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A novel microbial biosensor system based on *C. tropicalis* yeast cells for selective determination of L-Ascorbic acid

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ABSTRACT

In this study, a novel microbial biosensor was developed for the selective determination of L-Ascorbic acid. In the construction of the microbial biosensor, lyophilized *Candida tropicalis* yeast cells were immobilized with o-aminophenol by forming a film layer on a platinum electrode surface using electropolymerization. L-Ascorbic acid was quantified on the basis of both amperometric and differential pulse voltammetry (DPV) methods using the biosensor. The measurements were made at +0.24 V (*vs* Ag/AgCl) for amperometric studies and between 0.0 V and +0.7 V for DPV studies based on the oxidation of L-Ascorbic acid to dehydro-L-Ascorbic acid by ascorbate oxidase which takes place within the catabolic metabolic pathway of *C. tropicalis* yeast cells. According to the results obtained from the two methods, the response of the biosensor depends linearly on L-Ascorbic acid concentration between 100 and 1500 μM. The detection limit was 62 μM and 59 μM for amperometric and DPV measurements, respectively. The response time of the microbial biosensor was 14 s and 5 s for DPV and amperometric measurements, respectively. In the optimization studies of the biosensor, some parameters such as the optimum amount of the microorganism, o-aminophenol concentration, pH and temperature were determined. For the characterization of the biosensor, reproducibility, storage stability and the effect of interferences were determined.

1. Introduction

The use of biosensors generated by combining the specificity of biological species with the sensitivity of analytical systems is increasing day by day. Their response time is very fast and they have high storage stability, repeatability and also specificity for the analytes [1,2].

L-Ascorbic acid (L-AA), also known as vitamin C, is used as an antioxidant in some foods, pharmaceutics, and especially cosmetics [3]. It also plays an important role in some biological processes such as free radical metabolism, cancer prevention and immunity improvement [4]. In the central nervous system, L-AA has important regulatory effects on some neurotransmitters and enzymes. Due to the physiological importance of L-AA and also its use in the food industry, a fast, specific and sensitive method for the determination of L-AA is necessary not only for clinical chemistry and diagnostics, but also for the food industry [5]. Some different methods, such as fluorescence [6], chemiluminescence [7,8], liquid chromatography [9,10], UV spectroscopy [11] and also electrochemical methods [12–17] have been developed for the determination of L-AA. Amongst these, electrochemistry is considered to be one of the most suitable approaches because of its capacity for high sensitivity and simplicity.

The determination of L-Ascorbic acid by amperometric methods is based on its electrochemical oxidation at platinum or glassy carbon at potentials above +500 mV *vs* Ag/AgCl. This overpotential can be lowered by using chemically modified electrodes. Such electrodes, based on ferrocene [18], ferricyanide [19] and 7,7,8,8-tetracyanoquinodimethane (TCNQ) [20] in conjunction with tetrathiafulvalene have been proposed as efficient routes to a reduced L-AA oxidation potential.

Some biosensors based on ascorbate oxidase enzyme which catalyze the oxidation of L-Ascorbic acid have been developed. Most of these biosensors are amperometric and different immobilization methods are used for the ascorbate oxidase enzyme in biosensor fabrication [21–29]. A potentiometric biosensor was developed by Fernandes et al. [30] based on ascorbate oxidase immobilized on ethylene-vinylacetate membrane.

Biosensors which use microorganisms as the biocomponent are generally called microbial sensors. Microorganisms used in the biosensors can be prokaryotic like bacteria or eukaryotic like yeast. They can be used in the construction of the biosensors as dead or live cells. Measurements can be made through the respiration or

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metabolic activation of the microorganisms. In the case of dead microorganisms, measurements are made using extra or intracellular enzymes which the microorganism contains and the reactions that they catalyze [31–35].

Candida tropicalis is one of the most important yeast *Candida* yeast species. It is considered to be one of the most virulent, exceeded only by *C. albicans* in this regard. They can be found on plants and also in the digestive system of mammals such as mucocutaneous membranes of humans. *C. tropicalis* is considered to be an osmotolerant yeast and they are able to survive in a high salt concentration medium [36]. It has ascorbate oxidase enzyme activity which provides for the transformation of L-AA. There are some advantages to using yeast in biosensor construction, such as speed of growth, easy manipulation and growth using on a variety of different carbon sources. They are particularly robust with tough cell walls and wide environmental tolerance, e.g. to pH, temperature and ionic strength [33].

This paper describes a novel and the first microbial biosensor based on *C. tropicalis* immobilized with o-aminophenol by forming a film layer on a platinum working electrode surface using a electropolymerization method for selective and fast determination of L-Ascorbic acid.

Biosensors, in particular amperometric biosensors, are fast and cheap smart devices able to selectively detect and quantify organic acids, such as those in the wine industry. They can be competitors for conventional techniques, representing an attractive alternative for the wine industry. Some amperometric biosensors for wine analysis have been developed using platinum printed electrodes with immobilized enzymes such as alcohol, glucose, and lactate oxidase. These biosensors demonstrate a linear response to ethanol, glucose, and lactate within the concentration ranges of 0.3–20 mM, 0.04–2.5 mM, and 0.008–1.0 mM, respectively [37]. A multi-enzymatic biosensor was developed for the selective and simultaneous determination of D- and L-lactic acid in wine. The biosensor was based on the catalytic activities of the enzymes; L-lactate oxidase, D-lactate dehydrogenase and horseradish peroxidase. L-malic acid was also measured using a bi-enzymatic biosensor system developed by coupling the enzymes, L-malic dehydrogenase and horseradish peroxidase. In both cases, the enzymes were immobilized on an oxygen selective Clark electrode [38]. Some amperometric biosensors based on the corresponding dehydrogenases were also developed for the determination of ethanol, glucose and glycerol in wine samples. These biosensors showed sensitive responses to ethanol, glucose and glycerol within the concentration ranges of 2.5–250, 20–800, and 1–200 μM, respectively [39]. A biosensor based on the immobilization of tyrosinase onto a glassy carbon electrode modified with electrodeposited gold nanoparticles was reported for the analysis of polyphenols such as phenol, catechol, caffeic acid, chlorogenic acid, gallic acid and protocatechualdehyde in wine samples [40].

2. Experimental

2.1. Apparatus

In the experiments, a three electrode system from CH Instruments (USA) contained a CHI 111 model Ag/AgCl reference electrode, a CHI 115 model platinum wire counter electrode and a CHI 102 platinum working electrode. Gilson P1000 automatic pipettes (France), Yellow-Line magnetic stirrer (Germany) and Nuve model thermostat (TR) were used in the experiments. All measurements were done with a PalmSense Instrument system (Netherland). pH measurements were made by using a pH meter (Hanna Insturements HI 221- Italy) and sonication was done with an Ultrasonic LC 30 (Germany).

2.2. Chemicals and reagents

Candida tropicalis yeast cells were obtained from the Japan Collection of Microorganisms (JCM-Japan) in lyophilized form. o-Aminophenol was obtained from Aldrich, L-Ascorbic acid, citric acid, uric acid, glucose, potassium-phosphate monobasic, sodium hydroxide, hydrochloric acid and all other chemicals were purchased from Sigma Chemical Corporation (USA). All solutions used in the experiments were prepared just before use. All chemicals were prepared with bi-distilled water.

2.3. Microorganism and cultivation

Lyophilized *C. tropicalis* were first activated in a yeast medium agar (pH 6.3) containing glucose (10.0 g/L), peptone (5.0 g/L), yeast extract (3.0 g/L), malt extract (3.0 g/L) and agar (20.0 g/L) for 24 h at 25 °C. After this process, *C. tropicalis* cells were inoculated into 50 ml of growth medium, that contained the same substances of culture medium of the cells given below without catecholamines at 25 °C and 200 rpm for 24 h. Finally, the cells were incubated in 50 ml of the culture medium containing yeast extract (3.0 g/L), $Na_2HPO_4{\cdot}2H_2O$ (3.0 g/L), $NaH_2PO_4{\cdot}2H_2O$ (3.0 g /L), $(NH_4)_2SO_4$ (10.0 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), $CaCl_2$ (0.1 g/L), peptone (0.5 g/L), glucose (5.0 g/L), KCl (1.0 g/L) and catecholamines (25 ml/L) for 24 h at 25 °C and 200 rpm. Cells in logarithmic growth phase were collected for the biosensor preparation.

2.4. Preparation of microbial biosensor

The surface of the platinum electrode was polished with 1.0 and 0.05 μm alumina slurry on a microfiber cloth to produce a mirror surface. Following this, it was thoroughly rinsed with ultra-pure water and sonicated in absolute ethanol and ultra-pure water for 10 min to remove adsorbed particles. It was then left to dry at room temperature. The electrode was transferred to an electrochemical cell for cleaning using cyclic voltammetry between −1.0 and +1.0 V *vs* Ag/AgCl at 50 mV/s in 0.1 N HCl until a stable cyclic voltammetry (CV) profile was acquired.

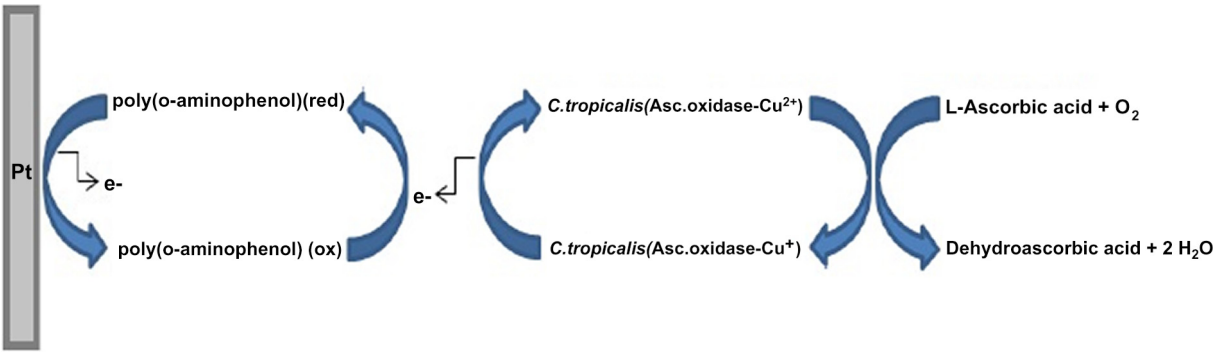
In the next step, lyophilized *C. tropicalis* cells were dissolved in an Eppendorf tube (in 300 μL phosphate buffer, pH 7.0; 50 mM) and added to 5.0 mM o-aminophenol solution (prepared in phosphate buffer, pH 7.0, 50 mM). By using the clean platinum electrode, the electropolymerization was carried out by using cyclic voltammetry at potentials between −0.1 and +0.9 V *vs* Ag/AgCl with a 10 mV/s scan rate [41].

2.5. Activity measurement

For enzyme activity measurement, *C. tropicalis* cells, especially those in the logarithmic growth phase, were collected and centrifuged at 4 °C for 20 min at 3000g. Then 1.0 g wet weight of cells was added to 10 ml buffer solution (phosphate buffer, pH 7.0, 50 mM) and subjected to ultrasonic disruption at 4 °C for 5 min. The supernatant was collected after centrifugation at 3000 rpm and 4 °C for 20 min. The ascorbate oxidase activity of the cells was measured by using a titrimetric method developed by Akyilmaz and Dinckaya, 1996 [42]. The method uses 2,6-dichloroindophenol as oxidant and is based on the reducing characteristics of L-Ascorbic acid.

2.6. Measurements

Electrochemical detection of L-Ascorbic acid is based on the catalytic reaction of ascorbate oxidase enzyme. *C. tropicalis*, incorporates ascorbate oxidase enzyme that catalyzes the oxidation of ascorbate to dehydroascorbate for metabolic purposes.



Schematic 1. Proposed measurement principle of the microbial biosensor.

Measurements were made at +0.24 V for amperometric studies. DPv studies were done between 0.0 and +0.7 V potentials *vs* Ag/AgCl at 10 mV/s scan rate in the presence of oxygen in phosphate buffer (50 mM pH 7.0) at 30 °C. The proposed measurement principle of the microbial biosensor is given in Schematic 1. According to this, L-Ascorbic acid added into a reaction cell is oxidized to dehydroascorbic acid by the ascorbate oxidase enzyme of *C. tropicalis* cells. Catalysis nvolves the conversion of Cu (II) atom in the active center of the enzyme to Cu (I) [43,44]. As a result of the generation of Cu (I), which is toxic to the cells, an electron is released and transferred to the electrode surface by o-aminophenol.

In order to examine the direct effect of L-Ascorbic acid on measurements, an electrode was prepared without *C. tropicalis.* Measurements were also undertaken with and without L-Ascorbic acid in the reaction cell by the DP method. The same measurements were made with a biosensor containing *C. tropicalis* cells at the electrode surface and results were compared (Fig. 1). As can be seen from the figure, the effect of L-Ascorbic acid on measurement was found to be very low, in the absence of the *C. tropicalis* cells. However, in the presence of *C. tropicalis* there was a marked biosensor response which resulted from the metabolic activity of the cells.

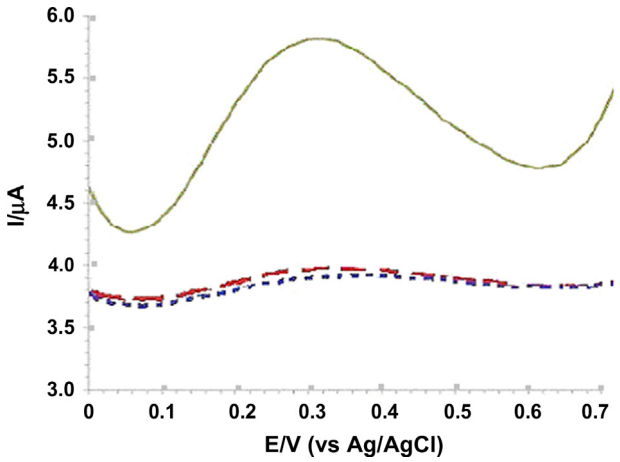


Fig. 1. Selectivity of the microbial biosensor and detection of the effect of L-Ascorbic acid on the responses (Phosphate buffer; pH 7.0, 50 mM; T: *30* °C), - - -: baseline, — — —: 500 μM L-Ascorbic acid without *C. tropicalis,* ———: 500 μM L-Ascorbic acid with *C. tropicalis.*

3. Results and discussion

3.1. Immobilization

Electropolymerization of o-aminophenol was carried out with lyophilised *C. tropicalis* cell (1.0 mg/mL) in the phosphate buffer. Hence, cyclic voltammograms were made at a potential range between −0.1 and +0.9 V by using bare Pt electrode in 5.0 mM o-aminophenol solution to form the polyaminophenol (POAP) conductive polymer on the Pt electrode surface. The thickness of the electrochemically synthesized POAP films plays an important role in the current response of the microbial biosensor. When the number of cycles is lower than 12, the amount of the cells entrapped in the POAP film gradually increases with an increase in the polymerization cycle number. However, when the number of cycles is higher than 12, the cells are covered by the POAP film resulting in a decrease in the available amount of *C. tropicalis* yeast cells at the biosensor surface. The cyclic voltammograms appearing during the formation of the conductive polyaminophenol and the immobilization of the *C. tropicalis* cells are given in Fig. S1.

3.2. Optimization of bioactive layer of the microbial biosensor

In this part of the study, the effects of some parameters such as the amount of *C. tropicalis* and the concentration of the o-aminophenol on the microbial biosensor response were investigated.

3.2.1. The effect of the amount of C. tropicalis on the biosensor response

Here, the effect of the amount of the cells in the bioactive layer on the biosensor responses was examined. For this purpose, the amount of the *C. tropicalis* cells was changed to 0.5, 1.0 and 2.0 mg/mL for biosensor preparation. In experiments, the differential pulse method was used between 0.0 and +0.7 V potential *vs* Ag/AgCl reference.

Fig. 2 illustrates the results obtained from this study. According to the figure, the best relation between the microbial biosensor response and the amount of cells and the most suitable linear range were obtained when 1.0 mg/mL of *C. tropicalis* was used. An increase in *C. tropicalis* to 2.0 mg/mL led to a very low biosensor response. This was an expected result owing to the diffusion arrier of the bioactive layer related to the amount of the microorganism. Although the highest responses were obtained with 0.5 mg/mL *C. tropicalis*, biosensor linearity was not at the desired level. Therefore, 1.0 mg/mL was chosen to be the optimum amount of *C. tropicalis* in the biosensor preparation.

3.2.2. The effect of concentration of o-aminophenol on the microbial biosensor response

To investigate changes in the biosensor responses due to different o-aminophenol concentrations, electropolymerization was

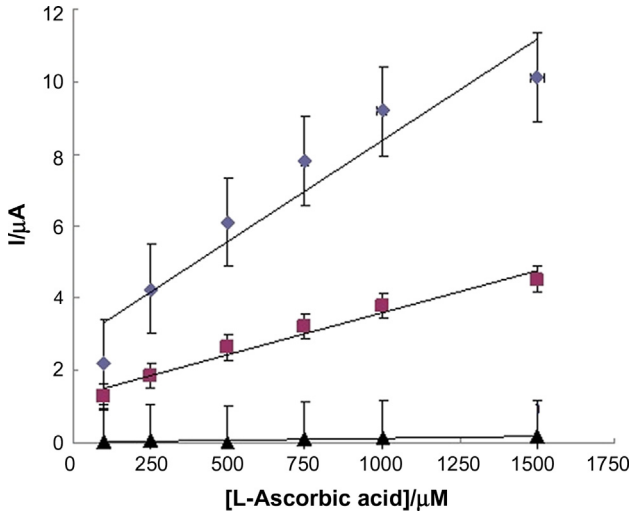


Fig. 2. The effect of the amount of the *C. tropicalis* on the biosensor response (Phosphate buffer; pH 7.0, 50 mM; T: *30* °C), (◆) 0.5 mg/mL (R^2 = 0.9113); (■) 1.0 mg/mL (R^2 = 0.9692); (▲) 2.0 mg/mL (R^2 = 0.7250).

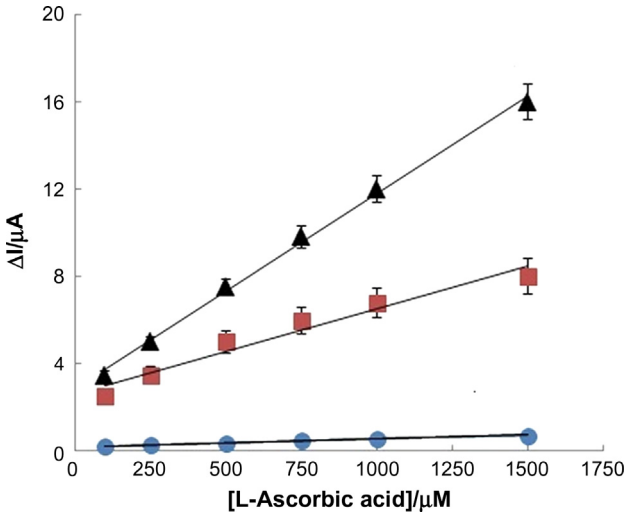


Fig. 3. The effect of o-aminophenol concentration on the microbial biosensor responses (Phosphate buffer; pH 7.0, 50 mM; T: *30* °C), (■) 2.5 mM (R^2 = 0.9548); (▲) 5.0 mM (R^2 = 0.9975); (●): 10.0 mM (R^2 = 0.9670).

carried out by 2.5, 5.0 and 10.0 mM o-aminophenol. In the experiments, the differential pulse method was used between 0.0 and +0.7 V potentials *vs* Ag/AgCl. Results are given in Fig. 3.

According to the figure, the highest biosensor responses were obtained using 5.0 mM solution of o-aminophenol in the preparation of the biosensor system. In addition, the most suitable linear curves were obtained by using this concentration so this concentration was used in the preparation of the biosensor. Due to the thickness of the surface of the biosensor prepared using 10.0 mM o-aminophenol, difficulty in substrate diffusion and deformation of the bioactive layer of the biosensor was observed. Despite the fact that the linearities of the biosensors that were prepared by using 2.5 mM and 5.0 mM of o-aminophenol were good, responses of the biosensor that was constructed using 5.0 mM o-aminophenol were the highest, so was used as the most suitable concentration for biosensor preparation.

3.3. Optimizations of the working conditions of the microbial biosensor

3.3.1. Effect of working temperature and pH on the microbial biosensor response

To look into the effect of the working pH on the microbial biosensor responses, experiments were carried out using 50 mM phosphate buffer with different pH values (pH 6.0, 6.5, 7.0, 7.5 and 8.0) and 50 mM Tris/HCl buffer (pH 8.5). The current response of the microbial biosensor increased with increase of the pH value, and the highest response was observed at pH 7.0. The responses increased from pH 6.0 to 7.0, but decreased above pH 7.0. The high response at pH 7.0 was attributed to the *C. tropicalis* cells in the poly o-aminophenol (POAP) film, which were more active in neutral solution (Fig. S2-A).

To optimize the working conditions of the microbial biosensor, the DP method was used in the studies of temperature optimization, experiments were done at 20, 25, 30, 35 and 40 °C. The responses of the microbial biosensor increased almost linearly from 20 °C to 30 °C, but decreased linearly from 30 °C to 40 °C. In iew of these results, 30 °C was chosen to be the working temperature. An increase in temperature from 30 °C to 40 °C showed a decrease in microbial biosensor responses, resulting from the deactivation of *C.tropicalis* yeast cells in this temperature range (Fig. S2-B).

3.4. Analytical characteristics of the biosensor

3.4.1. Linear range of the microbial biosensor

For the determination of the linear range for L-Ascorbic acid, amperometric and differential pulse methods were used under optimum working conditions (pH 7.0 and 30 °C). From results obtained by these methods, the responses of the biosensor depend linearly on L-Ascorbic acid concentration between 100 and 1500 µM. Differential pulse voltammograms, amperometric measurements and standard curves from these measurements are shown in Figs. 4 and 5, respectively. Detection limits obtained from the experiments were 62 µM and 59 µM for amperometric and DP measurements, respectively. Detection limits were calculated by using the equation (LOD = $3S_a/b$), where S_a is the standard deviation of the response and b is the slope of the calibration curve.

3.4.2. Repeatability

The repeatability of the microbial biosensor was determined for 500 µM L-Ascorbic acid (n = 6) by using the amperometric method with an applied potential of +0.24 V *vs* Ag/AgCl. From the experiments, the average value (*X*), standard deviation (SD) and coefficient of variation (CV %) were calculated to be 497 µM, ± 2.013 µM, and 0.405%, respectively. The same experiments were performed for all L-Ascorbic acid standards (n = 5). The results of the assays are shown in Fig. S3. From the figure, it can be stated that accurate and sensitive results were obtained for each standard. The RSD values for the L-Ascorbic acid standards were close to each other and ranged between 0.405 and 0.42%.

3.4.3. The effect of scan rate on the microbial biosensor response

Cyclic voltammetry is the best technique to probe chemical changes that occur as a result of electron transfer in a chemical reaction, because many combinations of the electron transfer and chemical reaction steps are possible. The influence of the scan rate on L-Ascorbic acid oxidation at the microbial biosensor is an important parameter for electrochemical kinetics. Thus, CVs at different scan rates were recorded in Phosphate buffer (pH 7.0; 50 mM) containing 500 µM of L-Ascorbic acid. The effect of scan rate on the microbial biosensor was studied by changing the scan rates from 10 to 200 mV/s. Fig. 6 shows a series of cyclic voltammograms recorded at different scan rates. According to the figure, an increase in scan rate results in an increase in current values, not

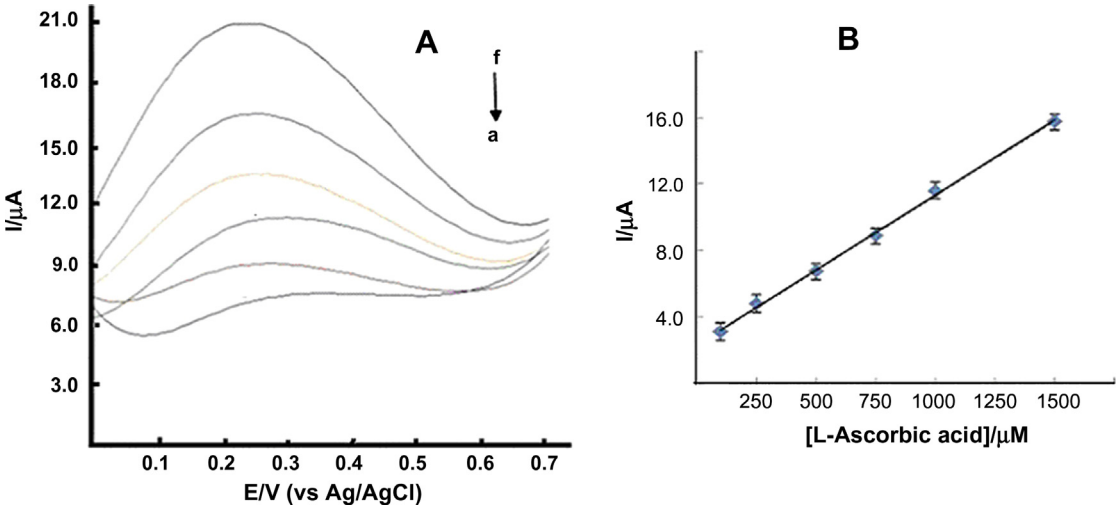


Fig. 4. Typical DP responses of the biosensor in a stirred phosphate buffer (50 mM, pH 7.0). Applied potential was between 0.0 and 0.7 V *vs*. Ag/AgCl. (A) DP responses of (a) 100 μM, (b) 250 μM, (c) 500 μM, (d) 750 μM, (e) 1000 μM, and (f) 1500 μM L-Ascorbic acid and (B) calibration curve between the current and the concentration of L-Ascorbic acid (R^2 = 0.9981).

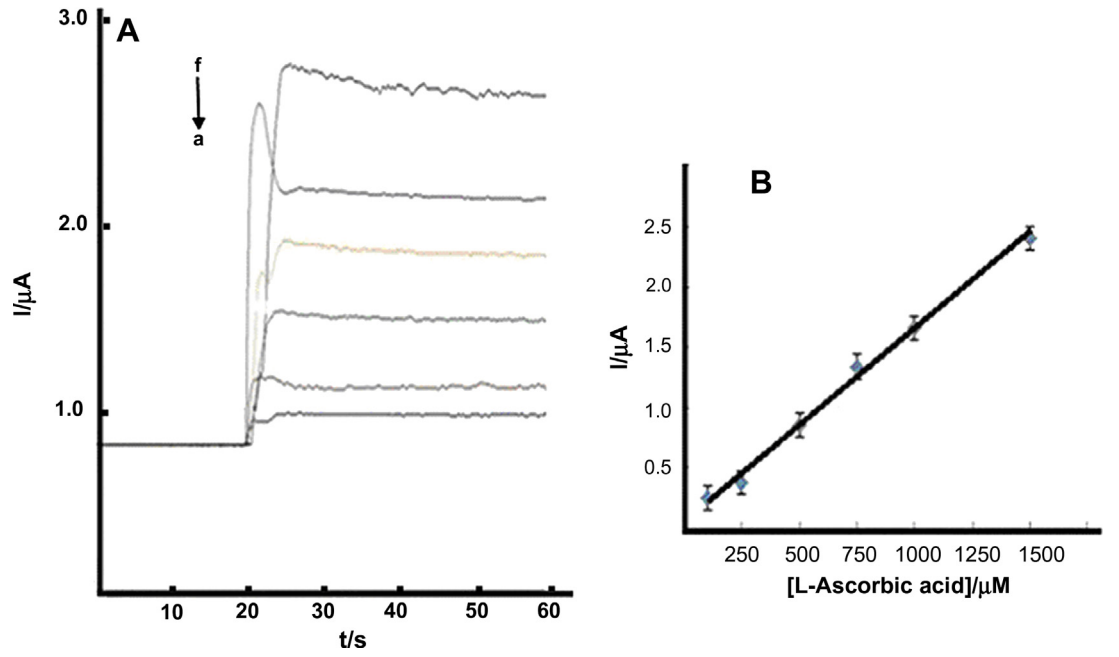


Fig. 5. Typical amperometric responses of the microbial biosensor in a stirred phosphate buffer (50 mM, pH 7.0). Applied potential: + 0.24 V *vs*. Ag/AgCl. (A) Amperometric response of (a) 100 μM, (b) 250 μM, (c) 500 μM, (d) 750 μM, (e) 1000 μM, and (f) 1500 μM L-Ascorbic acid and (B) calibration curve between the current and the concentration of L-Ascorbic acid (R^2 = 0.9965).

only in cathodic but also in anodic areas. From the experiments, a plot of peak current *vs* the square root of the scan rate reveals a linear relationship which is a characteristic of a diffusion-controlled process.

3.4.4. Substrate selectivity of the microbial biosensor

Selectivity is one of the most important characteristics of a microbial biosensor. The response of the microbial biosensor should be specific to L-Ascorbic acid and should not involve other electroactive substances in the sample. In this section, the selectivity of the biosensor was determined by using compounds such as citric acid, uric acid, glucose, and DOPA. For this purpose, 100, 250 and 500 μM concentrations of L-Ascorbic acid and a 500 μM concentration of the other compounds were used in the experiments. All the measurements were done at +0.24 V *vs* Ag/AgCl with a newly prepared microbial biosensor in phosphate buffer (pH 7.0; 50 mM) and at 30 °C. Fig. 7A shows the results obtained from the experiments. According to the figure, it can be said that the microbial biosensor shows a good response that is related to L-Ascorbic acid concentration and there are also weak and negligible interference effects of the other compounds on the microbial biosensor. From these results, the selectivity of the biosensor is seen to be very high. The selectivity experiment shown in Fig. 7A was repeated by adding the different compounds after waiting for current stabilization as in Section 3.4.1. Results obtained from these experiments were given in Fig. 7B. As is shown in the figure, the results confirm the findings from the previous experiment. In addition, five different wine samples containing

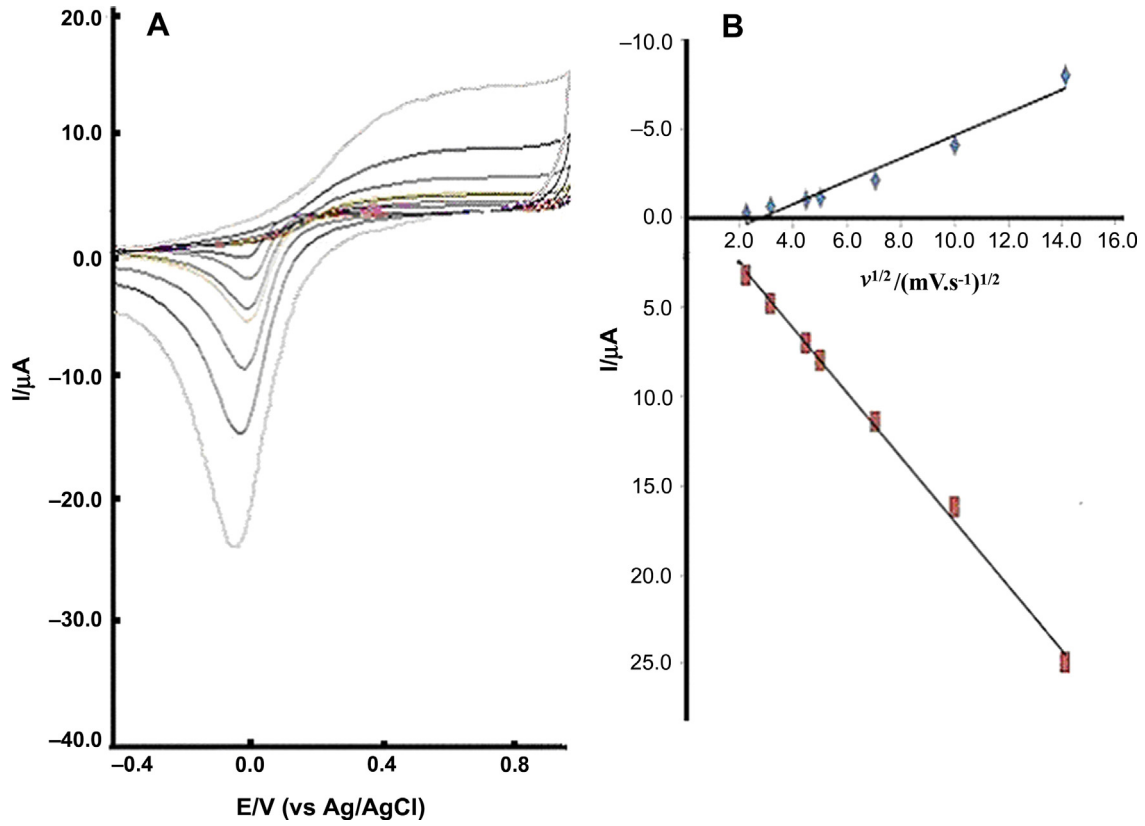


Fig. 6. (A) CV at different scan rates (from inside to outside): 5, 10, 20, 25, 50, 100, 200 mV/s, respectively. (B) Plot of cathodic and anodic peak currents versus the square root of scan rate ($mv^{1/2}$). Conditions: (Phosphate buffer; pH 7.0, 50 mM; *T: 30 °C*). L-Ascorbic acid: 500 μM.

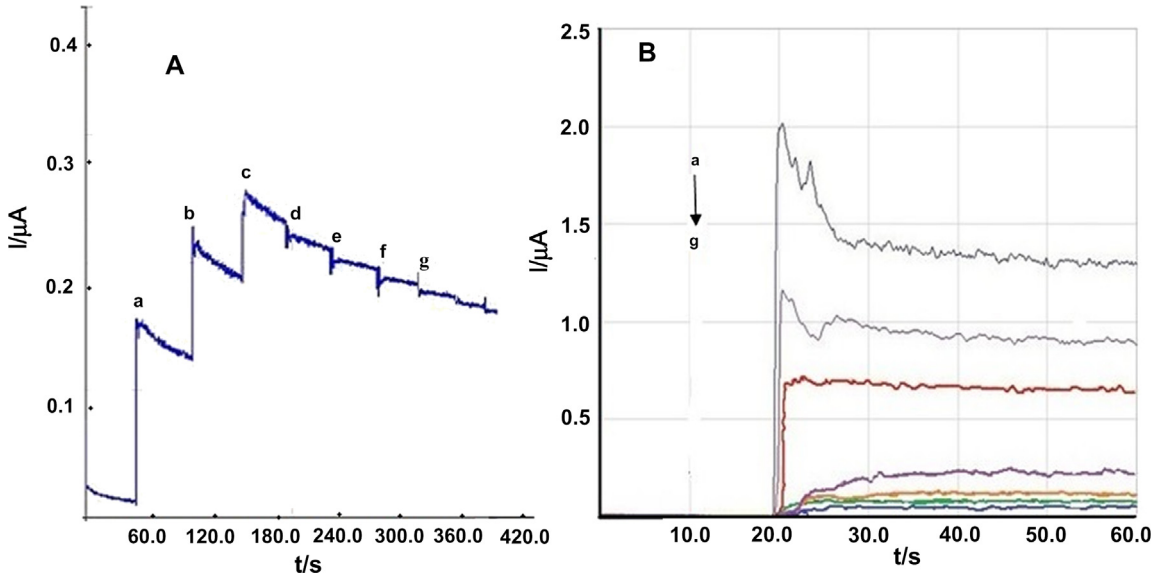


Fig. 7. Substrate selectivity of the microbial biosensor. A: sequential, B: waiting for the current stabilization, Applied potential: +0.24 V *vs.* Ag/AgCl, a: 500 μM L-Ascorbic acid, b: 250 μM L-Ascorbic acid, c: 100 μM L-Ascorbic acid, d: 500 μM glucose, e: 500 μM citric acid, f: 500 μM DOPA, g: 500 μM uric acid. (Phosphate buffer; pH 7.0, 50 mM; *T: 30 °C*).

L-Ascorbic acid were used as the real industrial samples to determine the selectivity of the developed microbial biosensor. Samples were purchased from a local market and used directly in experiments without or with alcohol levels between 8% and 15%. Table 1 shows the results obtained from the experiments. According to the Table, the biosensor shows good selectivity performance in the complex matrix of real industrial samples. Wines generally include organic acids such as tartaric, malic, citric, succinic and fumaric

Table 1
Selectivity determination of the microbial biosensor for wine samples.

Sample	Microbial biosensor* (mg/L)	S.D. (mg/L)	C.V. (%)
Wine 1	45.5	± 0.8463	1.86
Wine 2	53.7	± 1.0418	1.94
Wine 3	50.4	± 0.9526	1.89
Wine 4	60.1	± 1.2140	2.02
Wine 5	48.8	± 0.8686	1.78

* Average of five measurements.

Table 2
Comparison of the microbial biosensor with the other biosensors.

Biomaterial	Transducer	Linear range (μM)	Detection limit (μM)	Response time	References
Ascorbate oxidase	Screen printed electrodes	5–150	3	–	[28]
Ascorbate oxidase	Oxygen electrode	10–400	10	10 s	[23]
Ascorbate oxidase	Graphite/epoxy electrode	8–450	8	5 min	[30]
Peroxidase	ISFET	500–2000	500	–	[45]
Ascorbate oxidase	ZnO nanorods	1–50000	1	10 s	[46]
Ascorbate oxidase	Pt Disc electrode	50–20000	15	20 s	[26]
Ascorbate oxidase	Graphite electrodes	2–3300	1.5	20 s	[25]
C. tropicalis	Pt electrode	100–1500	62 (DPV) 59 (Amp.)	14 s (DPV) 5 s (Amp.)	This study

Table 3
L-Ascorbic acid determination in fruit juices and Vitamin-C tablets by using the microbial biosensor and DCIP methods.

Sample	Microbial biosensor			DCIP method		
	Average (mg/100 g) (n = 5)	SD	CV (%)	Average (mg/100 g)(n = 5)	SD	CV (%)
Orange	43.22	±0.8039	1.86	39.35	±0.9680	2.46
Lemon	40.08	±0.7094	1.77	35.48	1.0570	2.98
Grapefruit	34.46	±0.6616	1.92	28.97	±0.8749	3.02
Sample	Microbial biosensor			DCIP method		
	Average (mg/tablet) (n = 5)	SD	CV (%)	Average (mg/tablet) (n = 5)	SD	CV (%)
Tablet 1	498.79	±5.1874	1.04	508.12	±11.8392	2.33
Tablet 2	1002.03	±11.323	1.13	1011.23	±24.5245	2.48

acid and it can be expected that these organic acids will have interference effects on L-Ascorbic acid determination. However, the results indicate that the enzymatic induction and adaptation of *C. tropicalis* cells to L-Ascorbic acid is high and that cells selectively metabolise L-Ascorbic acid, even if there are similar organic acids in the medium.

3.4.5. Storage stability of the microbial biosensor

In order to investigate the storage stability of the biosensor, amperometric measurements were made. Measurements were made with the same electrode in the presence of 500 μM L-Ascorbic acid for 15 days. During this period, the biosensor was stored in phosphate buffer (50 mM, pH 7.0) at 4 °C when not in use. After the 15 days of storage, the biosensor response decreased to % 71 of the first day response. This result is highly acceptable for a microbial biosensor. So, it is clear that the biosensor developed is long-lasting and also that the yeast cell immobilization method used is efficient. We also compared the characteristics of the microbial biosensor with already existing biosensors as given in Table 2. The advantages of the microbial biosensor system developed are high sample frequency, good re-useability (approx. 75 samples can be measured with one microbial electrode with slight signal decrease), low detection limit, fast response and easy preparation. The response time of the microbial biosensor is extremely short compared to enzyme-based biosensors, which is an indication that *C. tropicalis* cells can metabolize L-Ascorbic acid effectively and in a very short time. On the other hand, with the developed microbial biosensor, measurements can be made using either amperometric or differential pulse methods. Considering the parameters given in the Table, although other biosensors have used specific enzymes such as ascorbate oxidase or peroxidase, it can be said that the microbial biosensor developed is relatively fast, effective and sensitive for L-Ascorbic acid determination.

3.4.6. Reproducibility

For the determination of the reproducibility of the microbial biosensor, 3 different biosensors prepared in the same manner and experiments were made with L-Ascorbic acid standards using the DP method. Fig. S4 shows the results obtained from the experiments. As shown in the figure, the responses to the L-Ascorbic acid standards were very close to each other. Similar, sensitive and repeatable results were obtained with the three microbial biosensors. For all microbial biosensors, a linear relationship to the L-Ascorbic acid concentration was obtained between 100 μM and 1500 μM with a correlation coefficient of 0.9946, 0.9978 and 0.9985, respectively.

3.4.7. Sample analysis

In this section of the study, L-Ascorbic acid was determined in three different fruits and two different vitamin C tablets for the purpose of sample application of the microbial biosensor. A solution of each tablet was prepared in phosphate buffer (pH 7.0, 50 mM). For the determination of L-Ascorbic acid in fruit juices orange, grapefruit and lemon were used. 100 g of the fruits were peeled and homogenized. After that, they were filtered through cheese-cloth to remove cellulose residue and then the collected filtrate was centrifuged at 8.000 rpm for 10 min at 4 °C. The supernatant obtained was kept in the dark at 4 °C and measurements were carried out on the same day. In the same samples, L-Ascorbic acid was determined by the DCIP reference method [42] and results compared. The average value, standard deviation and coefficient of variation were determined for each method (Table 3). As shown on the Table, the L-Ascorbic acid content of both fruit juices and vitamin-C tablets can be determined more quantitatively and more accurately with the microbial biosensor. If we compare the results with the microbial biosensors with results obtained by the reference method, it is possible to say that the microbial biosensor give more accurate and precise results.

4. Conclusion

In this research, we developed a novel microbial biosensor for the determination of L-Ascorbic acid. *C. tropicalis* is the first microorganism that is used in the construction of a microbial biosensor for this purpose. *C. tropicalis*, incorporates ascorbate oxidase enzyme in its metabolism converting L-Ascorbic acid to dehydro-L-Ascorbic acid. The biocomponent was immobilized with

o-aminophenol by forming a film layer on platinum electrode surface using electropolymerization. There has not been any microbial biosensor for L-Ascorbic acid determination until now, and this study is the first to render this function. Results of the characterization studies indicate that the microbial biosensor is fast, effective and sensitive for L-Ascorbic acid determination.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2019.107420.

References

[1] C.N. Kotanen, O. Karunwi, F. Alam, C.F.T. Uyehara, A. Guiseppi-Elie, Fabrication and in vitro performance of a dual responsive lactate and glucose biosensor, Electrochim. Acta 267 (2018) 71–79.
[2] Y. Xu, E. Wang, Electrochemical biosensors based on magnetic micro/nano particles, Electrochim. Acta 84 (2012) 62–73.
[3] C. Andre, I. Castanheira, J.M. Cruz, P. Paseiro, A. Sanches-Silva, Analytical strategies to evaluate antioxidants in food: a review, Trends Food Sci. Technol. 21 (2010) 229–246.
[4] P. Kalimuthu, S.A. John, Electropolymerized film of functionalized thiadiazole on glassy carbon electrode for the simultaneous determination of ascorbic acid, dopamine and uric acid, Bioelectrochemistry 77 (2009) 13–18.
[5] J. Ping, J. Wu, Y.X. Wang, Y.B. Ying, Simultaneous determination of ascorbic acid, dopamine and uric acid using high-performance screen-printed graphene electrode, Biosens. Bioelectron. 34 (2012) 70–76.
[6] X. Wu, Y. Diao, C. Sun, J. Yang, Y. Wang, S. Sun, Fluorimetric determination of ascorbic acid with o-phenylenediamine, Talanta 59 (2003) 95–99.
[7] N. Anastos, N.W. Barnett, B.J. Hindson, C.E. Lenehan, S.W. Lewis, Comparison of soluble manganese(IV) and acidic potassium permanganate chemiluminescence detection using flow injection and sequential injection analysis for the determination of ascorbic acid in Vitamin C tablets, Talanta 64 (2004) 130–134.
[8] H. Chen, R.B. Li, L. Lin, G.S. Guo, J.M. Lin, Determination of L-ascorbic acid in human serum by chemiluminescence based on hydrogen peroxide-sodium hydrogen carbonate-CdSe/CdS quantum dots system, Talanta 81 (2010) 1688–1699.
[9] L. Novakova, D. Solichova, P. Solich, Hydrophilic interaction liquid chromatography–charged aerosol detection as a straightforward solution for simultaneous analysis of ascorbic acid and dehydroascorbic acid, J. Chromatog. A 1216 (2009) 4574–4581.
[10] K.E. Hubbard, A. Wells, T.S. Owens, M. Tagen, C.H. Fraga, C.F. Stewart, Determination of dopamine, serotonin, and their metabolites in pediatric cerebrospinal fluid by isocratic high performance liquid chromatography coupled with electrochemical detection, Biomed. Chrom. 24 (2010) 626–631.
[11] L. Fraisse, M.C. Bonnet, J.P. deFarcy, C. Agut, D. Dersigny, A. Bayol, A colorimetric 96-well microtiter plate assay for the determination of urate oxidase activity and its kinetic parameters, Anal. Biochem. 309 (2002) 173–179.
[12] S.A. Kumar, P.H. Lo, S.M. Chen, Electrochemical selective determination of ascorbic acid at redox active polymer modified electrode derived from direct blue 71, Biosens. Bioelectron. 24 (2008) 518–523.
[13] D. Ragupathy, A.I. Gopalan, K.P. Lee, Electrocatalytic oxidation and determination of ascorbic acid in the presence of dopamine at multiwalled carbon nanotube–silica network–gold nanoparticles based nanohybrid modified electrode, Sens. Act. B 143 (2010) 696–703.
[14] D. Wen, S.J. Guo, S.J. Dong, E.K. Wang, Ultrathin Pd nanowire as a highly active electrode material for sensitive and selective detection of ascorbic acid, Biosens. Bioelectron. 26 (2010) 1056–1061.
[15] S. Qiu, S. Gao, Q. Liu, Z. Lin, B. Qiu, G. Chen, Electrochemical impedance spectroscopy sensor for ascorbic acid based on copper(I) catalyzed click chemistry, Biosens. Bioelectron. 26 (2011) 4326–4330.
[16] D. Lakshmi, M.J. Whitcombe, F. Davis, P.S. Sharma, B.B. Prasad, Electrochemical detection of uric acid in mixed and clinical samples: a review, Electroanalysis 23 (2011) 305–320.
[17] F. Prieto, B.A. Coles, R.G. Compton, Mechanistic determination using arrays of variable-sized channel microband electrodes: the oxidation of ascorbic acid in aqueous solution, J. Phy. Chem. B 102 (1998) 7442–7447.
[18] L. Fernandez, H. Carrero, Electrochemical evaluation of ferrocene carboxylic acids confined on surfactant–clay modified glassy carbon electrodes: oxidation of ascorbic acid and uric acid, Electrochim. Acta 50 (2005) 1233–1240.
[19] J.M. Zen, D.M. Tsai, A.S. Kumar, Flow injection analysis of ascorbic acid in real samples using a highly stable chemically modified screen-printed electrode, Electroanalysis 15 (2003) 1171–1176.
[20] J. Kulys, E.J. D'Costa, Printed electrochemical sensor for ascorbic acid determination, Anal. Chim. Acta 243 (1991) 173–178.
[21] X. Wang, H. Watanabe, S. Uchiyama, Amperometric l-ascorbic acid biosensors equipped with enzyme micelle membrane, Talanta 74 (2008) 1681–1685.
[22] E. Akyilmaz, E. Dinçkaya, A new enzyme electrode based on ascorbate oxidase immobilized in gelatin for specific determination of l-ascorbic acid, Talanta 50 (1999) 87–93.
[23] N. Chauhan, T.P. Dahiya, C.S. Pundir, Fabrication of an amperometric ascorbate biosensor using egg shell membrane bound Lagenaria siceraria fruit ascorbate oxidase, J. Mol. Catal. B: Enzym. 67 (2010) 66–71.
[24] M. Liu, Y. Wen, D. Li, R. Yue, J. Xu, H. He, A stable sandwich-type amperometric biosensor based on poly(3,4-ethylenedioxythiophene) – single walled carbon nanotubes/ascorbate oxidase/nafion films for detection of L-ascorbic acid, Sens. Act. B Chem. 159 (2011) 277–285.
[25] T. Dodevska, E. Horozova, N. Dimcheva, Electrochemical behavior of ascorbate oxidase immobilized on graphite electrode modified with Au-nanoparticles, Mater. Sci. Eng. B 178 (2013) 1497–1502.
[26] M. Liu, Y. Wen, J. Xu, H. He, D. Li, R. Yue, G. Liu, An amperometric biosensor based on ascorbate oxidase immobilized in poly(3,4-ethylenedioxythiophene)/multi-walled carbon nanotubes composite films for the determination of L-Ascorbic acid, Anal. Sci. 27 (2011) 477–482.
[27] Y. Wen, J. Xu, M. Liu, D. Li, L. Lu, R. Yue, H. He, A vitamin C electrochemical biosensor based on one-step immobilization of ascorbate oxidase in the biocompatible conducting poly(3,4-ethylenedioxythiophene)-lauroylsarcosinate film for agricultural application in crops, J. Electroanal. Chem. 674 (2012) 71–82.
[28] G. Csiffáry, P. Fűtő, N. Adányi, A. Kiss, Ascorbate oxidase-based amperometric biosensor for l-ascorbic acid determination in beverages, Food Technol. Biotechnol. 54 (2016) 31–35.
[29] D.K. Kannoujia, S. Kumar, P. Nahar, Covalent immobilization of ascorbate oxidase onto polycarbonate strip for L-ascorbic acid detection, J. Biosci. Bioeng. 114 (2012) 402–404.
[30] J.S.B. Fernandes, L.T. Kubota, N.G. de Oliveira, Potentiometric biosensor for l-ascorbic acid based on ascorbate oxidase of natural source immobilized on ethylene–vinylacetate membrane, Anal. Chim. Acta 385 (1999) 3–12.
[31] M.J. Villanueva, M.D. Tenorio, M. Sagardoy, A. Redondo, M.D. Saco, Physical, chemical, histological and microbiological changes in fresh green asparagus (Asparagus officinalis, L.) stored in modified atmosphere packaging, Food Chem. 91 (2005) 609–619.
[32] S.P. Arya, M. Mahajen, A rapid and sensitive method for the determination of ascorbic acid using iron(III)-ferronate complex, Anal. Sci. 11 (1995) 853–855.
[33] E. Akyilmaz, E. Dinçkaya, An amperometric microbial biosensor development based on Candida tropicalis yeast cells for sensitive determination of ethanol, Biosens. Bioelectron. 20 (2005) 1263–1269.
[34] E. Akyilmaz, A. Erdoğan, R. Öztürk, I. Yasa, Sensitive determination of L-lysine with a new amperometric microbial biosensor based on Saccharomyces cerevisiae yeast cells, Biosens. Bioelectron. 22 (2007) 1055–1060.
[35] E. Akyilmaz, I. Yasa, E. Dinckaya, Whole cell immobilized amperometric biosensor based on Saccharomyces cerevisiae for selective determination of vitamin B1 (thiamine), Anal. Biochem. 354 (2006) 78–84.
[36] D.L. Zuza-Alves, W.P. Silva-Rocha, G.M. Chaves, An update on candida tropicalis based on basic and clinical approaches, Front Microbiol. 8 (2017) 1–25.
[37] B.T. Goriushkina, A.P. Soldatkin, S.V. Dzyadevychj, Application of amperometric biosensors for analysis of ethanol, glucose, and lactate in wine, Agric. Food Chem. 57 (2009) 6528–6535.
[38] F. Mazzei, F. Botre, G. Favero, Peroxidase based biosensors for the selective determination of D, L-lactic acid and L-malic acid in wines, Microchem. J. 87 (2007) 81–86.
[39] M. Niculescu, R. Mieliauskiene, V. Laurinavicius, E. Csoregi, Simultaneous detection of ethanol, glucose and glycerol in wines using pyrroloquinoline quinone-dependent dehydrogenases based biosensors, Food Chem. 82 (2003) 481–489.
[40] V. Carralero Sanz, M. Luz Mena, A. Gonzelez-Cortes, P. Yanez-Sedeno, J.M. Pingarron, Development of a tyrosinase biosensor based on gold nanoparticles-modified glassy carbon electrodes.Application to the measurement of a bioelectrochemical polyphenols index in wines, Anal. Chim. Acta 528 (2005) 1–8.
[41] L. Liu, Z. Yin, Z. Yang, A L-cysteine sensor based on Pt nanoparticles/poly(o-aminophenol) film on glassy carbon electrode, Bioelectrochemistry 79 (2010) 84–89.
[42] E. Akyilmaz, E. Dinckaya, A. New, Titrimetric method for the activity determination of partially purified ascorbate oxidase from cucumber (Cucumis sativus L.), Hacettepe Bull. Nat. Sci. Eng. 25 (1996) 135–145.
[43] A.J. Augustine, C. Kjaergaard, M. Qayyum, L. Ziegler, D.J. Kosman, K.O. Hodgson, B. Hedman, E.I. Solomon, Systematic perturbation of the trinuclear copper cluster in the multicopper oxidases: The role of active site asymmetry in its reduction of O_2 to H_2O, J. Am. Chem. Soc. 132 (2010) 6057–6067.
[44] L. Quintanar, C. Stoj, A.B. Taylor, P.J. Hart, D.J. Kosman, E.I. Solomon, Shall we dance? How a multicopper oxidase chooses its electron transfer partner Acc, Chem. Res. 40 (2007) 445–452.
[45] V. Volotovsky, N. Kim, Ascorbic acid determination with an ion-sensitive field effect transistor-based peroxidase biosensor, Anal. Chim. Acta 359 (1998) 143–148.
[46] Z.H. Ibupoto, M.U.A. Syed, K. Khun, M. Willander, L-ascorbic acid biosensor based on immobilized enzyme on ZnO nanorods, J. Biosens. Bioelectron. 2 (2011) 1–7.