biosensor response was just about 50% of its initial activity.

These results showed that the pH of the working buffer could be regarded as an important factor for determining sensitivity of the biosensor toward 2,4-D. Consequently, the optimum pH for the biosensor was decided to be pH 7.0. The results are given in Figure 2.

Analytical Characteristics of the Biosensors

Figures 3a and 3b show the calibration curves of two biosensor types. Both biosensors had almost the same linear ranges. The response times for the biosensors were found to be different. A measurement with type I biosensor should be done in 10 min while the response time of type II biosensor was just 200 s.

Some analytical characteristics of the biosensors are also summarized in Table 1.

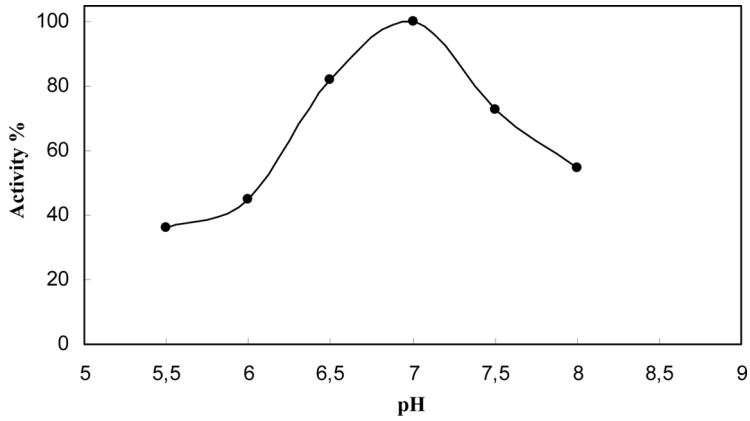

Figure 2. pH dependence of the biosensor. (Working conditions: For type I biosensor; 2,4-D concentration used was 80 μM, amounts of *Pseudomonas putida* cells-gelatin mixture and percentage of glutaraldehyde were kept constant as 250 μL and 2.5%, respectively. All buffers were 0.05 M phosphate buffer solutions with different pHs, T = 30°C).

Thermal Stabilities

Thermal stabilities of the biosensors were also tested. Type I electrode lost about 10% of its initial activity at 30°C after 5 hours; type II biosensor retained 74% of its activity when it was operated at the same conditions for 5 h.

Substrate Specificities

Interference effects of various phenolic compounds and other potential interfering compounds on the response of the proposed biosensors were studied. The results obtained with these studies are given in Table 2.

Sample Analysis

To demonstrate the feasibility of the biosensors based on *P. putida* cells for 2,4-D analysis, samples of herbicides were analyzed. In these experiments, instead of substrate, herbicide samples with appropriate dilutions, were added to the reaction cell. Sample analysis results are summarized in Table 3.

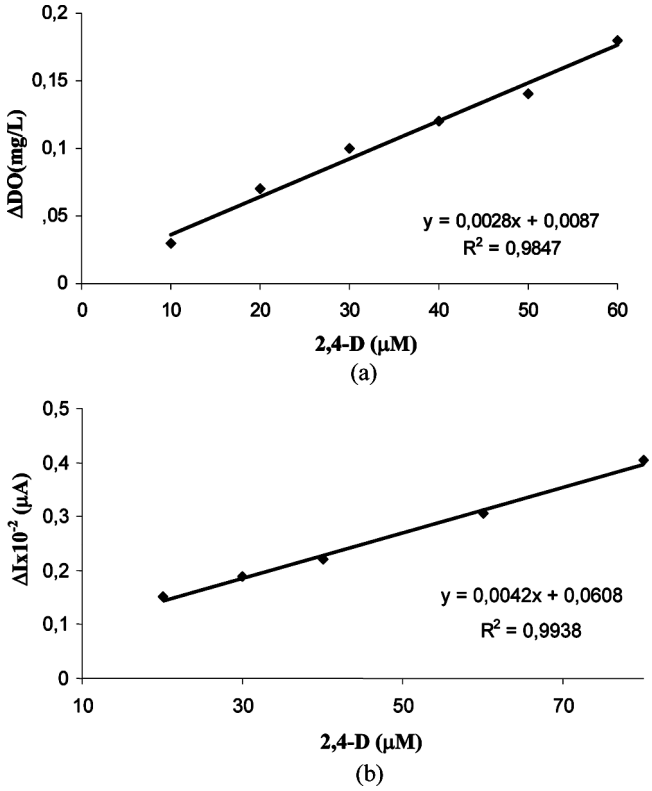

Figure 3. a) Calibration curve of type I biosensor for 2,4-D (Working conditions: Amounts of *Pseudomonas putida* cells-gelatin mixture and percentage of glutaraldehyde were kept constant as 250 μL and 2.5%, respectively. pH 7.0, 0.05 M phosphate buffer was used as working buffer solution. T = 30°C); b) Calibration curve of type II biosensor for 2,4-D (Working conditions: 2.5 μL of *Pseudomonas putida* -gelatin mixture was used for type II biosensor. Percentage of glutaraldehyde was 2.5%. pH 7.0, 0.05 M phosphate buffer was used as working buffer solution. T = 30°C, working potential was −0.7 V.)

Table 1. Analytic characteristics of the biosensors

Biosensor	Linear Range (μM)	Equation**	R^2	S.D* (± μM)	C.V*(%)
Type I	10–60	y = 0.0028x + 0.087	0.9847	1.756	3.96
Type II	20–80	y = 0.0042x + 0.061	0.9938	1.1	3.6

*These values were calculated by the 6 subsequent measurements

**X shows μM. y shows mg/L and μA for Type I and II systems, respectively.

Table 2. Determination of different compounds with the biosensors

Biosensor	Substrate	Linear	R^2 range (μM)
Type I	2,4-D	10–60	0.9847
	Phenol	20–100	0.9865
	2-chlorophenol	100–500	0.9887
	4-nitrophenol	50–200	1.000
	Glucose	50–400	0.9908
	Ethanol	50–250	0.9952
	Methanol	100–600	0.9797
Type II	2,4-D	20–80	0.9380
	Phenol	1–5	0.9918
	2-chlorophenol	20–200	0.9990
	4-nitrophenol	20–160	0.9798
	Glucose	1–10	0.9873
	Ethanol	10–80	0.9830
	Methanol	20–200	0.9990

Table 3. Determination of 2,4-D in an herbicide sample with the biosensors

Biosensor	Added 2,4-D (μM)	Found 2,4-D (μM)	n
Type I	33	34±1.8	6
	43	45±1.9	6
Type II	30	31.5±1.1	5
	40	40±0.6	5

*Results are expressed as the average ± S.D.

CONCLUSION

The results presented in this work demonstrate that microbial cell biosensors based on *Pseudomonas putida* are feasible for the detection of 2,4-D. The biosensors are readily prepared from inexpensive materials through a general method. At the end of this work, the biosensors showed good performance for detection of 2,4-D. A linear response range between 10 and 60 μM of 2,4-D for type I biosensor and between 20 and 80 μM of 2,4-D for type II biosensor were verified. Another important parameter for the present biosensors was measurement time. Total measurement time was about 10 minutes for type I biosensor. However, this period for type II biosensor was just 200 s. Moreover, the immobilization procedure used for two types of biosensors was very easy. Consequently, the simplicity in the bioactive material immobilization demonstrates a considerable

economic advantage because of its low cost, which could be desirable in routine analysis of foods. In this respect, the systems described are simple and inexpensive methods for monitoring of 2,4-D.

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