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Voltammetric determination of epinephrine by White rot fungi (*Phanerochaete chrysosporium* ME446) cells based microbial biosensor

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ABSTRACT

The lyophilized biomass of White rot fungi (*Phanerochaete chrysosporium* ME446) was immobilized in gelatine using glutaraldehyde crosslinking agent on a Pt working electrode. The fungal cells retained their laccase activity under entrapped state. The immobilized cells were used as a source of laccase to develop amperometric epinephrine biosensor. The catalytic action of the laccase in the biosensor released an epinephrinequinone as a result of redox activity, thereby causing an increase in the current. The optimal working conditions of the biosensor were carried out at pH 4.5 (50 mM acetate buffer containing 100 mM $K_3Fe(CN)_6$), and 20 °C. The sensor response was linear over a range of 5–100 μM epinephrine. The detection limit of the biosensor was found to be 1.04 μM. In the optimization and characterization studies of the microbial biosensor some parameters such as effect of fungi and gelatine amount, percentage of glutaraldehyde on the biosensor response and substrate specificity were carried out. In the application studies of the biosensor, sensitive determination of epinephrine in pharmaceutical ampules was investigated.

1. Introduction

Microorganisms, including microscopic fungi, are widely used in a variety of medical, biotechnological and industrial applications. Using microorganisms in biosensor construction is cheaper and easier than using isolated enzymes (Fakhrullin et al., 2009; Akyilmaz and Dinçkaya, 2005).

Laccase (EC 1.10.3.1, *p*-benzenediol: oxygen oxidoreductase) belongs to a family of multi-copper oxidases that catalyse the oxidation of a range of inorganic and aromatic compounds (particularly phenols) with the concomitant reduction of molecular oxygen to water. A few laccases have been isolated from plant sources (such as lacquer, sycamore and tobacco), however most known laccases have fungal origins (e.g. White rot fungi) (Revankar and Lele, 2006; Brondani et al., 2009).

Epinephrine or adrenaline [1-(3,4-dihydroxyphenyl)-2-methyloamino-ethanol] is one of the most important neurotransmitters in mammalian central nervous systems and exists in the nervous tissue and body fluid in the form of large organic cations (Ni et al., 1999; Yang et al., 2007). This compound controls the nervous system in its performance for a series of biological reactions and nervous chemical processes. The changes of its concentration may result in many diseases. Medically, epinephrine has also been used as a common emergency healthcare medicine. Therefore, the determination of epinephrine has attracted much attention from researchers (Felix et al., 2006; Shahrokhian et al., 2009; Ren et al., 2006).

The estimation of low endogenous concentrations of free catecholamines and related compounds in biological samples requires highly specific and sensitive procedures. There have been some reports for the determination of epinephrine such as HPLC with MS (Carrera et al., 2007), electrochemical (Wang et al., 1999) or fluorescence (Fotopoulou and Ioannou, 2002) detection, flow-injection chemiluminescence determination (Michalowski and Halaburda, 2001; Zheng et al., 2001; Du et al., 2003), flow-injection amperometric determination (Mateo and Kojlo, 1997; Garrido et al., 1997), flow-injection spectrophotometric determination (Solich et al., 2000), electrochemical EP-imprinted sol–gel film (Hsu and Yang, 2008), optic fibre biosensor coupled to chromatographic separation (Silva et al., 2009), bi-enzymatic system laccase-peroxidase in voltammetric biosensor (Leite et al., 2003), biosensor based on platinum nanoparticles dispersed in ionic liquid and laccase (Brondani et al., 2009), capillary electrophoresis with amperometric detection (Chicharro et al., 2004). There have been some reports which investigated the electrochemical response of EP on the various CMEs, such as triazole SAM modified gold electrode (Sun et al., 2006), poly(taurine) modified glassy carbon electrode (Wang and Chen, 2009), 2,3-dimercaptosuccinic acid self-assembled gold electrode (Kang et al., 2009), nano-Au self-assembled glassy carbon electrode (Yang et al., 2007), glassy carbon electrode modified

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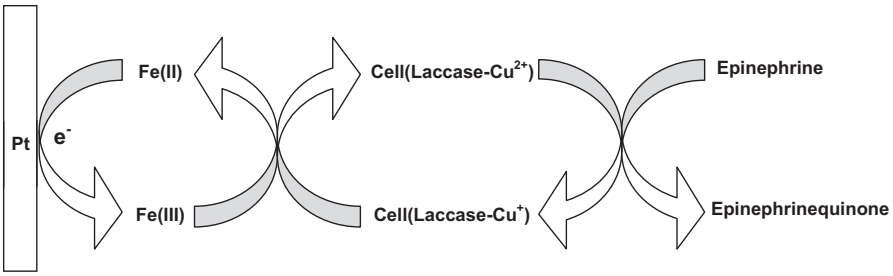

Scheme 1. Suggested mechanism of the microbial biosensor.

with caffeic acid (Ren et al., 2006). However, some of these methods are very expensive or complicated and need extraction or derivatization. Biosensor developed using White rot fungi cells in combination with transducers offers a good alternative compared with biosensors based on isolated enzymes as well as others analytical techniques for epinephrine determination. These biosensors have some advantages, such as low cost, simplicity of construction and needles of a co-factor for enzyme regeneration. One of the limitations of using cells in a sensor is the low sensitivity and specificity due to the permeability barrier and unwanted side reactions catalysed by other enzymes within the cell. These interferences can be minimized by cell permeabilization which leads to essential cofactor loss or heat inactivation (Jha et al., 2009; Banik et al., 2008). White rot fungal cells with a high content of laccase enzymes were employed in the construction of the biosensor system intended for voltammetric detection of epinephrine (Hakala et al., 2005).

In this study, a novel microbial biosensor based on White rot fungi (*Phanerochaete chrysosporium* ME446) is developed to determine epinephrine. White rot fungi (*P. chrysosporium* ME446) is a new and effective microorganism that has a laccase enzyme activity and also it is a new microorganism used in the construction of a microbial biosensor for a cheap and sensitive determination of epinephrine. The results obtained from the new proposed biosensor were compared with a spectrophotometric method. The modified electrode showed good stability and can be applied for selective determination of epinephrine in pharmaceutical and clinical samples.

2. Experimental

2.1. Chemicals and apparatus

White rot fungi (*P. chrysosporium* ME446) cells were obtained from Ege University Faculty of Science Biology Department in a lyophilized form, epinephrine, potassium hexacyanoferrate(III), gelatine, glutaraldehyde (25%) and all other chemicals were purchased from Sigma Chemical Co. (USA). All solutions used in experiments were prepared just before their use.

Experiments were carried out using Palm Sens (Netherland) potentiostat connected to a computer. A conventional three electrode system was used with the biosensor as the CHI 102 model Pt working electrode, a CHI 111 model Ag/AgCl as the saturated reference electrode and a CHI 115 model platinum wire as the counter electrode.

2.2. Microorganism and cultivation

P. chrysosporium ME446 was used in this study. The strain was grown in 500 ml Erlenmeyer flasks containing a liquid medium composed of (g/l): glucose: 10.5; yeast extract: 5.0, $(NH_4)_2SO_4$: 2.0, K_2HPO_4: 0.5, $MgSO_4{\cdot}7H_2O$: 0.5, $FeSO_4$: 0.02; $CaHPO_4$: 0.3, $ZnSO_4$: 0.2, $MnSO_4$: 0.2, $CuSO_4$: 0.25 with the final pH adjusted to 6.5 and inoculated with five cylindrical plugs (6 mm in diameter) of active mycelia on potato dextrose agar for five days. Flasks were incubated at 25 °C on an orbital shaker at 200 rpm for 10 days.

2.3. Determination of laccase activity

After incubation, fungal mycelia was filtered with Whatman No. 1 filter paper and then lyophilized. 20 mg lyophilized mycelia in a final reaction volume of 900 μl was shaken for 3 min and mycelia were immediately separated by centrifugation at 11,000 × *g* and 4 °C. Laccase activity in lyophilized mycelium was measured by oxidation of 5.0 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Sigma) in 100 mM sodium acetate buffer, pH 5.0, at 420 nm and using an extinction coefficient of 36,000 M^{-1} cm^{-1} (Svobodova et al., 2008; Kalmış et al., 2008). One unit of laccase activity was defined as units per mg of dry weight (DW) of the fungal mycelium necessary to oxidize 1 μmol of substrate per minute at 25 °C. Cell-bounded laccase activity was found to be approximately 16.5 ± 2.5 U/mg DW after 10 days incubation.

2.4. Cleaning procedure of the working electrode

Prior to coating, Pt electrode surface was polished with alumina slurries on microfiber cloth to obtain a mirror surface. After that, it was thoroughly rinsed with water and sonicated first in absolute ethanol and then in bi-distilled water for 10 min to remove adsorbed particles. Afterwards the electrode was thoroughly rinsed and checked if it was clean enough for immobilization or not by measuring CV at potential between −0.3 V and +1.1 V in 0.1 M sulfuric acid.

2.5. Preparation of bioactive layer

Immobilization of the cells on the transducer system is a key factor in the construction of a microbial biosensor. First of all White rot fungi (*P. chrysosporium* ME446) cells were taken into 500 μl of phosphate buffer (pH 7.5, 50 mM) and homogenized for 3 min. In the microbial biosensor construction to prepare the bioactive layer of the biosensor the lyophilized White rot fungi cells (*P. chrysosporium* ME446) (10 mg) and gelatin (5 mg) were mixed and dissolved in 300 μl phosphate buffer (pH 7.5, 50 mM) at 38 °C. 200 μl of the solution was spread over the Pt electrode, and then the bioactive layer was allowed to dry at 4 °C for 30 min. After that, the bioactive layer was treated with a cross-linking agent, glutaraldehyde (2.5%) (in phosphate buffer; pH 7.5, 50 mM) for 4 min to immobilize the cells on the surface of the Pt electrode.

2.6. Principle of measurements

Principle of measurements was based on monitoring decreases in current on reduction potential of potassium hexacyanoferrate(III) related to enzymatic reaction of laccase with epinephrine. Cyclic voltammograms were taken at a potential range between −0.4 and 0.8 V vs. Ag/AgCl reference electrode and correlation of

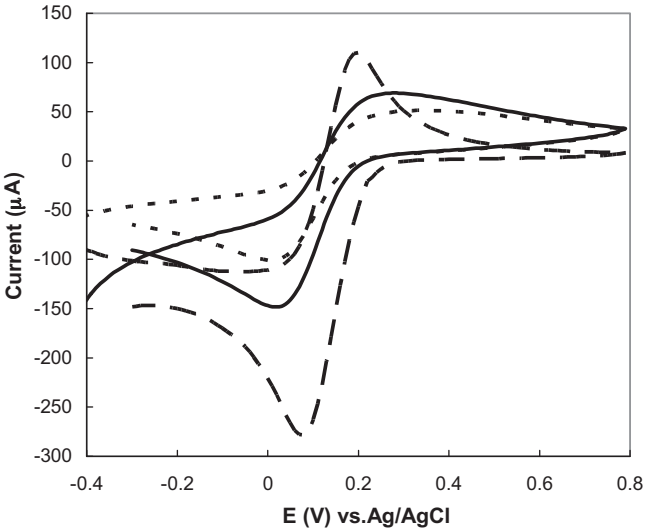

Fig. 1. Electrochemical behaviours of White rot fungi (*P. chrysosporium* ME446) modified electrode (acetate buffer; pH 4.5, 50 mM containing 100 mM $K_3Fe(CN)_6$; *T*: 20 °C). Bare Pt electrode (. . .), White-rot fungi modified platin electrode in the absence of EP (—), in the presence of 50 μM EP (– – –).

decreases in currents were monitored. Scheme 1 shows the measurement principle of the microbial biosensor. According to the Scheme it can be said that White rot fungi cells (*P. chrysosporium* ME446) use laccase enzyme activity to metabolism epinephrine. Laccase enzyme has Cu^{2+} as a cofactor and uses it for activity. As a result of the enzymatic reaction Cu^{2+} is reduced to Cu^{+} and potassium hexacyanoferrate(III) plays an effective role in the regeneration of the cofactor of the enzyme.

3. Results and discussion

3.1. Electrochemical behaviours of White rot fungi (P. chrysosporium ME446) modified electrode

Preliminary experiments to elucidate the catalytic activity of the cells of White rot fungi modified electrode for EP were performed with CV. The voltammograms were recorded using unmodified and modified Pt electrode. The CVs of 50 μM solution of EP were obtained in the potential range between −0.4 and +0.8 V using acetate buffer (pH 4.5; 50 mM) in the presence and absence of 100 mM $K_3Fe(CN)_6$. Fig. 1 shows the results obtained from the experiments.

As can be seen in Fig. 1 electrochemical oxidation of EP on the surface of modified electrode in pH 4.5 acetate buffer containing 100 mM $K_3Fe(CN)_6$ shows reversibility at peak potentials (+0.28 V red. and +0.14 V ox. vs. Ag/AgCl). In the absence of epinephrine there is no peak observed. The results obviously represent the catalytic action of laccase facilitating the kinetics of the electron transfer on the surface of the modified electrode.

3.2. Optimization of bioactive layer

For the determination of the effects of some parameters on the microbial biosensor response such as amount of White rot fungi cells (*P. chrysosporium* ME446), gelatine and percentage of glutaraldehyde were investigated.

3.2.1. Effect of the amount of White-rot fungi cells (P. chrysosporium ME446) on the biosensor response

In order to determine the effect of the amount of lyophilized White-rot fungi cells on the biosensor response the amount of gelatin (5 mg) and the percentage of glutaraldehyde (2.5%) on the bioactive layer were kept constant, however, the amount of White rot fungi cells was changed as 5, 10, and 15 mg in the biosensor preparation. According to the results obtained from this study, the best relation between the microbial biosensor response, the amount of cells biomass, and also the most suitable linear range were obtained when 10 mg cells of White rot fungi was used. This was an expected result because of the diffusion barrier of the bioactive layer of the biosensor related to the amount of the microorganism.

3.2.2. Effect of gelatine amount and glutaraldehyde percentage on the biosensor response

After detection, the effects of White-rot fungi amount on the biosensor response other parameters such as effects of gelatine and percentage of glutaraldehyde on the biosensor response were investigated, respectively. For this purpose, the amount of White rot fungi and glutaraldehyde were kept constant as 10 mg and 2.5%, however, different amounts of gelatine (2.5, 5, and 10 mg) were used in the biosensor construction. According to the results obtained, the most suitable biosensor responses were obtained when 5 mg gelatine was used in the biosensor construction. When higher and lower amounts of gelatine were used in the biosensor construction, decreases in the biosensor response were observed. These observations resulted from fungi migration from bioactive layer of the biosensor when 2.5 mg gelatine was used, and formation of a steric structure on the bioactive layer of the biosensor when 10 mg gelatine was used. The migration of the cells from the bioactive layer of the biosensor caused decrease in biosensor response. On the other hand, the steric structure did not give any permission for the substrate diffusion from the reaction medium to the electrode and as a result the biosensor responses decrease. After the optimal amounts of White rot fungi and gelatine were determined as 10 mg and 5 mg, respectively, for the investigation of the effect of the cross-linking agent glutaraldehyde, on the biosensor response the amount of White rot fungi and gelatine were kept constant as 10 mg and 5 mg and each of the newly prepared microbial biosensors were treated with 0.5, 1.0, and 2.5% glutaraldehyde. When 0.5 and 1.0% glutaraldehyde was used, a slight decrease was observed on the biosensor response due to inadequate gelatine–gelatine cross-links that caused deformation of the bioactive layer which would lead to decrease in biosensor response.

3.3. Optimization of working conditions

3.3.1. Effect of temperature on the biosensor response

For the determination of temperature effect on the microbial biosensor response, the experiments were carried out between 20 and 45 °C under the conditions obtained from the optimization studies. According to the results, optimum temperature was found to be 20 °C for laccase. As a result of increase in temperature, some defect and deformations occurred on bioactive layer of the biosensor as well as on the activities of the enzymes, thus 20 °C was chosen to be the working temperature for the biosensor.

3.3.2. Effect of pH on the biosensor responses

To determine the effect of pH value on the biosensor responses, different buffer solutions were prepared. For this purpose, 50 mM acetate (pH 4.0–5.5), citrate (pH 6–6.5) and phosphate (pH 7–8) buffers containing 100 mM $K_3Fe(CN)_6$ were used in the experiments. The pH effect on the biosensor performance was also investigated by measuring the current response to 50 μM epinephrine in the potential range between −0.4 and +0.8 V using the buffers containing 100 mM $K_3Fe(CN)_6$. From the experiments it can be shown that the peak current increased from pH 4.0 to 4.5,

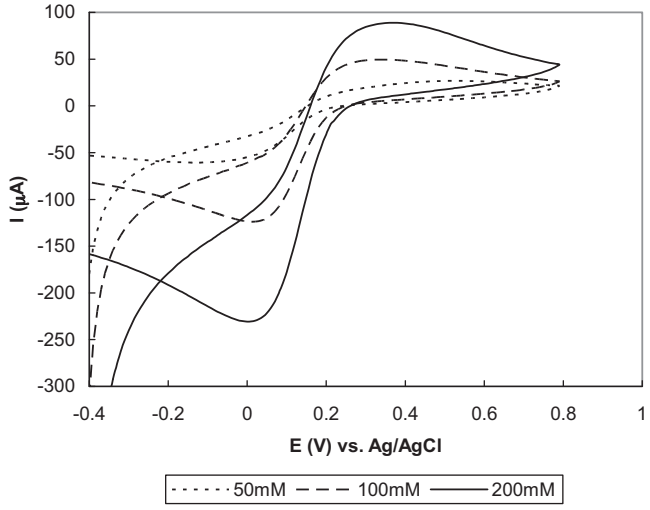

Fig. 2. Cyclic voltammograms to detect the effect of the concentration of $K_3Fe(CN)_6$ on the biosensor response (acetate buffer; pH 4.5, 50 mM containing 100 mM $K_3Fe(CN)_6$; *T*: 20 °C). The amount of cells of White-rot fungi, gelatine and percentage of glutaraldehyde were kept constant at 10.0 mg, 5.0 mg, 2.5%, respectively. Scan rate is 25 mV s^{-1} and potentials referred to Ag/AgCl reference electrode.

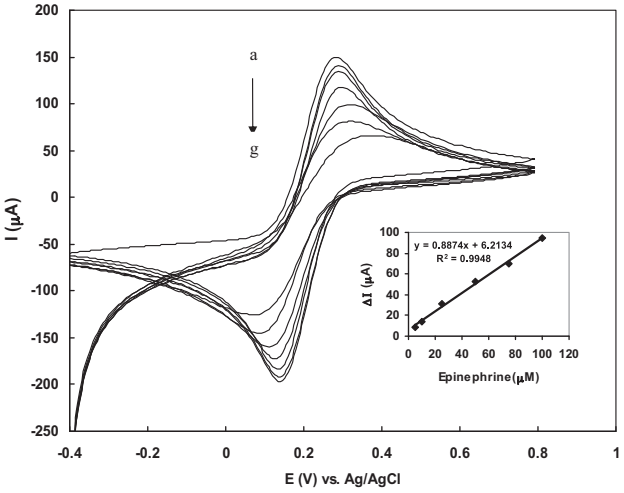

Fig. 3. Cyclic voltammograms obtained from the experiments and standard curve of the biosensor (acetate buffer; pH 4.5, 50 mM containing 100 mM $K_3Fe(CN)_6$ *T*: 20 °C). From a to g: baseline, 5, 10, 25, 50, 75 and 100 μM EP. The amount of cells of White-rot fungi, gelatine and percentage of glutaraldehyde were kept constant at 10.0 mg, 5.0 mg, and 2.5%, respectively. Scan rate is 25 mV s^{-1} and potentials referred to Ag/AgCl reference electrode.

and the maximum peak current was obtained at pH 4.5. At pH's greater than 4.5, the peak current gradually decreased. In order to obtain maximum sensitivity pH 4.5 was selected as the optimum.

3.3.3. *Effect of concentration of $K_3Fe(CN)_6$ on the biosensor response*

Different concentrations of $K_3Fe(CN)_6$ (50, 100 and 200 mM) were used for the determination of the effect of concentration of $K_3Fe(CN)_6$ on the biosensor responses. Especially in higher $K_3Fe(CN)_6$ concentrations, increase in oxygen diffusion to the bioactive layer could be possible and higher biosensor responses could be explained by the higher efficiency of substrate oxidation due to the increase of $K_3Fe(CN)_6$ in the layer of immobilized cells. The highest peak currents were obtained using 100 and 200 mM of $K_3Fe(CN)_6$. 100 mM $K_3Fe(CN)_6$ was found to be the most suitable mediator concentration in terms of electrode sensitivity and stability. Above and below this concentration, lose of sensitivity and decrease in stability of biosensor responses were observed. Using 50 and 200 mM of $K_3Fe(CN)_6$ there were no any reproducible results obtained from the measurements for different concentrations of epinephrine. Results achieved from these experiments are shown in Fig. 2. Due to the sensitivity and reproducible results gained using 100 mM $K_3Fe(CN)_6$, this concentration was chosen and used in further experiments.

3.4. *Analytical characteristics*

3.4.1. *Linear range of the microbial biosensor*

For the determination of a linear range for epinephrine, cyclic voltammetry and also differential pulse methods were used. According to the results obtained from both methods, responses of the biosensor depend linearly on epinephrine concentration between 5.0 and 100 μM. Cyclic voltammograms and the standard graphs related to the CVs are shown in Fig. 3. The limit of detection was calculated according to ($3S_b/m$) formula, where *m* is the slope of the calibration curve and S_b is the standard deviation ($n = 10$) of the response from 5.0 μM epinephrine.

Detection limits of the biosensor were found to be 1.04 μM for cyclic voltammetric experiments.

The cyclic voltammograms indicated that when concentrations of epinephrine were increased, anodic peak current of the biosensor decreased correlately at reduction potential of $K_3Fe(CN)_6$. From the cyclic voltammograms it can be said that $K_3Fe(CN)_6$ is an effective mediator in the regeneration of the enzyme.

3.4.2. *Reproducibility*

The reproducibility of the biosensor was determined for 50 μM epinephrine ($n = 5$). From the experiments, the average value, standard deviation (SD) and coefficients of variation (CV %) were calculated to be 48.45 μM, ± 1.85, and 3.83%, respectively.

3.4.3. *Substrate selectivity*

For the determination of substrate selectivity 50 μM of standards of various compounds such as pyrocatechol, phenol, L-tyrosine, L-ascorbic acid and D-glucose were examined using the biosensor. The biosensor response obtained for epinephrine was accepted as 100% and compared with the biosensor responses obtained for the other substances. Results obtained from the experiments are shown in Table 1.

From the results of the experiments the biosensor showed different responses for the substances because of the wide substrate selectivity of laccase enzyme, however the maximal responses were obtained for epinephrine. In the second part of the experiments, interference effects of these substances were investigated on the determination of epinephrine by the biosensor. For this purpose, using the biosensor, substances that were at the same concentration with epinephrine were tested in the presence of epinephrine. From the results it is obvious that the substances showed much less interference effects on the biosensor responses

Table 1
Substrate selectivity and interference effect of some compounds on the biosensor response.

Substrate[a] (μM)	Response[b] (%)	Substrate[a] (μM)	Response[b] (%)
Epinephrine (EP)	100.0	Epinephrine (EP)	100.0
Catechol	78.5	EP + catechol	124.5
Phenol	64.0	EP + phenol	106.6
L-Tyrosine	50.5	EP + L-tyrosine	89.4
L-Ascorbic acid	38.5	EP + L-ascorbic acid	75.2
D-Glucose	30.0	EP + D-glucose	63.6

[a] Concentration of the substrates is 50 μM.
[b] Average of four measurements.

Table 2
Comparison of the proposed method with other methods for the determination of epinephrine.

Modification	Linear range (mol L^{-1})	Detection limit (mol L^{-1})	Reference
Caffeic acid modified GCE	2.0×10^{-6}–8.0×10^{-5}	2.0×10^{-7}	Ren et al. (2006)
Graphite powder-Nujol-Pt. BMIPFE6-laccase modified CPE	9.99×10^{-7}–2.13×10^{-4}	2.93×10^{-7}	Brondani et al. (2009)
Bi-enzymatic system laccase-peroxsidase modified CPE	6.1×10^{-6}–1.0×10^{-4}	2.5×10^{-8}	Leite et al. (2003)
Graphite powder-mineral oil-*L. Chinensis* modified CPE	5.0×10^{-5}–3.5×10^{-4}	1.5×10^{-5}	Felix et al. (2006)
Carbon nanotube/nafion composite Electrode	2.0×10^{-7}–20×10^{-6}	4.0×10^{-8}	Ou et al. (2009)
Gelatine-glutaraldhyde-*P. chyrsosporium* modified Pt electrode	5.0×10^{-6}–1.0×10^{-4}	1.04×10^{-6}	This work

in the presence of epinephrine. This can be result from the substrate selectivity of the microorganism. Because, before the measurements the microbial biosensor was waited in epinephrine solution for 1 h to adapt it to epinephrine. The results of the experiments are shown in Table 1.

3.4.4. Applications

In order to apply the microbial biosensor for real samples some experiments were made. For this purpose some ampules that contain different concentrations of epinephrine were used in the experiments. The ampul samples were diluted to contain 50 μM epinephrine before the determination by the biosensor. After that assays were realized using the biosensor for each sample. Measurements were repeated five times. According to the results obtained from the experiments, the average values, standard deviations, and also coefficients of variant (%) were calculated to be 50.72 and 51.48 μM, ±0.15 μM and ±0.14, and 0.30% and 0.20% for ampul 1 and ampul 2, respectively.

4. Conclusion

The development of a biosensor method able to measure epinephrine will be useful to studies seeking to understand the total level of this compound in pharmaceuticals. This study also demonstrates the successful application of cells of White-rot fungi on a platin electrode for construction of a microbial biosensor. The biosensor based on cells of White-rot fungi has the advantages of short measurement time, linear range and also low detection limit (Table 2).

The total analysis time of a measurement takes only 30 s. Using the cells as a biocomponent in biosensor construction was an advantage in terms of the cost of the biosensor. Moreover, the immobilization procedure used was very easy. Consequently, the simplicity in the bioactive material immobilization demonstrates a considerable economic advantage because of low cost, which is desirable in routine analysis. In this respect, the microbial biosensor system developed is a simple and inexpensive method for monitoring of epinephrine in pharmaceuticals.

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