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Monitoring of the phenol biodegradation process with an electrochemical biosensor

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Monitoring of the water (domestic and/or industrial) pollution continuously and on regular bases is a quite severe issue, which still demands proper solution. Here we report on development of an amperometric biosensor for detection of phenol degradation in water and we compare the analytical data with those obtained from the most common analytical method: HPLC (High Performance Liquid Chromatography).

The described biosensor consists of an enzyme electrode, comprising a porous hydrophobic carbon electrode with immobilized enzyme, namely: Tyrosinase from mushroom (EC 1.14.18.1). In accordance to study the efficiency of tyrosinase amperometric sensor for the determination of phenol concentration, experiments at constant potential of 0.0V vs. Ag/AgCl reference electrode in phosphate buffer solution (pH 7.0) are performed. The response time is not exceeding 3 min and the amperometric response increased linearly up to 2 μM phenol concentration. The tyrosinase electrode exhibited a sensitivity of 330 nA μM^{-1} and the apparent Michaellis constant was calculated to be K^{app}_M = 12.6 μM with detection limit for phenol of 0.4 μM. Moreover, the tyrosinase electrode exhibited a good reproducibility and stability during 35 days of periodical measurements.

The tyrosinase electrode investigated in our study is applied efficiently for the detection and determining of the phenol concentration in real water samples.

Keywords: biodegradation of phenol, tyrosinase, gas-diffusion electrode, *Klebsiella oxytoca* strain

INTRODUCTION

Development of new, advanced technologies for removing wastewater contaminations is crucial issue regarding worldwide environmental pollution as result from human activities. Xenobiotics are one of the most toxic wastes from chemical, petrochemical, pharmaceutical, textile, and coke-chemical industry. Phenol and its derivatives are usually presented in higher than officially limited (restricted) concentrations in effluents from these industries [1].

The methods of wastewater treatment are various – physical [2, 3, 4], chemical [5, 6], physicochemical, thermal. The most advantageous among them are the biochemical methods, but phenol derivatives are among the most resistant compounds to biodegradation by microorganisms. However, here are known possibilities for degradation of phenol compounds by eukaryotic and prokaryotic organisms, despite their toxic effects in some cases. These organisms are capable of using such a phenolic compounds as a carbon and energy sources. This process involves a series of biochemical reactions, varying between different strains of bacteria and yeast. Phenol degrading microorganisms belong to different taxonomic groups - bacteria, fungi, yeasts and algae and the very first example of biodegradation of phenols has been reported by Störmer (1908). Since than, over the decades of extensive research, were found many strains of microorganisms capable of degradation of phenol. Among them the most commonly used are: *Bacillus stearothermophilus* [7]; *Pseudomonas putida* [8]; *Agaricus bisporus*, [9], *Pseudomonas sp*. [10], *Fusarium sp.* [11], *Alcaligenes sp*. [12], *Klebsiella oxytoca* [13] and others. These microorganisms posses a multienzyme systems to break down organic compounds, converting them into harmless products. They are highly specific at very low energy consumption.

As we mentioned before, the most common methods for determination of phenolic compounds are colorimetric, gas or liquid chromatography and spectrophotometric analyses. However, some of these techniques are expensive, time consuming and they are unsuitable for on site or field based analyses [14, 15].

The use of electrochemical biosensors has been shown to be a suitable alternative with respect to conventional well established analytical techniques for the monitoring of phenolic compounds content

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in local environment. Advantages of the presented analytical approach are its simplicity, relatively low cost and high selectivity [16].

The first report for polyphenoloxidases application in biosensors was published in 1977 by Macholan and Schanel [17]. Enzymes with wider substrate tolerance such as laccase, tyrosinase, peroxidase along with more specific enzymes, for e.g. phenolhydroxylase and catecholoxidase, could be used for enzyme electrode construction [18]. The phenoloxidizing enzymes mechanisms and some examples of their use in the construction of phenolselective biosensors are nicely reviewed by Peter and Wollenberger [19]. The biosensors based on the immobilization of tyrosinase on different electrode materials can be found in recent literature. Thus different materials [20-24] have been used to prepare tyrosinase electrochemical biosensors. In spite of the big amount of literature two general limitations need to be solved yet in order to improve their practical usefulness - the stability and high sensitivity of the biosensors [16].

The enzyme tyrosinase catalyzes phenols oxidation through hydroxylation with oxygen to catechols and subsequent dehydrogenation to the respective quinines compounds (1, 2). The electrochemical reduction of the product o-quinone is used as indicating reaction (3):

$$\text{Phenol} + O_2 \xrightarrow{\text{tyrosinase}} \text{Catechol} \quad (1)$$

$$\text{Catechol} + O_2 \xrightarrow{\text{tyrosinase}} \text{o-quinone} \quad (2)$$

$$\text{o-quinone} + 2H^+ + 2e^- \longrightarrow \text{Catechol} \quad (3)$$

The catechol produced in the electrochemical reaction (3) is also taking place in the enzymatic reaction (2), so that an “enhancing effect” is observed [25].

The goals of this work is to study the feasibility of a biosensor for determining the phenol concentration during biodegradation of phenol using *Klebsiella oxytoca* VA 8391.

EXPERIMENTAL

The experiment of phenol biodegradation

Klebsiella is a genus of fixed, gram-negative, oxidase-negative, rod-shaped bacteria.

Inoculum of *Klebsiella oxytoca* strain is obtained on medium with a composition (Meat extract ("Fluka") - 10 g/l, Peptone ("Fluka") - 10 g/l, NaCl - 5 g/l and distilled water was added to 1 liter total volume, which is seeded from agar medium. Cultivation – on a shaker "WiseCube" at 30 °C, speed - 150 rpm, pH 7.0, duration of the process - 24 hours.

Experiments of phenol biodegradation were conducted in a thermostated glass reactor with a total volume of 200 ml with stirring (thermostat "MLW UH").

Medium used for biodegradation of phenol was $MgSO_4.7H_2O$ - 0.2 g/l, $CaCl_2.2H_2O$ - 0.2 g/l, NaCl - 5 g/l, phenol - 1 g/l and phosphate buffer (pH 7.0) with the following parameters: pH 7.0; revolution speed - 200 rpm; temperature 30 °C; inoculum aged 24 hours, pH ~ 7.0; volume of seed culture - 20 ml; volume of medium – 200 ml; temperature 30 °C; revolution speed = up to 100 rpm.

The biomass concentration was determined by measuring the absorbance of a sample at wavelength λ = 590 nm on spectrophotometer Specol (CARL Zeiss, Jena, Germany). The biomass concentration of the bacterial strain *Klebsiella oxytoca* VA 8391 is calculated according to the equation (4):

$$C = (A - 0.0223) / 1.0019 \quad (4)$$

wherein C - biomass concentration g/l, A - absorption.

The concentration of phenol in the culture broth was determined by HPLC. The HPLC system was composed from a quaternary pump "Knauer S - 1000", UV - detector "Knauer", software for the collection and processing of data ("Eurochrom"), and C18 column "Discovery" (Shimadzu). As the mobile phase 10 % methanol in phosphate buffer pH 3.0 was used. The determination was carried out at a wavelength λ = 280 nm at flow rate 1 ml/min.

Periodically, samples were taken to determine the biomass and the amount of the phenol in the culture medium.

Enzyme electrode preparation

The investigated electrode comprises a porous carbon supporting layer placed in a conical plastic tube. Enzyme tyrosinase from mushroom (EC 1.14.18.1) is immobilized on the carbon electrode surface from the narrow end of the conic tube to form the enzyme electrode. For the enzyme immobilization the carbon electrode was treated with tyrosinase solution (4 mg/ml) and with neutralized Nafion 117 (Fluka) (5 weight %), solution. A porous carbon electrode made by pressing from a composite carbon material (acetylene black treated with polytetrafluorethylene (PTFE) by a special technology [26]) was used. This carbon electrode possesses a very high porosity (0.95 cm^3/g) combined with a high hydrophobicity and electronic conductivity. The

collection of current is achieved by a Ni wire pressed into the porous carbon electrode.

The enzyme gas-diffusion electrode is mounted in the electrochemical cell in a way providing contact between its enzyme layer and the electrolyte. The opposite side of the working electrode is out of the cell and is in contact with the air.

The electrochemical measurements were performed in a two-electrode cell (5 cm^3). A chlorinated silver wire was used both as reference Ag/AgCl and counter electrode respectively. Constant potential 0.00 V was maintained between the investigated and the reference electrodes.

Tyrosinase by "Sigma-Aldrich" – lyophilized powder, ≥ 1000 unit/mg solid was used in all experiments. The substrate, phenol, was purchased from "Sigma". 0.1 M phosphate buffer solution containing 0.1 M KCl with pH 7.0 was used as main background electrolyte.

The experimental set up for measurements includes a two-electrode cell with stirring, potentiostat connected with voltmeter and recording device for the plotting electrode signal vs. time.

RESULTS AND DISCUSSION

HPLC analysis

Batch process of phenol biodegradation by *Klebsiella oxytoca* strain at an initial concentration in the medium 0.200 g/l is presented on **Fig. 1**. The experiment was conducted using the methodology described in the experimental part above. Samples were taken every 30 minutes. They were analyzed chromatographically.

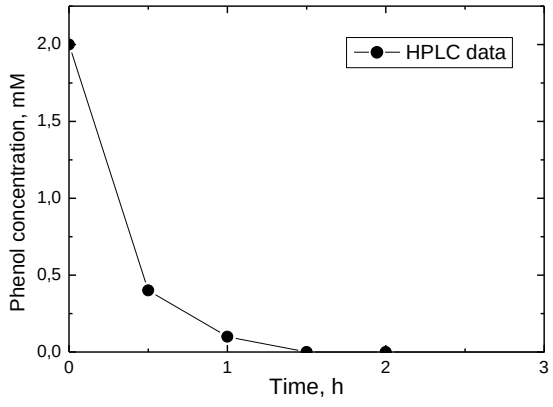

Fig. 1. HPLC analyses of the phenol biodegradation using *Klebsiella oxytoca.*

It is noteworthy that the time for biodegradation in these experiments is very short – 60-90 minutes.

Enzyme electrode analysis

After a stable background current is obtained a definite amount of phenol was added to the electrolyte in the cell and the amperometric response of the tyrosinase electrode was recorded. From our previous studies [25] the value of 0.00V versus Ag/AgCl reference electrode has been accepted for the working potential of the investigated tyrosinase electrode. The background current at this potential is practically zero. At this low potential interferences from easily oxidizable species are minimized. The amperometric signal of the investigated tyrosinase electrode (the steady-state current at a constant potential) is presented in **Fig. 2 (a)** as a function of the phenol concentration in the electrolyte. The experimental points are obtained by consecutive addition of aliquots of 0.1 mM phenol stock solution to the electrolyte in the cell with simultaneous registration of the current. The response time (defined as the time when 95% of the steady-state current is reached) was less than 3 min. It is seen that the amperometric signal increases linearly with the increase of the phenol concentration in the electrolyte up to 2 μM. The detection limit for phenol was found to be 0.4 μM calculated as being three times the signal-to-noise ratio (S/N = 3). The values of the steady-state current are presented in the other part of the **Fig. 2 (b)** as a function of the sensitivity, expressed by the ratio between the steady-state current and the phenol concentration at which it is obtained (electrochemical Eadie-Hofstee plot). The biosensor sensitivity is calculated to be 330 nA μM^{-1}. It is seen that the sensitivity of the investigated electrode is practically constant up to concentration of phenol at which a calibration curve is linear

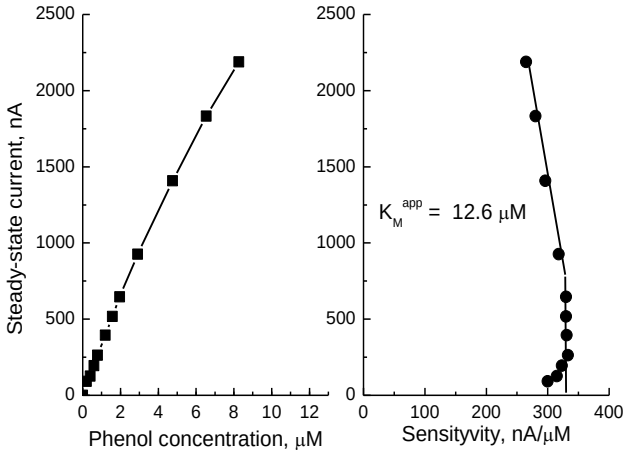

Fig. 2. (a) Steady-state current of the enzyme electrode as a function of the phenol concentration in the electrolyte. Experimental conditions: applied potential E = 0.00 V vs. Ag/AgCl; 0.1 M phosphate buffer solution containing 0.1 M KCl (pH 7.0); phenol concentration 0.1 mM. **(b)** Corresponding electrochemical Eadie-Hofstee plot.

(vertical part of the curve). In this region of phenol concentration diffusion control of the processes on the electrode is predominate. At higher phenol concentration kinetic control predominates in the enzymatic reaction. From the curve in this region the value (slanted part of the curve) of the apparent Michaellis constant of the enzymatic reaction is estimated as K^{app}_{M} = 12.6 μM.

The calibration curve obtained with a stock solution of phenol is used as a foundation for calculating the unknown phenol concentrations in real samples taken at hour 0, hour 1 and hour 4 since the beginning of the process of biodegradation with *Klebsiella oxytoca* strain.

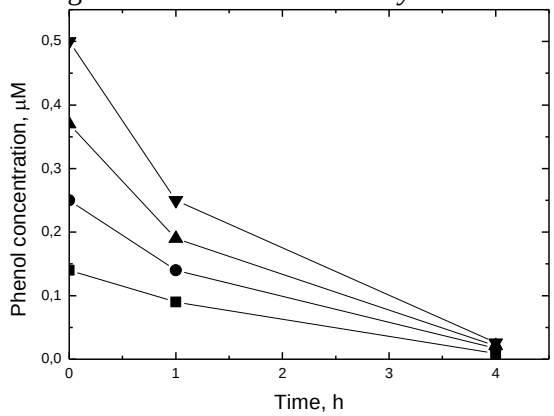

Fig. 3. Dependence of the phenol concentration in real samples on the biodegradation time. Experimental conditions: applied potential E = 0.00 V vs. Ag/AgCl; electrolyte – 0.1 M phosphate buffer solution containing 0.1 M KCl (pH 7.0); 150 μl (-■-), 250 μl (-●-), 350 μl (-▲-) and 450 μl (-▼-) diluted (1:9) solution of real samples.

The reducing of phenol concentration during the biodegradation process is illustrated on **Fig. 3**, which represents the dependence of phenol concentration from biodegradation time. Measurements are performed with the same electrode and the amperometric signals are obtained by addition of 150 μl, 250 μl, 350 μl and 450 μl of diluted (1:9) solution of real samples taken at hour 0, hour 1 and hour 4 since the onset of biodegradation. The unknown concentrations of phenol for real samples are obtained by recalculation based on the phenol calibration curve.

It is seen from the figure that over the first hour of phenol biodegradation, the phenol concentration decreases, compared with that, obtained from the 0 hours sample (i.e., immediately after "feeding" the microorganisms with phenol). Very low values of the steady-state current (and of the phenol concentration, respectively) are obtained with a sample taken on hour 4. This shows that the phenol concentration in the sample is drastically reduced of the 4-th hour, i.e. the process of phenol biodegradation by then is completed.

A well-known fact is that enzyme immobilization is one of the crucial problems in the investigation and development of enzyme electrodes. Carbon electrode materials provide wide range for enzyme immobilization. A various ways for improving immobilization of the tyrosinase enzyme were tested and used [27 and reference therein]. It is possible to combine cross-linking with other immobilization techniques to produce very reliable tyrosinase sensors.

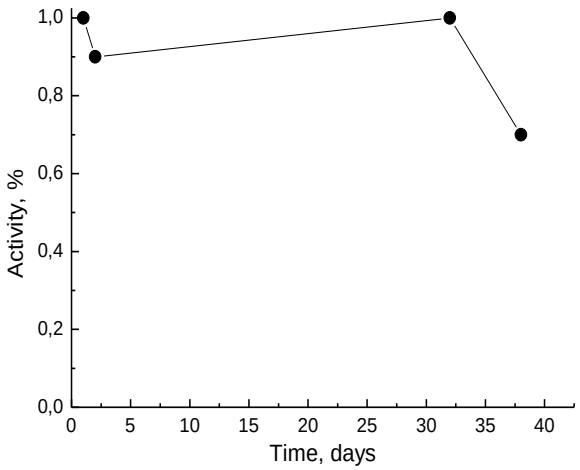

Fig. 4. Long-term stability of the tyrosinase electrode kept in buffer solution at 4 °C. The same experimental conditions as in Fig. 2.

To characterize the enzyme electrode stability, the response of the electrode to 0.8 μM phenol solution at constant potential 0.00 V vs. Ag/AgCl was periodically registered. In between the measurements the electrode is kept in 0.1 M phosphate buffer solution at 4 °C for more than 40 days. The relative response (percentage of the electrode response to the initially measured one) for tyrosinase electrode as a function of the storage times is presented in **Fig. 4.** Between the fifth and the thirteenth day measurements have been carried out with real samples, so there is no information on the storage stability during this period. It should be noted that the measurements made with this electrode have commenced after forty days stay in buffer at 4 °C. **Fig 4**. shows that tyrosinase electrode is still retained about 70 % of its original response after 35 days of storage. The investigations indicate that tyrosinase electrodes prepared by layering of pre-mixed mixed enzyme layer, containing carbon material, Nafion and tyrosinase shows relatively stable characteristics over long period.

CONCLUSIONS

The possibilities of wastewater treatment via biodegradation, and particularly of phenol contaminations, using the microbial strain *Klebsiella oxytoca* in a batch process are explored. The biodegradation was monitored by HPLC analyses and compare with an electrochemical analysis with enzyme immobilized electrodes. Electrochemical biosensor for determination of concentration of phenol in water solution used is based on an enzyme gas-diffusion electrode with enzyme tyrosinase. This investigation indicates that tyrosinase electrodes display relatively stable and reproducible characteristics after 40 days of storage and during 35 days of periodically measurements. The unknown concentrations of phenol for real samples are obtained by recalculation based on the phenol calibration curve.

The results obtained via both methods - HPLC analyses and electrochemical enzyme electrodes indicate that the phenol concentration in the sample is reduced over the 4-th hour, i.e. the process of phenol biodegradation by then is completed. For that reason biosensors for determining phenol concentration in biodegradation processes can find practical application. The future research will focus on extending the lifetime of the enzyme electrodes and on studies of other enzymes for the analysis of phenol in polluted waters.

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ПРОСЛЕДЯВАНЕ ПРОЦЕСА НА БИОДЕГРАДАЦИЯ НА ФЕНОЛ С ЕЛЕКТРОХИМИЧЕН БИОСЕНЗОР

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(Резюме)

Постоянният или периодичен контрол на степента на замърсеност на отпадъчни води (битови или индустриални) е доста сериозен въпрос, който все още не е решен. В тази статия докладваме за разработването на амперометричен биосензор за откриване на фенолна биодеградацията във вода и сравняваме аналитичните данни с тези, получени от най-често използвания аналитичен метод ВЕТХ (Високо Ефективна Течна Хроматография).

Описаният биосензор включва ензимен електрод, който се състои от порест хидофобен въглероден електрод с имобилизиран върху него ензим Тирозиназа (ЕС 1.14.18.1). За изследване ефективността на тирозиназния амперометричен сензор за определяне на фенолна концентрация, бяха проведени експерименти при постоянен потенциал 0.0V спр. Ag/AgCl сравнителен електрод във фосфатен буфер с pH 7.0. Времето за отклик на сензора не надвишава 3 мин. и амперометричния сигнал нараства линейно до концентрация на фенол 2 μM. Тирозиназният електрод показа чувствителност 330 nA μM^{-1}, изчислена е привидната Михаелисова константа K^{app}_M = 12.6 μM при откриваема граница за фенол 0.4 μM. Освен това, тирозиназния електрод показа добра възпроизводимост и стабилност по време на 35 дневни периодични измервания.

Като заключение, описаният тирозиназен електрод би могъл да намери приложение за ефективно откриване и определяне на фенолни концентраци в реални водни проби в реално време.