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Development of Tyrosinase-Based Biosensor and Its Application for Monitoring of Bioremediation of Phenol and Phenolic Compounds

JURAJ ŠVITEL[†,‡] AND STANISLAV MIERTUŠ*[,†,§]

POLY-tech, Area di Ricerca, Padriciano 99, I-34012 Trieste, Italy, and International Centre for Science and High Technology—UNIDO, Area Science Park, I-34014 Trieste, Italy

A tyrosinase-modified solid composite biosensor has been developed, and its application for the determination of phenol and related compounds in environmental samples was studied. The composite transducer for amperometric biosensor was based on graphite powder modified with tyrosinase and 2-hexadecanol used as a solid binding matrix. The response of a biosensor modified with 4% of tyrosinase was linear up to 2.5 μM, the sensitivity was 0.0225 μA/μM, and the detection limit 0.2 μM. Various parameters influencing biosensor performance have been also studied: working potential, buffer concentration, pH, and response with other compounds. The sensitivity of biosensor without surface renewal decreased to 20% of the initial value after 1 month. The sensitivity is restored after surface renewing. The biosensor was tested in laboratory-scale experiments for monitoring of phenol bioremediation in water and soil. The biosensor was also tested for analysis of other phenolic wastes: leachate from leather processing containing chlorophenols and waste from oil processing containing polyphenols.

Introduction

Phenols are widely used chemicals and industrial byproducts. Waste phenols are produced mainly by the wood-pulp industry (phenols are components of natural compounds, e.g., lignin and tannin). Phenolic wastes are also generated during production of synthetic polymers, such as phenolic resins and coking of coal operation of oil refineries. Phenols are released into the environment also by the degradation of pesticides with phenolic skeleton. Phenols and especially chlorophenols have been defined as dangerous pollutants due to their toxicity and persistency in the environment. For these reasons, many phenols are listed in the EC (European Commission) list of dangerous substances (*1*) and in the U.S. EPA (United States Environmental Protection Agency) list of priority pollutants (*2, 3*). Recent environmental legislation restricts the content of phenols. For example, the maximum concentration limit of phenol in water for the protection of public health is 3.5 μg/L (*4*), and maximum levels of phenolic compounds in surface water for drinking purposes are in the range 1−10 μg/L (*5*).

* Corresponding author phone: ++39 40 3756622; fax: ++39 40 9220016; e-mail: stano@goblin.icc.trieste.it.

† International Centre for Science and High Technology.

‡ Permanent address: Slovak Technical University, Radlinského 9, 81237 Bratislava, Slovakia .

§ POLY-tech.

Various types of phenol degradation processes have been reported previously. Phenol-containing wastes have been treated for many years using activated sludge process. Recently considerably high phenol loading rates have been achieved (*6*). An immobilized culture of *Pseudomonas* was reported to be able to degrade phenol or 4-chlorophenol (*7*). Phenols in soil are spontaneously bioremediated by phenooxidizing microorganisms and also were found to be able to undergo bioremediation by the plant root surface peroxidases (*8*). Studies on simulations of in-situ phenol remediations have recently been published (*9*).

Increasing legislative and environmental control has initiated the development of analytical techniques for fast monitoring of environmental pollutants. The determination of phenolic compounds in wastewater has been exhaustively reported recently (*10*). Conventional methods for phenols determination are GC (gas chromatography) and LC (liquid chromatography). Obligatory analytical procedures comprise sample pretreatment by extraction. Recently, the application of new techniques such as capillary electrophoresis, immunoassays, and mainly biosensors has expanded (*10*). The application of biosensors is favorable due to some generally claimed advantages: intrinsic specificity, low costs, fast analyses, and minimal requirements for sample pretreatment (*11*). However, widespread application of biosensors is still considerably hampered by the low stability of biosensors and insufficient detection limits. Enzyme-based amperometric biosensors based on tyrosinase, laccase, or peroxidase have been previously reported (see references see below). The most frequently used enzyme for phenol determination is tyrosinase.

Tyrosinase (EC 1.14.18.1) is now a well-characterized enzyme. Tyrosinase oxidizes phenol in two steps: phenol is oxidized to catechol (*o*-benzenediol), which is consequently oxidized (by tyrosinase) to *o*-quinone. Tyrosinase shows no activity for the oxidation of *p*- and *m*-benzenediols. Laccase, which catalyzes the oxidation of *o,m,p*-benzenediols to the corresponding *o,m,p*-quinones, is used for the detection of these benzenediols. Thus, coimmobilization of tyrosinase and laccase allows the detection of several phenolic compounds (*12*). The approach to enhanced sensitivity using coimmobilization of tyrosinase with horseradish peroxidase has been described (*13*). The application of peroxidase (*14*) and microbial cells (*15*) for monitoring phenol and related aromatic compounds has also been reported. The detection of phenols by amperometric biosensors is based on the direct electrochemical reduction of quinones produced by enzymatic reaction. A measurement principle based on the measurement of oxygen depletion by a oxygen probe was also reported (*16*). A variety of methods for immobilization or integration of tyrosinase with an electrochemical transducer has been reported: surface modification of electrodes (enzyme adsorption) (*12, 17*); enzyme incorporated (mixed) within a carbon paste (*18, 19*); and various bulk-modified carbon composite electrodes, e.g., activated graphite particles (*20*), Teflon−graphite composite (*21*), epoxy-based composite (*22*); immobilization in films, e.g., in electrochemically polymerized polypyrrole film (*13, 23*), poly(ester sulfonic acid) ionomer (*24*); and a biosensor based on the screen printing of tyrosinase containing carbon ink (*25*). The use of biosensors was also shown to be possible in conjunction with FIA (flow injection analysis) (*26*) or liquid chromatography (where chromatography performs separation and a biosensor is used as a specific detector) (*27*−*29*). Tyrosinase may be used also for the detection of phenolics in organic phase (*17, 30, 31*).

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Studies aimed at the development of biosensors are usually performed with pure compounds. Studies aimed at the analysis of phenols in real environmental samples rarely appear. The only known reports are on the application of a tyrosinase biosensor for the analysis of phenol in river water (*18*) and on the application of a peroxidase biosensor for the analysis of 2-amino-4-chlorophenol in surface water (*14*).

Recently, we have developed a new concept of biosensors based on solid components (*32*−*35*). Such compounds, called the solid binding matrix (SBM), have amphiphilic character and are solid at ambient temperature. The main advantages of biosensors based on SBM (e.g., hexadecanol, hexadecanone, cholesteryl oleate, etc.) are good stability and mechanical (possibility of modification with enzymes and mediators, easy fabrication) and electrochemical (fast response, low background currents, operational and storage stability) properties (*33*).

The aim of this study is to develop a tyrosinase biosensor based on SBM and to evaluate its application for fast monitoring of various phenolics in water and soil samples. The detailed goals are as follows: the development of a biosensor based on SBM bulk-modified with tyrosinase; the evaluation of analytical properties and biosensor performance; and its practical application for monitoring of phenols remediation by *Pseudomonas* sp. on the laboratory scale.

Materials and Methods

Biosensor Preparation. The graphite powder used for biosensor preparation (Aldrich) was first modified with tyrosinase (Sigma). The biosensor modified with 4% (w/w) of tyrosinase was prepared by the following procedure: 2 mg of tyrosinase was dissolved in 0.2 mL of distilled water, 24 mg of graphite was added, and the mixture was dried using a fan (approximately 20 min). The modified graphite was ground in a porcelain dish and mixed with 24 mg of 2-hexadecanol, and this mixture was melted at 55 °C. The composites containing 0.5% and 10% of tyrosinase have been prepared by analogous procedure using graphite and 2-hexadecanol in the ratio of 1:1. For the biosensor preparation, a PVC tip (inner diameter 2 mm, outer diameter 5 mm, length 10 mm) was used. Electrical contact was ensured by a brass rod (diameter 2 mm) inserted into the tip to create a cylindrical space. Melted composite material was filled into a preheated PVC tip and left to cool at room temperature. The end of the tip (active part) was ground with an emery paper and polished on a sheet of common paper before the first use. Surface renewing was done by the same procedure. In some experiments, the surface of the biosensor was covered with membrane Spectra/por, type 1 (Spectrum Medical Industries Inc.) and fixed with a rubber O-ring.

Measurement with Biosensor. Chronoamperometric measurements were performed with a potentiostat Amel Model 559 and a recorder Amel Model 868. All measurements were performed in a vessel equipped with magnetic stirrer (400 rpm) filled with 10 mL of 0.1 M sodium phosphate buffer at pH 6.0. Buffer was saturated with air before measurements. Measurements were performed at the potential −50 mV vs SCE (saturated calomel electrode). Standard solutions or samples were added during measurements in small volumes (10−100 μL). Standard solutions were prepared by dilution of phenol (Fluka) in demineralized water (Milli Q, Millipore). Phenol-containing water was measured directly without any predilution. Samples of soil (1 g) were mixed with distilled water (5 mL) and shaken for 5 min. Then extract was left to sediment for 5 min, and aqueous phase was analyzed. Phenol of analytical grade added to the extracts was used as an internal standard.

Microbial Strain and Cultivation Conditions. The strain used for remediation in this study was *Pseudomonas stutzeri* isolated from a long time PCB-contaminated soil. Celles were grown in a synthetic medium composed of five parts, A−E. Part A is KH_2PO_4, 84.5 g. Part B is $MgSO_4 \cdot 7H_2O$, 12.3 g. Part C is $(NH_4)_2SO_4$, 66.0 g. Part D is $Ca(CH_3COO)_2$ 10.0 g. Part E consists of $FeSO_4 \cdot 7H_2O$, 5.0 g; $ZnSO_4 \cdot 7H_2O$, 5.0 g; $MnSO_4 \cdot 5H_2O$, 5.0 g; $CuSO_4 \cdot 5H_2O$, 2.0 g; $CoSO_4 \cdot 7H_2O$, 1.0 g; $Na_2MoO_4 \cdot H_2O$ 1.0 g; and H_3BO_3, 0.5 g. Parts A−D were each dissolved in 500 mL of distilled water and autoclaved for 20 min at 120 kPa. Ingredients of part E were each dissolved in 100 mL of distilled water, sterilized separately and mixed in a 40:4:4:10:10:10:10 volume ratio . This solution (10 mL) was mixed with distilled water (1 L) to give the final solution E. The solutions A−E were then mixed in a 10:5:10:10:10 volume ratio. To 45 mL of this solution, 995 mL of distilled water was added. Final pH of this solution was adjusted to 7.2 with 5 M NaOH solution. All inorganic salts were of analytical grade purchased from Sigma. As a sole carbon source, phenol (Fluka) 2 g/L was used. The cells were cultivated 7 days on a rotary shaker at 28 °C. The concentration of cells was expressed as the concentration of dry cell weight (g_{DW}/L) after drying 2 h at 100 °C.

Contaminated Water and Soil. Contaminated water was prepared from tap water, which gives no response with a biosensor, by addition of pure phenol (Fluka) to obtain a phenol concentration of 1 mM. Contaminated soil was prepared from the soil collected from local source. Dry soil was sieved, and 0−2 mm particles fraction was used for the experiments. Water content of soil was adjusted to 15% (w/w). Phenol dissolved in water was added to obtain contamination 100 ppm (relative to dry weight). In the blank experiment (performed with pure soil), aimed at the evaluation of possible interferences by phenolic compounds released from lignine, no response of the biosensor was detected.

Bioremediation. Phenol bioremediation in water was tested using 50-mL closed test tubes that were filled with 10 mL of contaminated water. Tubes were inoculated with a grown *P. stutzeri* culture, cultivated on a rotary shaker, and sampled every 24 h. Phenol bioremediation in soil was tested in 50-mL test tubes filled with 20 g of soil. Tubes were cultivated statically and sampled every 24 h. Before each sampling, the soil was vigorously shaken. Blank experiments without cells and without phenol were done for both bioremediations in water and in soil.

Results and Discussion

Calibration of Biosensor. The composite biosensor was connected to the potentiostat, and the initial signal was allowed to stabilize for 5 min before each measurement. The typical response of the composite biosensor to successive injections of phenol dissolved in deionized water (in 0.5 μM steps) is shown in Figure 1. The calibration curve obtained for phenol showed a linear response up to 2.5 μM (Figure 2A). At high phenol concentrations, the response of the biosensor becomes nonlinear (Figure 2B).

Parameters of the Biosensor. The slope of the calibration curve measured with a biosensor modified with 4% of tyrosinase and presented in Figure 2A is 0.0225 μA/μM, and it is a measure of the sensitivity of the phenol biosensor. The detection limit, based on a signal-to-noise ratio of 3, is 0.2 μM (18.8 μg/L). In general, the selectivity of a biosensor depends on enzyme loading. For comparison, the biosensor modified with 0.5% of tyrosinase exhibited a sensitivity of 0.0011 μA/μM and a detection limit of 2 μM, while the biosensor modified with 10% of tyrosinase exhibited a sensitivity of 0.09 μA/μM and a detection limit of 0.05 μM (4.7 μg/L). The detection limits of phenol determination previously reported in the literature were 0.3 μM (*18*), 0.4 μM (*17*), 2.6 μM (*31*), and 9 μM (*24*). All further experiments were performed with a biosensor modified with 4% of tyrosinase, which is sufficient for analysis of phenol in

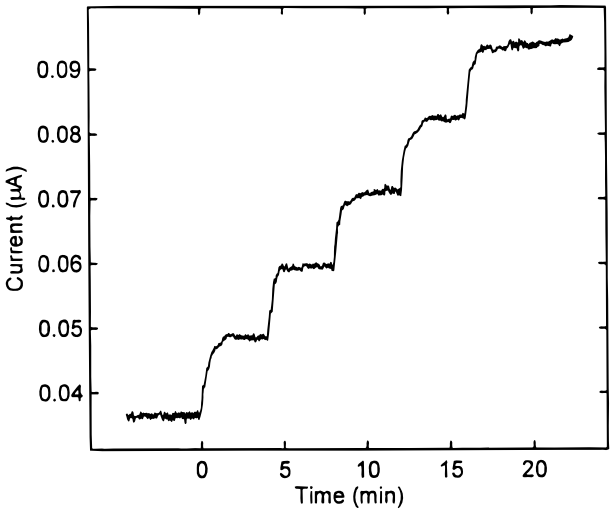

FIGURE 1. Current−time recording of the succesive increments of phenol. Phenol additions of 0.5 μM were done at equidistant time intervals of 4 min.

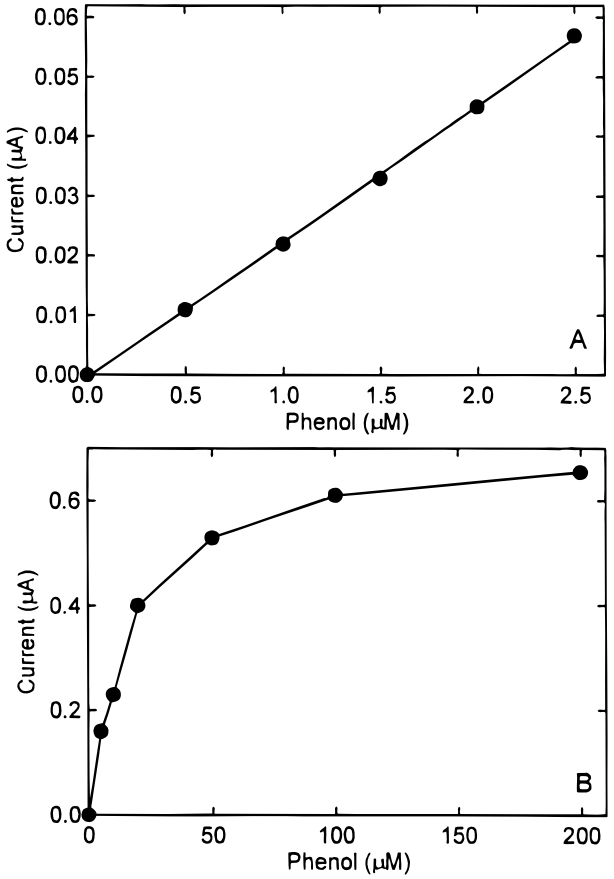

FIGURE 2. Calibration curves of biosensor. Data in panel A correspond to the measurement presented in Figure 1. Data in panel B were measured by an analogous experiment for the large concentration scale of added phenol.

environmental samples of water at micromolar level and analyses of soil at ppm levels. Further lowering of the detection limit if required, e.g., for analysis of drinking water, may be achieved by increasing the enzyme loading.

When an electrode is connected to a potentiostat, the signal decreases. After the time of stabilization of the initial signal, the current remains constant in the range of 0.02−0.05 μA (background current). In general, the stabilization time depends on the previous history of a sensor. The

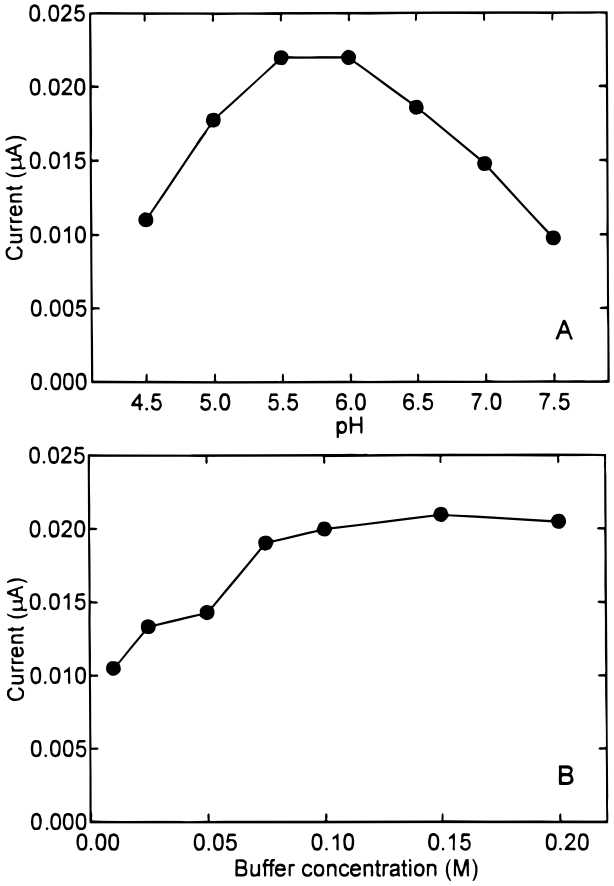

FIGURE 3. Effect of pH (A) and buffer concentration (B) on biosensor response. Measured as response to 1 μM phenol.

stabilization time of freshly prepared biosensors or biosensors after surface renewing is usually in the range of 3−5 min. The exposition of biosensor to concentrations up to 200 μM leads to considerably longer times of stabilization (up to 30−40 min, depending on the phenol concentration). The exposition of the biosensor to extremal concentrations, e.g., several minutes in 1 mM, practically extinguishs further use of a biosensor. According to our opinion, it may be caused by phenol penetration into the biosensor matrix. However, for the purposes of environmental analysis, lower concentrations ranges are expected, which is certainly favorable also from the point of view of minimal time of stabilization.

The performance of biosensors is influenced by parameters such as pH, buffer concentration, and working potential. Figure 3A shows the influence of pH. Maximum biosensor response was achieved in the interval of 5.5−6.0. This value differs from pH 7.0, which was found as optimum for tyrosinase in other matrixes (*20*, *23*, *26*).

The influence of buffer concentration was investigated up to 0.2 M using buffer at pH 6.0 (Figure 3B). Biosensor response is constant in the range 0.1−0.2 M. At buffer concentrations lower than 0.1 M, signal intensity depends on buffer concentration, and at concentrations lower than 50 mM, an increased noise also appears. A buffer concentration of 0.1 M was chosen for further work.

The effect of the working potential on biosensor response is documented in Figure 4. As can be seen, the biosensor response reaches a plateau in the interval −100 to −200 mV and decreases by 20% at the potential of 0 mV. It is not significant if considering the advantage of work at potentials close 0 mV. We observed that at lower potentials (up to −200 mV) the stabilization time of the initial signal is longer but simultaneously the biosensor response is faster. On the

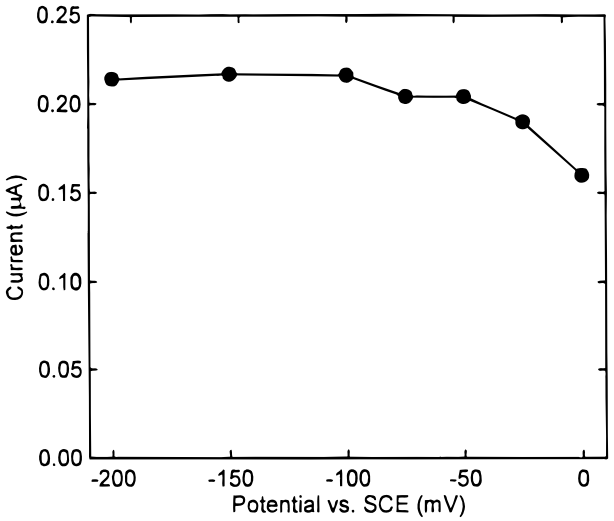

FIGURE 4. Effect of working potential on biosensor response. Responses are expressed as steady-state currents at 1 μM phenol.

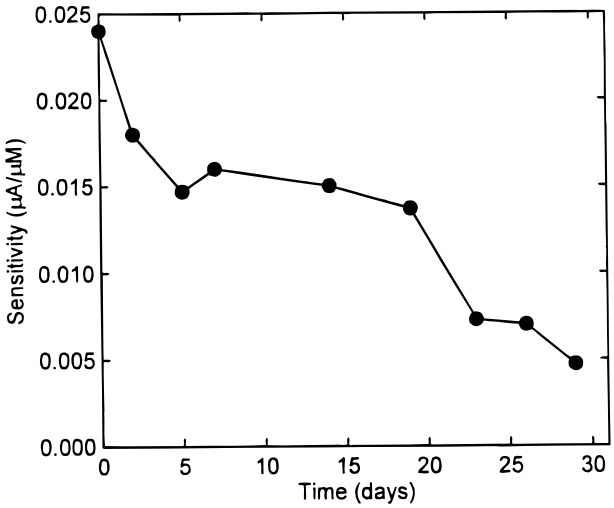

FIGURE 5. Stability of biosensor. The stability was tested by repetitive measurements without surface revewal. The sensor was stored at ambient temperature between measurements.

other hand, the biosensor operating at a potential around 0 mV should be much less subject to possible interferences. As a compromise, the value of −50 mV was chosen, and our further experiments were performed at −50 mV. Other authors have reported working potentials of 0 mV vs Ag/AgCl (*25*), −50 mV vs Ag/AgCl (*12*), 200 mV vs Ag/AgCl (*13*), and −200 mV vs SCE (*20, 23*).

The response to substrates other than phenol was also briefly investigated. The sensitivities of the biosensor, expressed as the slope of the calibration curve, with phenol, catechol, and tyrosine were 2.25×10^{-2}, 2.51×10^{-2}, and 7.57×10^{-4} μA/μM respectively, which corresponds to the response ratio of 1:1.16:0.03. This value differs from the ratios reported for tyrosinase by other authors, e.g., reported ratios of responses to phenol:catechol:L-tyrosine were 1:1.79:0.13 (*18*) and 1:4.29:0.01 (*23*). Tyrosinase-modified biosensors have been used mainly for the detection of phenol and catechol, and in this respect they are often experimentally studied. The substrate specificity of tyrosinase enables the detection of a wide range of compounds. Tyrosinase biosensors were reported to give response with various substituted phenols and catechols, e.g., dopamine, L-tyrosine, noradrenaline (*20*); *p*-cresol, L-DOPA (*23*); pyrogallol, tyramine, 3,4-dihydroxyphenyl alanine (*19*); *o*,*p*-chlorophenol (*12*); norinephedrine, dopamine, 3,4-dihydroxyphenylacetic acid, homovanilic acid (*27*); and 3,4-dihydroxybenzaldehyde and other catechols substituted in the same position by another group (*28*). The list of previously reported compounds that undergo oxidation by tyrosinase suggests that *o*,*m*-monophenols and *p*-catechols are detectable by tyrosinase biosensor. The responses to the individual compounds mentioned above differ in signal intensity. The results of analyses of complex samples of phenols should be expressed as a "phenol equivalent". The nonspecificity of the biosensor should be considered advantageous for the purpose of fast contaminant monitoring, especially if considering that legal limits are also expressed as total phenols (*1*−*3*). In addition, oxidative bioremediation of various aromatic compounds leads to the formation of catechol or substituted catechols. Thus, these bioremediation processes can be monitored by monitoring intermediate products.

Stability of the Biosensor. Stability is usually considered as one of the key factors considerably hampering the practical applicability of biosensors. The operational stability of the biosensor was tested by repetitive measurements during 1 month (without surface renewing). Figure 5 illustrates the sensitivity decrease and variations over the time. As can be seen, the sensitivity of a biosensor decreased to 20% of the initial value after 1 month due to the partial inactivation of the enzyme. The activity of the biosensor may be restored by surface renewing. According to our experiences, the sensitivity of biosensor after surface renewing varies in the 0.020−0.035 μA/μM range . This may be caused by nonhomogeneous distribution of components inside the matrix and by low reproducibility of surface renewing. Due to the same reasons, the stability of the enzyme inside the biosensor matrix cannot be evaluated accurately. Our repeated measurements (with surface renewing) after 3 months of storage at ambient temperature indicated no activity decrease. This excellent stability of the biosensor should be attributed to the protective effect of the solid binding matrix on enzyme (*32*).

Biosensor Application to Analysis of Real Samples and Bioremediation Monitoring. One of the aims of this study was to test prepared biosensors for the analysis of real samples. We performed a simple bioremediation experiment aimed at the bioremediation of phenol by the bacterial strain *Pseudomonas* in water and in soil. Results of the bioremediation process monitored with a biosensor are presented in Figure 6. As can be seen from the graphs in Figure 6B, bioremediation by *Pseudomonas* interferes with bioremediation by naturally occurring microflora. Some decrease of the content of phenol is caused by partial evaporation (see curve ○ on Figure 6B). Then, it should be concluded that this experimental setup involving minimal sample pretreatment should be considered as a fast method for phenol monitoring in water and soil samples.

Furthermore, we have tested the application of a biosensor for phenol determination in two types of industrial wastes: leachate from leather processing containing a mixture of chlorophenols and waste from olive oil processing containing an undefined mixture of polyphenols. For routine phenol monitoring in leachate, HPLC is currently used as a reference method. The result of biosensor analysis, expressed as phenol equivalents, (4.7 ppm) was lower as compared to reference analysis of chlorophenols (11 ppm). This can be attributed to the lower activity of tyrosinase with a majority of substituted phenols as it is supported by several citations in the Parameters of the Biosensor paragraph. The result of polyphenol analysis (0.12 g/L), also expressed as phenol equivalents, was not possible to compare with any reference analysis because no reasonable method for analyzing of this type of waste is available.

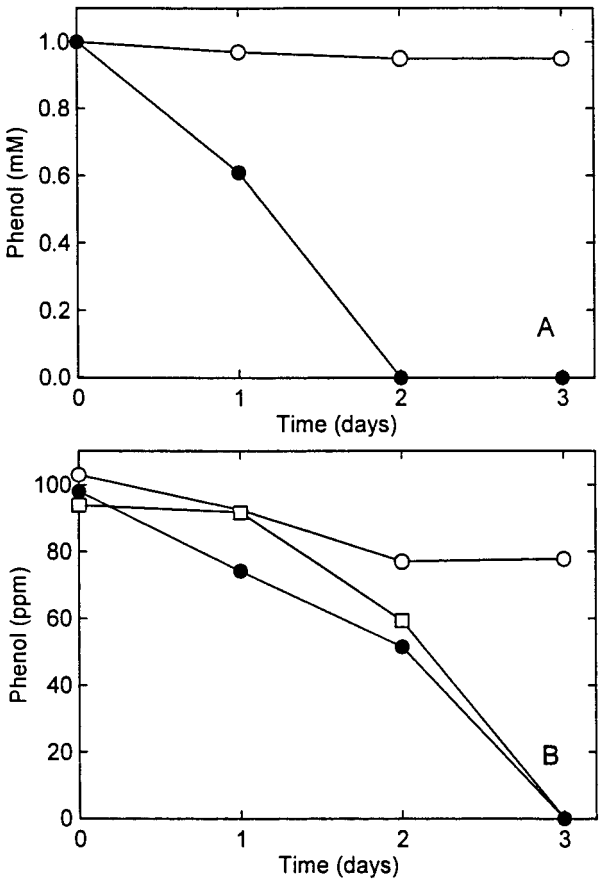

FIGURE 6. Bioremediation of phenol by *Pseudomonas stutzeri* in water (A) and soil (B). Legend (A): (●) water with the cells *Pseudomonas* (0.09 g/L), (○) blind experiment without cells. Legend (B): (●) soil with the cells *Pseudomonas* (0.045 mg/g of soil), (□) soil without cells, (○) sterilized soil without cells.

In the case of substituted phenols or polyphenols, analysis by biosensor may serve as a comparative empirical method for the evaluation of contamination. The potential of biosensor use resides in fast phenol monitoring, e.g., if a biosensor is incorporated into a portable monitoring device. It should be concluded that accuracy and selectivity of phenol biosensors cannot compete with standard laboratory techniques. The advantages of biosensor analysis are speed, simplicity, low cost instrumentation, and the possibility to perform measurements in the field (outside laboratory).

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