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Cell Biosensor for Detection of Phenol in Aqueous Solutions

E. I. RAININA,*[1] I. E. BADALIAN,[2]
O. V. IGNATOV,[3] A. YU. FEDOROV,[4]
A. L. SIMONIAN,[2] AND S. D. VARFOLOMEYEV[1]

[1]*Chemical Enzymology Department, Lomonosov Moscow State University, Lenin's Hills, Moscow, 119899, Russia;* [2]*Yerevan Physics Institute, Yerevan, Armenia;* [3]*Research and Technology Institute of Agricultural Biotechnology, Saratov, Russia; and* [4]*Genetics Scientific Research Institute, Saratov, Russia*

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ABSTRACT

A microbial sensor for concentration measurement of phenol in aqueous solutions has been developed. Phenol-utilizing cells *Pseudomonas putida* GFS-8 immobilized in poly(vinyl)alcohol cryogel were used as a biological transducer. Relationships between phenol concentration in the activating medium and endogenic cell respiration have been established. Cell respiration and phenol concentration in the assay solution positively correlated at a phenol concentration range of 0.1–2.0 mg/L and were linearly dependent in the range of 0.1–1.0 mg/L. A Clark membrane electrode was the physiochemical transducer. The assay may be completed within 5 min. The cells oxidize phenol, pyrocatechol, mesityl oxide, aniline, and do not react with a number of xenobiotics, sugars, and alcohol. With the exception of aniline, most components found in waste waters from phenol production affect neither the assay process nor the ability of these cells to use phenol as exogenic respiratory substrate. The immobilized cells retained their ability to utilize phenol as an exogenic respiratory substrate for up to 1 mo.

Index Entries: *Pseudomonas putida* GFS-8; immobilized microbial cells; biosensor; phenol detection; poly(vinyl)alcohol cryogel.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Phenol is a high-volume industrial chemical produced almost entirely as an intermediate for the preparation of other chemicals. These chemicals include synthetic polymers, such as phenolic resins, biphenol, and caprolactam plastics intermediates, and chlorinated and alkylated phenols. In general, natural phenol is produced by the cumene process (*1*). Phenolic wastes are also generated during the coking of coal, distillation of wood, operation of gas works and oil refineries, microbial decomposition of organic matter, and manufacture of livestock dips, and occur as a constituent of human and animal wastes (*2*). Although phenolic compounds play important biochemical and physiological roles in living systems, their accumulation in the environment as a result of intensive human activity may result in drastic ecological problems. The available data for phenol indicate that acute and chronic toxicity to fresh-water aquatic life occurs at concentrations as low as 10.2 and 2.56 mg/L, respectively, and to salt-water aquatic life at concentrations as low as 5.8 mg/L. Based on available toxicity data, the maximum concentration limit for the protection of public health, the derived level is 3.5 mg/L (*2*). From the above discussion, it is clear that a foremost task in environmental protection is the monitoring of aquatic resources and waste waters to quantify phenol and its related compounds.

In addition to chromatographic and spectrophotometric methods for phenol detection, analytical systems based on immobilized enzymes and cells have been gaining an ever-growing prevalence (*3–5*). The major advantages of such systems include the short time required for the assay, selectivity, simplicity of the operation, and feasibility to use it for multicomponent solutions under field conditions. Comparison of enzyme and cell biosensors shows that the enzyme-based analytical systems are more sensitive and afford a wider concentration range for phenol detection (*6*). However, such factors as enzyme inactivation by reaction products (e.g., polyphenol oxidase) (*7*) or requirement of external electron donors, such as FAD-containing phenol hydroxylase (*8*), complicate the assay and increase the cost. The application of microbial sensors in the place of enzyme sensors may be an appropriate alternative. Neujahr (*9*) reported on the application of whole cells of yeast *Tricosporon cutaneum* instead of the isolated enzyme phenol hydroxylase. The author obviated the cumbersome work of enzyme purification and ruled out the need for NADPH addition. Also, a cell-based biosensor using *Pseudomonas cepacia* for monitoring aromatic compounds was reported (*10*).

This article describes a sensor based on microbial cells immobilized in poly(vinyl)alcohol cryogel (crio PVA); its sensitivity is comparable with enzyme sensors. The biosensor was developed by use of phenol-utilizing cells of *Pseudomonas putida* GFS-8. The cell cultivation conditions were optimized, resulting in the production of biocatalysts with stable and high phenol-oxidizing activity. To assess the practicality of the biosensor,

phenol was measured in model multicomponent solutions similar to waste waters.

MATERIALS AND METHODS

Bacteria Strain and Inocula Preparation

Ps. putida GFS-8 were obtained from the Genetics Scientific Research Institute (Saratov, Russia). The culture was maintained consistently on Luria-Broth (LB) agar plates and synthetic medium (M-9) supplemented with phenol agar plates. The washoff of cells grown on M-9 agar plates for 48 h was used as inoculum.

Growth Medium

1. LB medium: 1% peptone; 0.5% yeast extract; 0.5% NaCl; pH 7.0;
2. Synthetic medium supplemented with yeast extract and phenol: 0.075% $(NH_4)_2\ SO_4$; 0.05% NH_4Cl; 0.05% $MgSO_4 \times 7H_2O$; 0.05% KH_2PO_4; 0.05% K_2HPO_4; 0.1% yeast extract; 0.025% phenol, pH 7.0; and
3. Synthetic medium M-9 supplemented with phenol: 0.6% $Na_2HPO_4 \times 12H_2O$; 0.3% KH_2PO_4; 0.05% NaCl; 0.1% NH_4Cl; 0.025% phenol, pH 7.0.

Growth of Microorganisms

The *Ps. putida* GFS-8 cells were grown on LB medium in 750-mL conical flasks, containing 100 mL medium to the end of the logarithmic growth phase (180 rpm, 30°C). Biomass was then separated from the medium and transferred to activating medium (M-9 medium supplemented with phenol) for 24 h (180 rpm, 30°C).

Measurements of Phenol Oxidizing Activity

Phenol oxidizing activity was measured amperometrically with a Clark membrane electrode using the method described by Karube (*11*). The measurements were made in a temperature-controlled 5-mL amperometric unit, containing a cell suspension or immobilized cells, and 50 m*M* potassium phosphate buffer, pH 7.0, at 30°C. The reaction was initiated by injecting a phenol solution (0.1–5.0 mg/L). The initiation of the reaction by phenol allowed us to take into account the endogenic respiration of free or immobilized cells. The specific activity of freely suspended cells was defined as oxygen quantity (μM) consumed by 1 mg cells (wet wt) for 1 min. The specific activity of immobilized cells was defined as oxygen quantity (μM) consumed by 1 mg cells entrapped in PVA cryogel.

Phenol Determination

The dynamics of phenol concentration through cells incubation in the M-9 medium supplemented with phenol was measured spectrophotometrically at 270 nm (*12*).

Cell Immobilization

The immobilized biocatalyst was obtained by entrapment of *Ps. putida* GFS-8 cells in PVA cryogel by the procedure reported previously (*13,14*). The diameter of the beads was 0.8–1 mm. The content of the biomass in the beads was 10% (w/w) (wet wt).

Reagents and Apparatus

Reagents: yeast extract and Bacto-agar (DIFCO, US), peptone (SERVA, Germany), phenol, pyrocatechol (Sigma, St. Louis, MO), mesityl oxide, acetophenone, hydroquinone, 3,4-dihydroxybenzoic acid, 3-methoxyphenol, 4-methoxybenzoic acid, 2,6-dimethylphenol, dimethylphenylcarbinol (Merck, Germany). Other reagents were of the Russian grade "chemically pure." In all experiments, a freshly prepared phenol solution was used.

Apparatus: centrifuge Beckman G2-21 (US), spectrophotometer Hitachi-21 (Japan), polarograph LP-7 (Czechoslovakia), incubator shaker Series 25 (New Brunswick, US), amperometric unit with Clark electrode (YerPhy, Armenia), and Cryomat for biocatalyst production (BioChemMak, Russia).

RESULTS AND DISCUSSION

The microbial degradation of phenol has been widely studied. The major intermediate product of microbial oxidation of phenol is catechol. Catechol is further metabolized by *ortho-* or *meta*-pathways to form the compounds necessary for vital cell activity (*15,16*). Irrespective of microbial metabolic pathways, the key steps of aerobic phenol degradation include oxygen utilization. The application of phenol-utilizing cells for the development of an amperometric biosensor for phenol determination is therefore possible. It was shown earlier that *Ps. putida* GFS-8 have been successfully applied for phenol degradation in purification of waste waters from phenol production by the cumene method (*27*).

To maintain a high phenol oxidizing activity, the cells were grown on LB medium and transferred into M-9 medium with phenol, as the only source of carbon and energy. There was no cell growth in M-9 medium in the course of these experiments, and the rate of phenol utilization by the cells had a positive dependence on aeration of the medium. Cells grown and activated in such conditions exhibited high and stable endogenic respiration equal to 1.48 μmol O_2/min × mg cells (wet wt).

Cell respiration did not increase when phenol was injected as an exogenic substrate in the amperometric unit. It is reasonably safe to suggest that the only reason for this cell behavior is the high concentration of endogenic intermediates/products, such as catechol, inside the cells. The concentration of endogenic respiratory substrates may be dependent on phenol concentration in the activating medium. To confirm this proposal, phenol concentration in M-9 was varied. Cells grown to the stationary phase in LB medium were transferred to M-9 supplemented with varying phenol concentrations, and incubation was carried out in a thermostat shaker at at 30°C and 180 rpm. During the course of cell incubation, samples were taken regularly. Cells were separated from the medium by centrifugation for 15 min at 10,000 rpm. Parameters that were monitored included phenol concentration, cell endogenic respiration, and cell respiration with phenol as exogenic substrate. Results obtained are presented in Fig. 1. One can see that the lowest rate of phenol utilization occurred when the phenol concentration was 0.45 g/L (Fig. 1A, curve 3). Cells incubated at this phenol concentration exhibited stable endogenic respiration, which slightly increased during incubation (Fig. 1A, curve 2). In this case, cells did not use phenol as exogenic respiratory substrate when tested in the amperometric unit (Fig. 1A, curve 1). It is conceivable that the cells accumulate metabolic intermediates during the incubation on the medium with a high phenol content. These intermediates hold the endogenic respiratory activity at a constant high level. Decrease of phenol concentration to 0.25 g/L in the activating medium was accompanied by an increase in the rate of phenol utilization (Fig. 1B, curve 3). Endogenic cell respiration appears to decrease during incubation (Fig. 1B, curve 2), whereas the cell's ability to utilize phenol as exogenic respiratory substrate significantly increased (Fig. 1B, curve 1) and reached the maximal value when practically all phenol in the medium was depleted. Cell incubation in the medium with 0.1 g/L phenol (Fig. 1C), showed that the tendency of this result was very similar to the data presented in Fig. 1B. As phenol was depleted during incubation, endogenic respiration decreased, whereas cell response to exogenic phenol increased. A phenol concentration of 0.1 g/L is probably too low for activation of the metabolic pathway to oxidize phenol. It must be emphasized that to produce cells with low endogenic respiration, not only is the phenol concentration in the activating medium important, but also the ratio of cell concentration to phenol concentration is important. The results demonstrated in Fig. 1 were obtained at a cell concentration of 10 g/L in activating medium. Decrease in the cell concentration to 0.2 g/L resulted in high endogenic respiration. Cell concentrations as high as 35 g/L resulted in 50% cell lysis during incubation.

From the results described above, it follows that for production of *Ps. putida* GFS-8 cells with low endogenic respiration and high ability to oxidase phenol, as an exogenic respiratory substrate, it is necessary to incubate 10 g/L cells in activating M-9 medium with 0.25 g/L phenol for 7–12

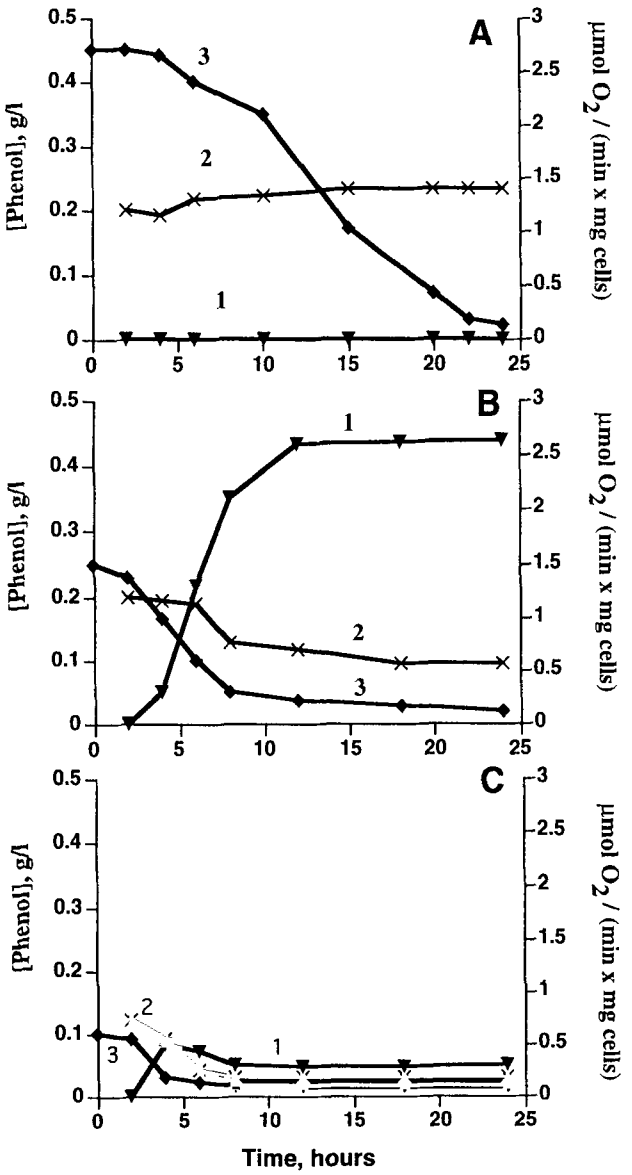

Fig. 1. Incubation of *Ps. putida* GFS-8 cells in M-9 media with various initial phenol concentrations: (**A**) 0.45 g/L; (**B**) 0.25 g/L; (**C**) 0.10 g/L; cell concentration was 10 g/L. 3—phenol content, 2—cell endogenic respiration, and 1—cell respiration with phenol as an exogenic substrate. Cell respiration was defined from oxygen consumption rate in the amperometric unit. The reaction was initiated by injecting of 100 μL phenol solution into 5 mL amperometric unit, contained 2 mg cells/mL 50 m*M* K-phosphate buffer, pH 7.0, 30°C.

h. The ability of these cells to use phenol as an exogenic respiratory substrate was reduced by 40% from the initial value after 3 d and was decreased to 0% in 6 d.

To simplify the preparation of active cells and to prolong their ability to oxidize phenol as exogenic substrate, the cultivation was conducted on

Table 1
Oxygen Consumption by *Ps. putida* GFS-8 Cells in Presence of Various Compounds

Substrate, 1 mg/L	Relative activity, %
Phenol	100
Pyrocatechol	170
Mesityl oxide	46
Acetophenone	0
Hydroquinone	0
Benzoic acid	0
Parachlorbenzoic acid	0
Ortochlorbenzoic acid	0
Phenilacetic acid	0
Dimetilphenylcarbinol	0
Benzaldehyde	0
3,4-Dihydroxybenzoic acid	0
3-Methoxyphenol	0
4-Methoxybenzoic acid	0
2,6-Dimethylphenol	0
Glucose	0
Saccharose	0
Lactose	0
Xylose	0
Arabinose	0
Ramnose	0
Dulcitol	0
Sorbitol	0
Inositol	0

synthetic medium supplemented with phenol and yeast extract as described by Spanning and Neujahr (*17*). However, this procedure was modified; we performed the batch cultivation with a single injection of phenol so that the initial phenol concentration in the medium was 0.25 g/L. The cells grew in this medium at a rate comparable to that of cells in LB medium. Furthermore, the cells retained their properties for up to 2 wk and lost only 15% of the initial activity on the 10th d of storage. Owing to the evident advantages of cell cultivation in synthetic medium supplemented with yeast extract and phenol, this method was used in further experiments to obtain the biomass. It was shown that *Ps. putida* GFS-8 cells can utilize not only phenol as respiratory substrate, but also pyrocatechol and mesityl oxide, and these cells do not use various xenobiotics, sugars, and alcohol as a substrate (Table 1). The high activity with pyrocatechol is the result of the fact that this compound is the main intermediate of phenol metabolism (*15,16*).

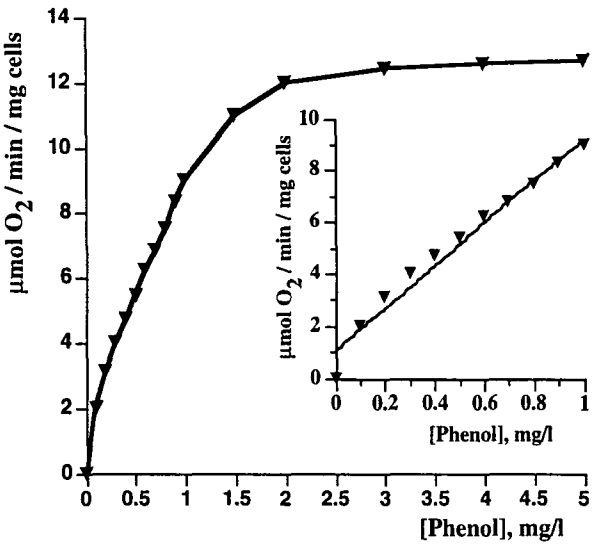

Fig. 2. The rate of oxygen consumption by free *Ps. putida* GFS-8 cells as a function of the phenol concentration in the amperometric unit. The inserted graph shows linear part of the curve ($y = 8.21x + 1.06$; $R^2 = 0.975$). The reaction was initiated with injection of 100 µL cell suspension into 5 mL amperometric unit contained phenol solution.

The most important criterion for the applicability of the cells to develop amperometric biosensor is the correlation between the rate of oxygen consumption and substrate concentration in the assay solution. We have shown that for these cells, a positive correlation between these two variables was observed in the phenol concentration range of 0.1–2 mg/L, and a linear dependence was detected in the range of 0.1–1.0 mg/L (Fig. 2). On optimization of the assay, we established that the cells showed a maximum respiratory activity with phenol at 30°C in 50 m*M* potassium-phosphate buffer, pH 7.0, and at a cell concentration in the amperometric unit in the range of 1.5–2.0 mg/mL (wet wt). The response time after substrate injection was 1 min. The total time required for analysis was approx 5 min.

In spite of the potential to use freely suspended cells as a biological transducer for biosensors in modern analytical systems, the immobilized cells are preferred since immobilization usually stabilizes the biocatalyst, ensures its multiple application, and improves the reproducibility of the results (*18,19*). Many different methods for immobilization of microbial cells have been reported (*19,20*). However, to choose the carrier and immobilization method is a difficult problem, and each case is resolved according to the objective and application purpose. If the reaction is catalyzed by intracellular enzymes involving cofactors, the immobilization conditions and carrier are selected to preserve the structural integrity of cells and to provide a minimal diffusional barrier created by the matrix. One of the methods ideally suited to the above requirements is the entrapment of cells in cryogel of PVA. A successful immobilization of microbial cells

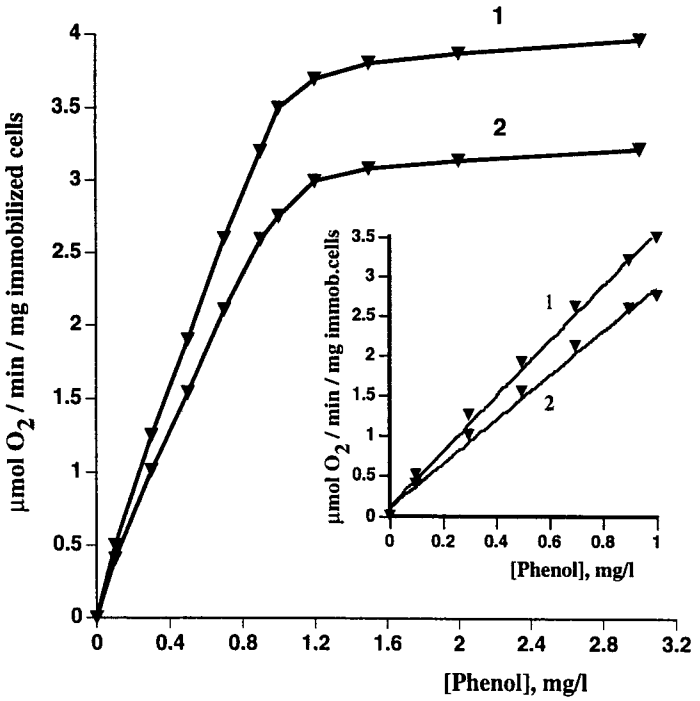

Fig. 3. Phenol calibration curve for cells immobilized in PVA cryogel; 1—1st d after immobilization; 2—30th d after immobilization. The inserted graph shows linear part of the curve ($y_1 = 3.44\times + 1.27$; $R_1^2 = 0.965$; $y_2 = 2.74\times + 1.14$; $R_2^2 = 0.944$).

by this method was reported (*13,21–25*). High mechanical strength and porosity of the resultant matrix allow for application of the biocatalyst in analytical systems. The immobilized biocatalyst was produced by freezing cells in an aqueous PVA solution. The resultant beads (0.8–1 mm in diameter) were thawed, placed in M-9 medium supplemented with 0.25 g/L phenol, and incubated at 30°C for 1 d. The biocatalyst was subsequently washed with 0.05*M* potassium-phosphate buffer, pH 7.2, and used to assay phenol. The amount of immobilized cells placed in the amperometric unit was the same as that for free cells and was approx 2 mg/mL (or 20 mg/mL of biocatalyst). It was shown that both immobilized cells and free cells show a linear dependence of respiratory activity on phenol concentration in an identical range of phenol concentration (Figs. 2 and 3). The response time and the total analysis time were also similar. During storage in phosphate buffer with a low phenol concentration (0.01 mg/L) at 4°C, the biocatalyst retained no less than 80% of its initial activity over a month (Fig. 3, curve 2).

The feasibility of this biosensor was assessed by studying the effect of compounds most frequently found in waste waters from phenol production (*1*) on the ability of the immobilized cells to use phenol as exogenic substrate and on parameters of this analytical system. The solutions of

Table 2
Respiratory Activity of Immobilized *Ps. putida* GFS-8 with Phenol as Exogenic Respiratory Substrate in the Presence of Some Components of Waste Waters

Substrate	Concentration, mg/L	Relative activity, %
Phenol	1.00	100
Aniline	1.00	71
Phenol + aniline	1.00	47
Acetophenone	0.26	0
Phenol + acetophenone	1.00 + 0.26	100
Dimethylphenylcarbinol	0.03	0
Phenol + dimethylphenylcarbinol	1.00 + 0.03	100
Acetone	0.70	0
Phenol + acetone	1.00 + 0.70	100
Cumene	0.06	0
Phenol + cumene	1.00 + 0.06	100
Phenol + $AlCl_3$	1.00 + 0.70	100

each compound, listed in Table 2, singly or in combination with phenol, were used in the amperometric unit as the respiratory substrates for immobilized cells. The concentrations of the test substances were selected according to their content in real waste waters. It should be noted that these compounds injected in the assay buffer without immobilized cells do not affect the electrode current value.

As illustrated in the Table 2, components of the waste waters, such as acetophenone, cumene, dimethylphenylcarbinol, acetone, and aluminum chloride, have no effect on phenol determination. However, when aniline was injected into the measurement unit containing the biocatalyst, the oxygen consumption rate was 71% of the oxygen consumption rate with phenol injection. At a simultaneous injection of equimolar concentrations of phenol and aniline, the overall change in oxygen consumption rate was less than with phenol alone. Scheller et al. (*26*) also described some influence of aniline on phenol determination.

Thus, the biosensor we have developed on the basis of *Ps. putida* GFS-8 allows for phenol determination in the concentration range of 0.1–1.0 mg/L. The total assay time is only approx 5 min. The same biocatalyst may be used to quantify phenol at least 10 times. After each use, it is necessary to wash the biocatalyst thoroughly with buffer for 10 min. The reproducibility of analysis was +/−5%. The biosensor retained, at least, 80% of the initial catalytic activity for up to 1 mo.

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