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Detection of phenolic compounds by thick film sensors based on *Pseudomonas putida*

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Abstract

Amperometric biosensors using bacterial cells were developed for the determination of phenolic compounds and the measurement was based on the respiratory activity of the cells. For this purpose, *Pseudomonas putida* DSM 50026 which is one of the well-known phenol degrading organisms, was used as a biological component. The cells were grown in the presence of phenol as the sole source of organic carbon. As well as phenol adapted cells, the bacterium which used the glucose as the major carbon source, was also used to obtain another type of biosensor for the comparison of the responses and specificities towards different xenobiotics. The commercial oxygen electrode was used as a transducer to test the sensor responses for both induced and non-induced cells. Our results showed that the adaptation step enable us to obtain biosensor devices with different substrate specificity. Moreover, *P. putida* was immobilized on the surface of thick film working electrodes made of gold by using gelatin membrane cross-linked with glutaraldehyde. The biosensors were calibrated for different phenolic substances. Furthermore, phenol detection was performed in synthetic wastewater samples.

Keywords: Microbial biosensor; Thick film electrodes; *Pseudomonas putida*

1. Introduction

The intensive increase in the release of a diverse range of hazardous compounds into the environment has made their detection of paramount importance. In particular, this is necessary for successful clean-up of pollution from the environment and timely elimination of the consequences or a reduction of the scale of hazardous release [1]. Phenolic compounds are one of the major pollutants of industrial waste waters and, at the same time, the compounds have high toxicity to the human organism when present above certain concentration limits this require rapid, easy to operate and low-cost toxicity screening procedures. The trend towards simplification and automation of the analysis methods used in modern

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chemical and biochemical laboratories or in quality control of some industrial biosynthesis processes has led to setting up some electrometric procedures for determining phenol, based on biosensors [2–4]. The use of microbial biosensors to determine the concentrations of substances is based on the presence of specific enzyme systems in microorganisms which transform certain chemical compounds. The transformation processes can be accompanied by the appearance of electrochemically active products or utilization of reaction co-substrates, which enable the use of standard electrochemical techniques—amperometry or potentiometry [5]. As judged by their sensitivity, time of response and stability of signals, microbial sensors are similar to enzyme based sensors but are less selective. This may be due to the complexity of the elements of the enzyme apparatus of cells. Insignificant amount of biomass as well as high stability make the use of microbial sensors preferable in some cases compared to enzyme sensors. This is especially true in the detection of a pool of toxic compounds showing similar composition, or the assessment of comprehensive indices of the condition of the environment as, for instance, biological oxygen demand (BOD) [6]. In this study, amperometric biosensors using bacterial cells were developed for the detection of phenolic compounds. *P. putida* DSM 50026 was used as a biological component and the measurement was based on the respiratory activity of the cells. For this purpose, the cells were grown in the presence of phenol as the sole source of organic carbon. As well as phenol adapted cells, the bacterium which used the glucose as the major carbon source, was also used to obtain another type of biosensor for the comparison of the responses and specificities towards to different xenobiotics.

2. Experimental

2.1. Reagents

All chemicals were commercially avaible and of reagent grade. L-DOPA, L-tyrosine, syringic acid, caffeic acid, resorcin, picric acid, hydroquinon, 2,6-dihydroxybenzoic acid, benzoic acid were products of FLUKA AG (Switzerland). Phenol was from Merck AG (Darmstadt, Germany).

Mineral salts medium (MSM) with the following composition [7] was used as a growth medium: 0.1% NH_4NO_3, 0.05% $(NH_4)_2SO_4$, 0.05% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.15% K_2HPO_4, 0.05% KH_2PO_4, 0.0014% $CaCl_2 \cdot 2H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$ and trace element solution (1 ml/l). The pH of the medium is 6.9. Trace element solution was prepared according to Ref. [8].

Synthetically concocted waste water compositions: Sample type 1 composed of $(NH_4)_2SO_4$ (0.5 g), $MgSO_4$ (0.1 g), $MnSO_4$ (0.01 g), $FeSO_4$ (0.0005 g) and known amounts of phenol in tap water (100 ml), pH 7.2 [9]. Sample type 2 included NaCl (50 g/l) and phenol (100 g/l) in 1 M HCl solution [10].

2.2. Microorganism and culturing conditions

P. putida DSM 50026, which is a gram negative, polar, flagelled unicellular bacteria was sub-cultured on Nutrient Agar.

To obtain non-adapted, control cells which use glucose as a carbon source, the microorganism was inoculated into 50 ml of MSM containing 250 mg/l glucose and incubated at 28 °C on an orbital shaker at 150 rpm. After 24 h, when cells were grown, the biomass was harvested by centrifugation at 10 000 rpm and suspended in MSM and then re-centrifuged. The supernatant was removed and the cellular paste was used for making biosensor.

Induction of the cells to phenol (250 mg/l) was performed with the following steps; the wild type microorganism was inoculated to the MSM medium containing gradually increasing phenol and decreasing glucose concentrations by daily inoculations until the medium reached 250 mg/l of phenol. After adaptation was completed, the cellular paste was obtained as described above.

Cell growth was followed spectrophotometrically by measuring optical density at 560 nm and the relationship between optical density and the living cells was also investigated [11]. In all experiments, log-phase bacterial cells ($OD_{560} = 0.450$) were used.

2.3. Apparatus

Clark electrode (Oxiliquid mod.22 IDRONAUT, Brugherio MI, Pt diameter; 1 mm) covered with a Teflon membrane (Beckman, no 670597) and ceramic thick film sensors (BVT Technologies a.s., Czech Republic) with gold working, Ag/AgCl reference and Pt auxiliary electrodes were used, respectively. The changes in current were measured with a Potentiostat (Metrohm 641, Herisau, Sweden).

2.4. Electrode preparation

P. putida cells which have 1.8×10^9 cell titer (50 μl) and 225 bloom gelatin (10 mg) were mixed at 38 °C in MSM (250 μl). 50 μl of mixed solution was spread over the membrane of the oxygen electrode and allowed to dry at 4 °C for 1 h. Finally, it was immersed in 2.5% glutaraldehyde in 50 mM phosphate buffer (pH 7.5) for 5 min [12]. Moreover, the procedure belong to thick film sensors was the same as the previous one, except 2.5 μl of mixed solution was placed on gold working electrode of ceramic thick films.

Daily prepared enzyme electrodes including fresh cells in logarithmic phase were used in all experimental steps.

2.5. Measurements of the respiratory activity by the microbial electrodes

All measurements were carried out at 28 °C which is the growth temperature of the bacteria. Various phenolic substrates were added individually to the reaction cell. When a respirable substrate was added, the oxygen concentration in the bioactive layer decreased until new steady state was reached within 5–10 min. The Working electrodes of both, commercial Clark and screen printed ceramic electrode, were polarized at −700 mV vs Ag/AgCl. The current changes were recorded. After completion of the measurement in the 5 min of reaction time, the electrode was rinsed with distilled water and allowed to equilibrate before another measurement. MSM, pH 6.9 was used as a working buffer.

3. Results and discussion

3.1. Optimization of the sensor response

The sensor response to phenol was tested in the presence of potassium phosphate buffer (50 mM, pH 6.9) and MSM (pH 6.9), respectively by adapted cells immobilized on Clark electrode (Fig. 1). The higher signal was observed in MSM which was also used as a growth medium for the bacteria and then, MSM was chosen as a working buffer solution in further experiments. During the measurements, to protect metabolic activity was the most important point. For this reason the other buffer system with different pH values and also different temperatures were not used. On the other hand, working temperature and pH were also similar with the previous works [13,14]. In these experimental conditions, the response time of microbial sensor based on Clark electrode and thick film were found to be 10 and 5 min, respectively.

10, 25, 50 and 100 μl of bacterial cell which have the same cell titer given in section 2.4 were also

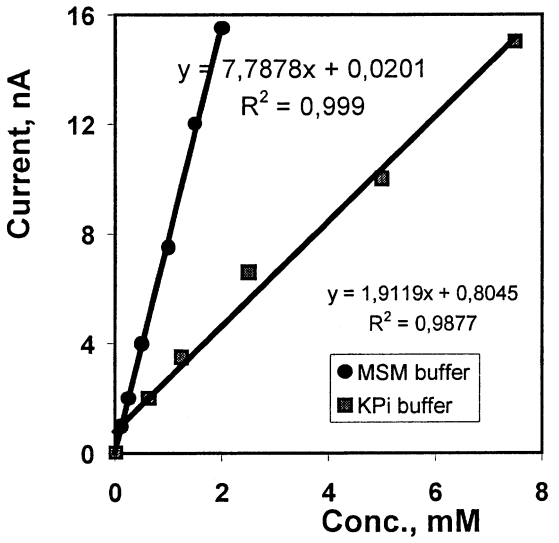

Fig. 1. Calibration for phenol in potassium phosphate (KP_i; 50 mM) and MSM, pH 6.9.

used to prepare the immobilization mixture and the sensor responses were measured in the presence of 1.0 mM phenol concentration by biosensor based on Clark electrode. Maximum response was found to be in 50 μl cell amount and higher amounts caused to decrease in signal because of the diffusion problem.

3.2. Substrate specificities

The substrate specificities of adapted and control cells to different phenolic compounds were tested by the oxygen electrode as a transducer and given in Table 1. As can be seen, both adapted and control cells showed the response towards phenol, 2,6-dihydroxybenzoic acid and benzoic acid. However, apart from these compounds, while adapted cells are giving the response to DOPA, tyrosine, syringic acid, resorcin, picric acid, methyl-parathion, no detectable signal was observed for these compounds by the biosensor including control cells. This circumstance can be explained by the adaptation effect that caused different substrate specificities in bacterial cells. In *P. putida*, phenol degradation depends on producing the phenol hydroxylase and further metabolic enzymes, produced only in the presence of their substrates. While phenol adapted cells can use phenol as a sole carbon source and metabolize it at high levels, non-adapted cells were exposed to phenol at quite low concentrations, their response to phenol can be more rapid than phenol adapted cells.

Table 1
Sensor response of adapted and control cells immobilized on Clark electrode to different phenolic substances[a]

Phenolic compound	Signal, current (I, nA)[b]	
	Adapted cell	Control cell
Phenol	6.7±0.3	61.5±0.3
Catechol	–	22.9±0.3
L-DOPA	23.6±0.3	1.9±0.2
L-Tyrosine	38.2±0.3	–
L-Syringic acid	30.3±0.3	–
Caffeic acid	–	–
Hydroquinone	–	34.2±0.6
Adrenaline	–	112.1±0.2
Benzoic acid	58.9±0.3	38.4±0.3
Resorcin	6.3±0.3	–
Picric acid	15.5±0.4	–
3-Hydroxytyramine	–	0.62±0.01
2,6-Dihydroxybenzoic acid	10.3±0.3	6.2±0.3
2,3-Dihydroxynaphthalene	–	27.8±0.3
Toluen	1.8±0.3	–
Methyl-parathion	11.4±0.3	–

[a] Results are expressed as mean ±S.D, $n=4$.
[b] The concentration of substances in reaction mixture; 1 mM.

3.3. Thick film sensors

In this part of the study, thick films with gold working electrode were used as a basic sensor for the determination of detection limits of phenolic compounds. Bacterial cell which are in same age were directly immobilized by means of gelatin cross linked with glutaraldehyde. Decrease of the current because of the oxygen consumption was easily detected without requiring the gas permeable selective membrane. Instead of gold surface we also used screen printed graphite electrode to test the sensor response. However, in this case surface modification with the cellulose acetate membrane was necessary to obtain better signals without noise (unpublished data).

The calibration curves for phenol, catechol, hydroquinone, benzoic acid and 1,2-dihydroxynaphthalene were obtained by control cells immobilized on gold working electrode on ceramic substrates (Table 2). For these compounds linear detection ranges were found (Table 2) and above these levels, inhibition was observed. On the other hand, adapted cells were used to obtain calibration graphs for phenol, L-tyrosine, L-DOPA, L-syringic acid and methyl-parathion (Table 2). Detection ranges for phenol and parathion-methyl were found to be 0.5–6.0 μM and 0.3–2.5 μM. Moreover, L-tyrosine, L-DOPA, L-syringic acid were determined in the same range (0.02–0.2 μM). In the higher concentrations of these compounds, sensor response remained steady. This could be due to the adaptation process which caused the higher resistance towards the xenobiotics in bacterial cells [15].

Table 2
Calibration curves obtained with thick film sensors with various phenolic compounds, with control and adapted cells

Cell type		Linear range (μM)	Slope ($mA \cdot M^{-1}$)	R^2
Control cells	Phenol	0.10–1.00	16.54	0.9986
	Catechol	0.50–5.00	5.07	0.9971
	Hydroquinone	0.25–1.00	15.20	0.9972
	Benzoic acid	0.10–0.75	15.82	0.9981
	1,2-Dihydroxynaphtalene	0.25–2.50	8.01	0.9992
Adapted cells	Phenol	0.50–6.00	5.03	0.9995
	L-Tyrosine	0.02–0.20	87.08	0.9988
	L-Syringic acid	0.02–0.20	37.01	0.9852
	L-DOPA	0.02–0.20	61.88	0.9989
	Methyl-parathion	0.30–2.50	3.64	0.9917

Operational life time of thick film sensors were also tested in MSM, 28 °C. In these conditions, during 6 h, 25 measurements could be performed without any decrease in sensor response. After that, reduced signals were observed. Because of this reason, to obtain similar results, each electrode was prepared daily.

The reproducibility of the thick films based on adapted and control cell were also searched. For adapted cell, 2.0 μM ($n = 7$) phenol concentration was tested and the S.D. and variation coefficient (C.V) were calculated as ± 0.09 μM and 4.4%. Moreover, these values were found as ± 0.02 μM and 3.9% for control cell in the presence of 0.5 μM ($n = 7$) phenol concentration, respectively.

3.4. Sample application

The microbial biosensors were applied in waste water samples. For this aim, two different types of synthetic waste water sample were prepared as described in material and methods. Both types of sample which included known amount of phenol were used as stock substrate solutions with different dilutions by working buffer and added to the reaction cell after equilibration and then the change in current was measured. The signals obtained from the waste samples were found to be very similar with the standard phenol solutions having the same concentration (Table 3). The results showed that, no sample matrix effect interfered in our measurements. Both type of cell could be used the presence of these kinds of waste water samples without requiring sample pretreatment.

Table 3
Application of the microbial thick film electrodes to the synthetic waste water samples

Phenol concentration (μM)	Detected amount (μM)	
	Adapted cell	Control cell
Sample type 1		
0.25		0.24 ± 0.01
0.50		0.52 ± 0.02
1.0	1.01 ± 0.02	
2.0	1.98 ± 0.05	
Sample type 2		
0.25		0.25 ± 0.01
0.50		0.51 ± 0.03
1.0	1.01 ± 0.02	
2.0	2.02 ± 0.03	

Results are expressed as mean ± S.D, $n = 7$.

Enzymes with a broad substrate spectrum, i.e. laccase, tyrosinase, polyphenol oxidase and peroxidase as well as specific enzymes, e.g. phenol hydroxylase and catechol oxidase, may be used for construction of phenol biosensors [16,17]. The tyrosinase electrodes are often unstable due to fouling of electrodes by accumulation of polymerization products formed by the resulting quinones. Another reason may be inactivation of enzymes by

these reactive products [18]. In spite of this fact, most biosensors for detection of phenols employ tyrosinase as biorecognition material. Another biosensor for phenol detection was based on phenol hydroxylase entrapped in a sol/gel matrix linked to a Clark-type oxygen electrode. The limit for detection of phenol demonstrated by the sensor was 2.5 mM and the injection of cofactor to the medium was required. Alternatively, whole cells and plant tissues have been combined with various electrodes. Microbial sensors are inexpensive and easily produced, possess the sensitivity and stability comparable with those of enzyme sensors and no additional efforts are needed for purification of enzyme [19].

From the practical point of view, to characterize the substrate selectivity of sensors is very important. The selectivity of microbial sensors is known to be generally rather low, and researchers are looking for new ways to improve it [20]. In our case, biosensors with different selectivity for detection of phenol based on whole cells were obtained by adaptation process. Our findings showed that, adaptation process affected to substrate specificity but, higher sensitivity was not observed by this way. In further studies, adaptation will be performed with different amounts of xenobiotics to obtain biosensors having different selectivity.

4. Conclusions

Actually biotechnological processes have been employed in several industrial productions, in biomedical applications and in environmental remediation. Interest was focused on biomediators as purified enzymes or metabolic pathways of cells, tissues etc. with specific biochemical properties for analytical and either production or degradation applications [21]. In this study, development of *P. putida* based biosensors for determining the phenolic compounds. Bacterial cells with and without adaptation steps enable us to get biosensor systems with different substrate specificities, thus it could be possible to analyze different phenolic substances individually in the mixed samples by using these sensors. On the other hand, in some cases adaptation may eliminate the necessity of using genetically manipulating microorganisms.

In previous studies, different types of microorganisms were used in biosensor systems [22–25]. In comparison to the others [20], our biosensors have higher sensitivity as microbial sensor. Furthermore, immobilization of microorganisms instead of pure enzymes on the thick film electrodes provided economic and practical disposable biosensors. The immobilization method was also useful for the protection of microbial activity. All our data showed that the obtained biosensors could be used as simple, rapid and direct methods of determining phenol in various media such as waste water samples with different compositions.

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