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A novel microbial biosensor based on *Circinella* sp. modified carbon paste electrode and its voltammetric application

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ABSTRACT

Microbial biosensors have been developed for voltammetric determination of various substances. This paper describes the development of a new biosorption based microbial biosensor for determination of Cu^{2+}. The developed biosensor is based on carbon paste electrode consisting of whole cells of *Circinella* sp. Cu^{2+} was preconcentrated on the electrode surface at open circuit and then cathodically detected with the reduction of Cu^{2+}. The voltammetric responses were evaluated with respect to percentage cell loading in the carbon paste, preconcentration time, pH of preconcentration solution, scan rate and interferences. The optimum response was realized by biosensor constructed using 5 mg of dry cell weight per 100 mg of carbon paste in pH 5.5 preconcentration solution. Under the optimum experimental conditions, the developed microbial biosensor exhibited an excellent current response to Cu^{2+} over a linear range from 5.0×10^{-7} to 1.0×10^{-5} M ($r^2 = 0.9938$) with a detection limit of 5.4×10^{-8} M (S/N = 3). The microbial biosensor had good sensitivity and reproducibility (R.S.D. 4.3%, $n = 6$). Finally, the applicability of the proposed microbial biosensor to voltammetric determination of Cu^{2+} in real sample was also demonstrated and validated with atomic absorption spectrophotometric (AAS) method.

1. Introduction

Biosorption is a process that uses removal of toxic heavy metals from industrial or other effluents. This process, which is applied with live or inactivated biomass, is a relatively novel technology and represents a potentially cost-effective way for the removal of heavy metals from aqueous solutions [1,2]. Various biomasses such as bacteria [3,4], yeast [5], fungi [6–9], algae [10–15], and mosses [16,17] for biosorption of metal ions have been widely used for a long time. The biosorption capacity of these biomasses may also be enhanced by chemical modifications [18].

Fungal biomasses are suitable biosorption materials, because they can be produced in an inexpensive way using simple fermentation techniques [19]. Fungal biomasses have been used to biosorption of various metal ions [20–25]. The biosorption mechanisms differ from each other depend on the species and the origin of the biomasses [26].

Fungal cell walls and their components play a major role in the biosorption. The cell walls have several functional groups (the acetamido group of chitin; amino and phosphate groups in nucleic acids; amino, amido, sulfhydryl and carboxyl groups in proteins; and hydroxyls in polysaccharides) [27]. These functional groups those are able to bind metal ions by adsorption, ion exchange or covalent bonding. Some fungi, such as *Absidia*, *Cunninghamella*, *Mucor* and *Rhizopus*, show excellent biosorption for metal ions [25,28–31] because of the high chitin and chitosan content of the cell walls [20].

Circinella sp. used in the study is a mucor-like species in the order Mucorales (Absidiaceae family). *Circinella* sp. take place the fungus class Zygomycetes and the main components of cell wall of Zygomycetes class are chitin, chitosan and polyglucronic acid. While chitin and chitosan are the sponge members of cell wall in Zygomycetes, glucronic acid and mannoproteins are gel-like polymers [32]. Comparative studies using *Circinella* species and other Mucorales members (*Rhizopus*, *Mucor*, *Absidia*) have been made for microbiological production of chitosan which is commercially produced from shrimp and used as chromatographic support material, chelating and antimicrobial agent [33]. However, there are no investigations about the use of *Circinella* sp. for the electrochemical determination of metal ions.

The biosorption process can be combined with electrochemical methods for sensitive determination of heavy metals. Biomolecules such as organelles, enzymes, receptors and micro-organisms have been used as modifying agents for the preparation of biosensor.

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Among them micro-organisms have advantages since they detect chemical substances in a wide range, broad operating pH and temperature [34]. In addition, microbial biosensors have importance for the detection of complex parameters for pollution such as toxicity, quality or load of water or soil [35]. There have been many reports about the usage of microbial sensor [34,36,37], but only a few of them are related to determination of heavy metal ions [38–40].

Voltammetric determination and environmental control of copper is of importance due to the fact that copper is an essential element and causes toxic effect on humans and other living organisms at high-level concentration. Voltammetric determination of copper using carbon paste electrodes (CPEs) modified with ion exchangers [41–44], complexing agents [45–51], and micro-organisms such as algae [52,53] and amperometric determination of copper using oxygen electrode modified with yeast membranes [38,40] have been reported.

The purpose of the present study is to develop a novel biosorption based biosensor for voltammetric determination of Cu^{2+}. A type of fungal micro-organism, *Circinella* sp., was firstly used as a sensor modifying agent. Biocatalytic properties of micro-organisms have been used recently for the preparation of microbial biosensors but they are not based on preconcentration and voltammetric measurement of metal ions. In our study, *Circinella* sp. is incorporated into carbon paste in a simple preparation method and Cu^{2+} is voltammetrically determined followed by preconcentration. The present method is reasonably selective and has low detection limit. It is also easy and inexpensive way for the determination of Cu^{2+}. The developed sensor can also be used to determine Cu^{2+} in real samples.

2. Materials and methods

2.1. Chemicals and reagents

All solutions were prepared with high purity water ($18\,M\Omega\,cm^{-1}$) from a USF ELGA UHQ water purification system. Fine graphite powder, 1–2 μm, (Sigma–Aldrich, St. Louis, MO, USA) and mineral oil (Sigma–Aldrich, St. Louis, MO, USA) were used for the carbon paste electrode (CPE). Stock solutions of Cu^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+} and Pb^{2+} (1.0×10^{-3} M) were prepared from the corresponding analytical grade metal nitrates (Merck, Darmstadt, Germany). Standard solutions of metal ions were prepared freshly by diluting stock solutions. Electrolyte solution was prepared from analytical grade $NaNO_3$ (Merck, Darmstadt, Germany). Potato Dextrose Agar medium (PDA; Merck, Darmstadt, Germany) was used for cultivation. Other chemicals used for cultivation medium were also prepared from analytical grade. The effect of pH on preconcentration solution was studied. The pHs of preconcentration solutions were set using either 0.1 M KOH (Merck, Darmstadt, Germany) or 0.1 M HNO_3 (Riedel-de Haën, Seelze, Germany). Bakosel (Sanofi Dogu Medicine Corporation, Turkey) is a vitamin and mineral combination capsule, which is used for animals, containing vitamin E (500 IU), copper sulphate (10.00 mg), cobalt sulphate (12.50 mg), sodium selenite (2.50 mg), di-calcium phosphate (150.00 mg).

2.2. Micro-organism and culture conditions

The micro-organisms used in this study were *Circinella* sp. TEM was obtained from Culture Collection of Industrial and Molecular Microbiology Research Laboratories, Biology Department, Ege University, Turkey.

Potato Dextrose Agar medium (PDA; Merck, Darmstadt, Germany) was used for cultivation. After incubation for 5 days at 25 °C, spores were extracted by Na-lauryl sulphate (1%) and counted with Thoma Lam. In 500 mL Erlenmeyer flasks, *Circinella* sp, was cultured in 125 mL of liquid medium comprising (g/L): bacteriological peptone, 10; sucrose, 20; KH_2PO_4, 1; $NaNO_3$, 1; $MgSO_4{\cdot}7H_2O$, 0.5. 1.0×10^7 spores were inoculated to the medium. Cultures were grown at 25 °C on an orbital shaker at 150 rpm for 5 days. The biomass was readily separated from the broth by decanting, washed three times with 250 mL aliquots of distilled, de-ionized water, and then lyophilized (Edwards Freeze Dryer, UK). The resultant biosorbent was homogenized using a Sorvall Omni-mixer [31].

2.3. Preparation of sample (Bakosel capsule)

The preparation of sample before voltammetric analysis was made as follows and also given in our previous reports [42,53]. The inner part of the Bakosel capsule was transferred into a beaker and 10.0 mL of ultra-pure de-ionized water and 0.1 mL of concentrated HNO_3 were added. Then, the mixture was transferred into a separation funnel and 15 mL of chloroform was added and shaken for 4 min. The addition of chloroform was repeated for times. After that, aqueous phase was separated and put in a 100.0 mL of volumetric flask and its volume was completed with ultra-pure de-ionized water.

The content of sample was also analyzed and verified with AAS method. According to the AAS analysis, metal ions in the Bakosel capsule are 2.35 mg of Cu(II), 2.57 mg of Co(II), 0.58 mg of Na(I) and 26.43 mg of Ca(II) [52].

2.4. Preparation of microbial biosensor

The working carbon paste electrode (CPE) was prepared by using two pastes. The first paste was made by mixing 5.0 mg of cells with 65 mg of fine graphite powder and 30 mg of mineral oil. The other paste was prepared by thoroughly mixing pure fine graphite powder (0.70 g) and mineral oil (0.30 g). The carbon pastes were packed into the hole of the electrode body (glass cylindrical tube, i.d. 3.3 mm) and pressed with steal rod. Then, the electrode surface was smoothed on a weighing paper. The electrical contact between carbon paste electrodes and equipment was established with a copper wire, which was inserted in the second paste. The *Circinella* sp. modified biosensor was stored at 4 °C until use.

2.5. Experimental instrumentation and method

The preconcentration and voltammetric measurement experiments were established by following steps: first step was preconcentration of Cu^{2+} on the surface of microbial biosensor at open circuit via immersing the biosensor in 15.0 mL of stirring Cu^{2+} solution for selected preconcentration time. After the preconcentration step, biosensor was taken out the preconcentration solution and rinsed with ultra-pure de-ionized water. Finally, biosensor was placed in the voltammetric cell filling with 15.0 mL of 0.05 M $NaNO_3$ and preconcentrated Cu^{2+} was stripping from the biosensor surface by voltammetric measurement. The background current was also obtained after the preconcentration step in the absence of Cu^{2+} (blank solution) at open circuit and then voltammetrically stripping. The preconcentration step was conducted using a magnetic stirrer (Metrohm 728 Stirrer, Herisau, Switzerland) and solution temperature was controlled at optimal temperature (25 °C) with a thermostat (PolyScience, Chicago, USA). Differential pulse cathodic stripping voltammetry (DPCSV) experiments were performed with Metrohm (Herisau, Switzerland) 746 trace analyzer and 747 VA stand. The instrument equipped with three-electrode cell containing the supporting electrolyte, 0.05 M $NaNO_3$, purged by bubbling pure nitrogen for 40 s, before measurements. Differential pulse

cathodic stripping (DPCS) voltammograms were obtained towards cathodic direction in the range of 600 to −800 mV with a scan rate of 80 mV/s. The pulse amplitude was 50 mV, pulse time was 40 ms and measuring time was 20 ms. Working electrodes were carbon paste electrodes modified with *Circinella* sp. The counter electrode was a platinum wire and the reference electrode was a Ag/AgCl (saturated KNO_3).

2.6. SEM measurements

The surface morphologies of the developed microbial biosensor were investigated by scanning electron microscopy (SEM) instrument (JEOL JSM-6060 SEM) combined with energy dispersive X-ray spectroscopy (EDS). The samples mounted on the plate kept under vacuum and then coated with gold particles before the SEM measurements. SEM micrographs were recorded for both the surface of blank and copper loaded microbial biosensors.

3. Results and discussion

3.1. SEM characterization of the biosensor

The SEM micrographs of the surface of microbial biosensors before and after loading of 1.0×10^{-3} M of Cu^{2+} were given in Fig. 1. The results of elemental analysis were also given in Table 1. The results show that the difference between before and after loading of Cu^{2+} on the biosensor surface. According to the elemental analysis results, a significant amount of copper was loading on the biosensor surface via the sorption ability of micro-organism. Some elements such as Ca, Mg and P were also obtained in the analysis beside copper may be due to the cultivation medium of micro-organism and its cell wall components.

Table 1
Elemental analysis results of the microbial biosensor

Before loading of copper		After loading of copper	
Component	Conc. (wt.%)	Component	Conc. (wt.%)
C	82.671	C	77.917
O	11.939	O	7.612
Mg	1.626	Mg	0.739
P	2.913	P	4.132
Ca	0.796	Ca	0.232
Cu	0.056	Cu	9.368

(kV: 20.0; take off angle: 35.0°; elapsed live time: 100.0).

3.2. Cyclic behaviour of the biosensor

Cyclic behaviour of microbial the biosensor was investigated for 1.0×10^{-3} M Cu^{2+} in 0.05 M $NaNO_3$. Fig. 2 shows the cyclic behaviour of unmodified and modified sensor. As shown in Fig. 2b, the well-defined cathodic peak and anodic peak observed at around −300 and −35 mV, respectively. The difference between Fig. 2a and b obviously depicts the effect of *Circinella* sp. modification on improvement of electrode response for the determination of Cu^{2+}. It was clear that the ion exchange and adsorption character of functional groups of *Circinella* sp. caused the effective preconcentration of Cu^{2+} onto the electrode surface and improvement of the electrode response.

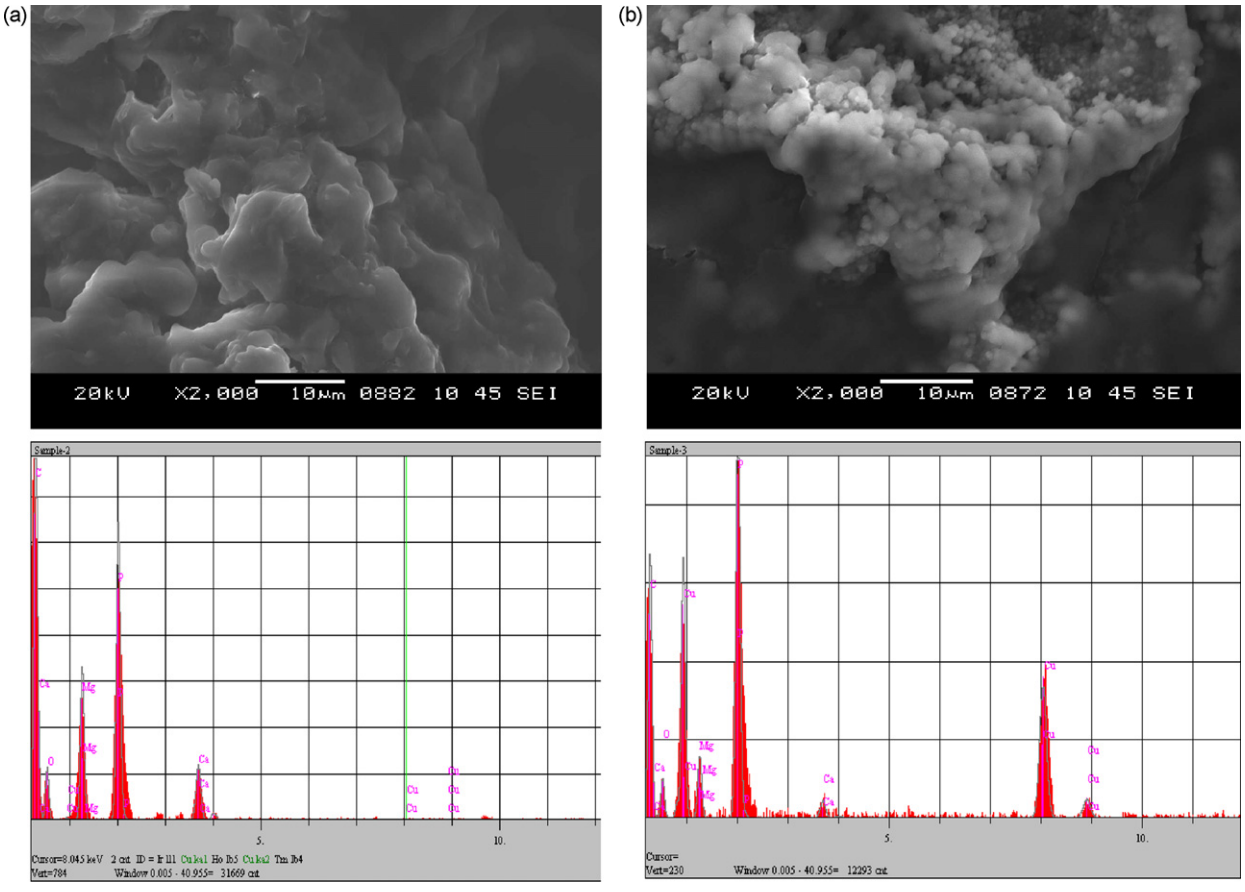

Fig. 1. SEM micrographs and spectrums (a) before loading of copper (b) after loading of copper onto biosensor surface.

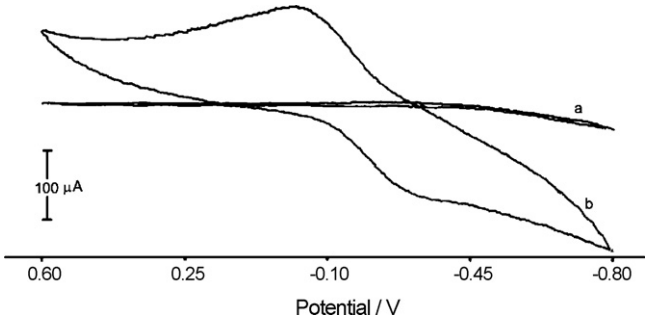

Fig. 2. Cyclic voltammograms with (a) unmodified CPE and (b) *Circinella* sp. modified CPE (concentration of Cu^{2+}: 1.0×10^{-3} M; detection: cyclic voltammetry in 0.05 M $NaNO_3$ with 80 mV/s scan rate; cell ratio in CPE: %5.0 (w/w); preconcentration time: 30 min).

3.3. The effect of scan rate

The effect of different scan rates on the peak current for the voltammetric determination of 1.0×10^{-5} M Cu^{2+} with the *Circinella* sp. modified microbial biosensor was studied by varying the scan rate between 15 and 100 mV/s. It was observed that the biosensor response increased linearly with an increase in scan rate up to 80 mV/s. The higher scan rate caused the decrease in peak current. As the highest sensitivity was achieved at 80 mV/s, all subsequent biosensor measurements were performed at 80 mV/s.

3.4. The effect of cell amount in carbon paste

The effect of various carbon paste compositions on the peak current for the voltammetric determination of 1.0×10^{-5} M Cu^{2+} with developed microbial biosensor was studied. Microbial biosensors were prepared using varying percentages of the *Circinella* sp. changing between 0 and 15% (w/w). The biosensor response increased with increasing percentages of the *Circinella* sp. in carbon paste up to 5.0% (w/w). Increasing percentages of the *Circinella* sp. in carbon paste beyond 5.0% (w/w) was not useful for electrode preparation since the background current was rather large and signal was noisy. Therefore, 5 mg of dry cell weight per 100 mg of carbon paste was selected for further experiments.

3.5. The effect of preconcentration time

To determine the effect of preconcentration time on the microbial biosensor response, various preconcentration times changing between 5 and 60 min was selected. The preconcentration time is an important factor for the biosorption of metal ions. The biosorption of metal ions onto the biomasses surface depending on the preconcentration time are related to the functional groups of cell wall and their sorption capacity. As shown in Fig. 3, peak current gradually increased up to 30 min, and then it reached the level off. Hence, 30 min was used as a preconcentration time at all further experiments.

3.6. The effect of preconcentration solution pH

To establish the optimum conditions, the effect of preconcentration solution pH on biosensor response was investigated from pH 2.0–8.0. Biosorption process occurs by means of adsorption, ion exchange, and covalent binding with the biosorptive sites of the fungal cell wall including hydroxyl, carboxyl, amino, sulfhydryl and amino functional groups. The main cell wall components of *Circinella* sp. used in the study is chitin, chitosan and polyglucronic acid and it has carboxylate, hydroxyl and amino functional groups.

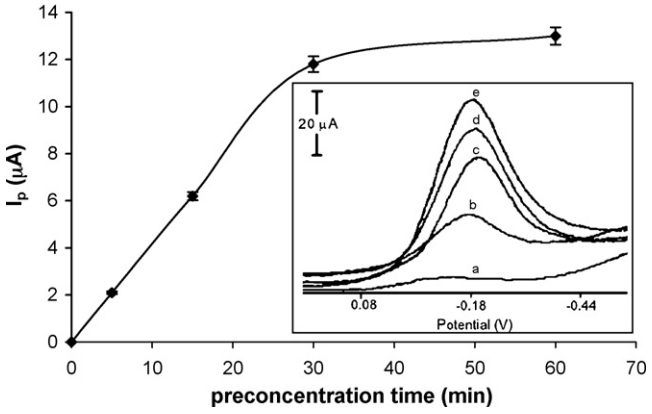

Fig. 3. A plot and voltammograms for the effect of preconcentration time on the microbial biosensor response (concentration of Cu^{2+}: 1.0×10^{-5} M; detection: DPCSV in 0.05M $NaNO_3$ with 80 mV/s scan rate; cell ratio in carbon paste: 5.0% w/w; scan rate: 80 mV/s; a: background current; b: 5; c: 15; d: 30; and e: 60 min). R.S.D. % values for each points of the preconcentration time curve are between 2.8 and 4.9 ($n = 5$).

The pH of preconcentration solution affects the solubility of metal ions and the ionization state of the functional groups (carboxylate, phosphate, and amino groups) of the fungal cell wall [6,54]. The negative charges of functional groups, carboxylate and phosphate, provide the biosorption of metal ions [54]. It was clear that the biosorption mechanism at preconcentration step was affected by the pH of preconcentration solution. The lower peak current at pH 2.0 is an indication of possible competition of protons for the exchange sites on the fungal cell wall [54]. At low pH values, functional groups of cell wall are surrounded by protons and occurring repulsive forces prevent the approach of metal ions to cell wall [2]. The deprotonation of the metal binding sites at higher pH causes to increasing negative charges and this led to improvement of the biosorption of metal ions [54]. However, the decrease can occur at higher pH values due to the formation of anionic hydroxide complexes [54]. The highest peak current was obtained at pH 5.5 with *Circinella* sp. modified biosensor for 1.0×10^{-5} M Cu^{2+}. Therefore, the pH of 5.5 was used for subsequent studies.

3.7. Effect of interferences

The response of microbial biosensor was evaluated in the presence of 1.0×10^{-6} M Cu^{2+} and the interfering metal ions, Cd^{2+}, Ni^{2+}, Zn^{2+} and Pb^{2+}, at concentrations of 1.0×10^{-6} to 1.0×10^{-4} M (Fig. 4). Interference studies were conducted by differential pulse

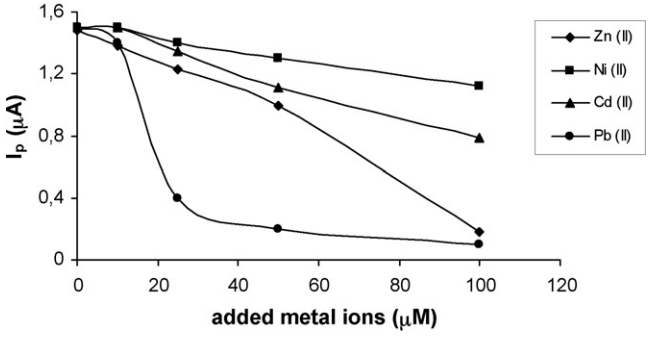

Fig. 4. Interference effects of some metal ions in presence of the preconcentration solution on the microbial biosensor response (concentration of Cu^{2+}: 1.0×10^{-6} M; cell ratio in carbon paste: 5.0% w/w; preconcentration time: 30 min; pH: 5.5; detection: DPCSV in 0.05 M $NaNO_3$ with 80 mV/s scan rate).

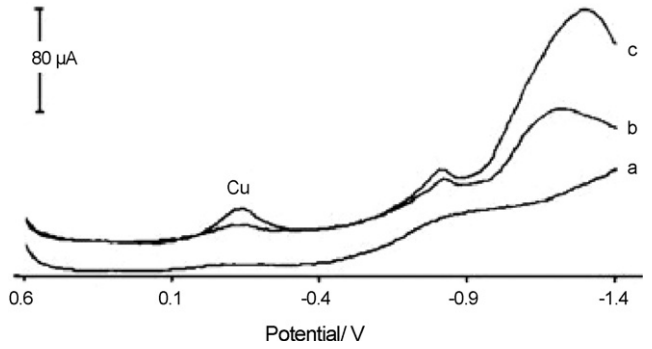

Fig. 5. DPCS voltammograms of the microbial biosensor in two different metal ion mixtures including Cu^{2+}, Cd^{2+}, Zn^{2+} and Ni^{2+} (a: background current; b: the mixture of 5.0×10^{-7} M of Cu^{2+}, Cd^{2+}, Zn^{2+} and Ni^{2+}; c: the mixture of 1.0×10^{-6} M of Cu^{2+}, Cd^{2+}, Zn^{2+} and Ni^{2+}; cell ratio in carbon paste: 5.0% w/w; pH: 5.5; preconcentration time: 30 min; detection: DPCSV in 0.05 M $NaNO_3$ with 80 mV/s scan rate).

cathodic stripping voltammetry (DPCSV) at identical conditions. As shown in Fig. 4, a 10-fold excess of Pb^{2+}, a 25-fold excess of Zn^{2+} and a 50-fold excess of Cd^{2+} and Ni^{2+} had no influence on the determination of 1.0×10^{-6} M Cu^{2+}.

Furthermore, the matrix effect on the response of microbial biosensor was investigated in two model solutions containing equal concentration of Cd(II), Zn(II), Ni(II) and Cu(II). Metal ion concentrations were chosen as both 5.0×10^{-7} and 1.0×10^{-6} M. The results indicated that the peak current intensity of Cu(II) was not affected by lower concentrations of these metal ions (Fig. 5). The higher concentration of interfering metal ions in the matrix causes decreasing peak current intensity of Cu^{2+}. Metal ions at high concentrations caused interfering effect because they are potentially competing for the binding sites with Cu^{2+}. The decrease can be also attributed that the saturation of binding sites at the biosensor surface. When presence of higher concentration of interfering ions in the mixture solution, the interference effect can be eliminated the standard addition of known concentration of copper ions in the matrix. Therefore, our findings are very promising since the *Circinella* sp. modified biosensor can be used in the determination of metal ion mixtures at low concentrations.

3.8. Analytical characteristics of the microbial biosensor

The calibration plot obtained using the optimum conditions was depicted in Fig. 6. The developed microbial biosensor has a linear range between 5.0×10^{-7} and 1.0×10^{-5} M Cu^{2+} (0.032 and 0.635 mg L^{-1}) ($r^2 = 0.9938$). Compared with the existing microbial biosensor [38,40] for determination of copper, our developed microbial biosensor has significant differences in the preparation of the biosensor and the determination method. Lehmann et al. developed a new system for the amperometric detection of Cu^{2+} using recombinant *Saccharomyces cerevisiae* strains as the biocomponent in the microbial biosensor. Their biosensor has a linear range between 5.0×10^{-4} and 2.0×10^{-3} M Cu^{2+} [38]. Tag et al. reported the amperometric detection of Cu^{2+} by *Saccharomyces cerevisiae* modified two biosensors using flow injection analysis. They found the linear ranges of both microbial biosensors as 1.6–6.4 and 0.05–0.35 mg L^{-1} Cu^{2+}, respectively [40].

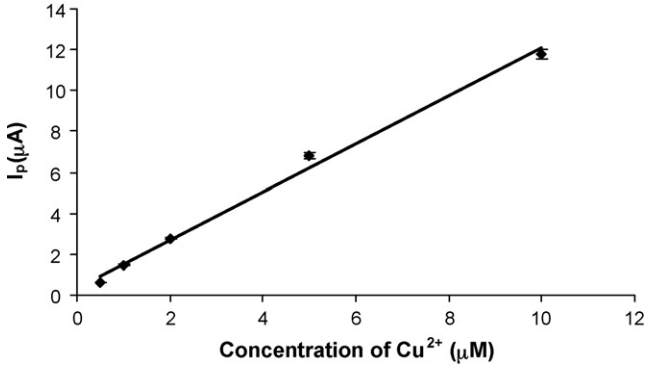

Fig. 6. Calibration plot of the developed microbial biosensor for 5.0×10^{-7} to 1.0×10^{-5} M Cu^{2+} under optimum experimental conditions. R.S.D.% values for each point of the calibration curve are between 1.8 and 3.2 ($n = 5$).

The developed *Circinella* sp. modified microbial biosensor had very good electrode-to-electrode reproducibility as evidenced by the low relative standard deviation of 4.3% ($n = 6$) in the response of six microbial biosensors prepared at different times to 5.0×10^{-6} M Cu^{2+}. The detection limit (defined as three times the standard deviation of the response obtained for a blank) was found 5.4×10^{-8} M (0.0034 mg L^{-1}). Tag et al. found the detection limits as 2.1 and 0.0067 mg L^{-1} Cu^{2+} for their developed biosensors [40].

Compared with the existing microbial sensors, our developed microbial biosensor provides a wider linear range of copper detection than those of references based on amperometric microbial biosensors modified by *Saccharomyces cerevisiae* [38,40]. Besides, the electrode-to-electrode reproducibility value of the developed *Circinella* sp. modified biosensor is better than amperometric microbial biosensor modified by *Saccharomyces cerevisiae* [40]. In conclusion, our biosensor has better advantages for determination of Cu^{2+} due to its sensitivity, reproducibility and simple preparation.

3.9. Sample application of microbial biosensor

The developed microbial biosensor was also used to determine Cu^{2+} in Bakosel capsule. The concentration of Cu^{2+} in the Bakosel capsule was determined with the standard addition method due to the matrix effect of the sample. The differential pulse cathodic stripping (DPCS) voltammograms were obtained from blank solution (Fig. 7a), sample solution (Fig. 7b) and the mixture of sample and known concentration of Cu^{2+} solution (Fig. 7c). These results were confirmed by AAS method. The concentration of Cu^{2+} in Bakosel capsule by using the microbial biosensor was consistent with the results found in AAS method (Table 2).

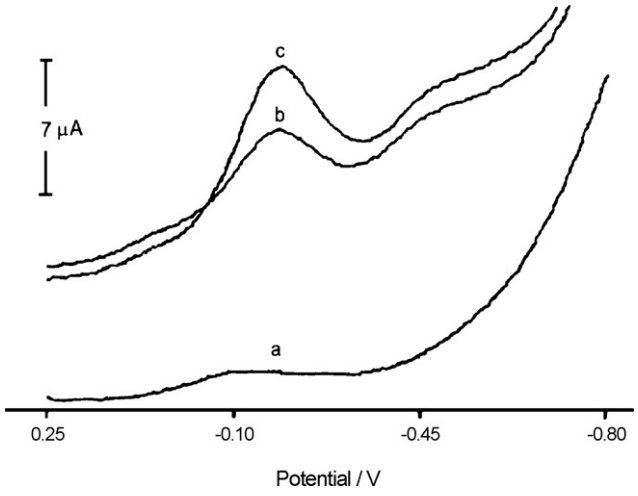

Fig. 7. DPCS voltammograms of the developed microbial biosensor (a) blank, (b) sample, and (c) the mixture of sample and known concentration of Cu^{2+} (cell ratio in CPE: 5.0% w/w; preconcentration time: 30 min; detection: DPCSV in 0.05 M $NaNO_3$ with 80 mV/s scan rate).

Table 2
Application of the *Circinella* sp. modified biosensor for sample

	DPCSV	AAS
Bakosel capsule (mg Cu/capsule)	2.29 ± 0.086	2.35 ± 0.00097

Results are expressed as mean ± S.D., $n = 3$.

4. Conclusion

The removal of heavy metals from aqueous media by using biosorption, biomasses including algae, micro-organism, lichen etc. have been widely used for a long time. The use of biomasses as modifying agents for the preparation of sensors has attracted much interest. A combination of biosorption with electrochemical methods is useful for developing sensitive determination techniques of various substances. Among these biomasses, fungal cells including functional groups such as phosphate, amide, carboxyl, sulfhydryl, thiol and hydroxide have been used to prepare microbial sensors. There are few studies about the use of them for the determination of metal ions. *Circinella* sp. shows a good biosorption capacity for heavy metals.

In the present work, a new biosorption based microbial biosensor was developed using *Circinella* sp., a fungal cell, for the determination of Cu^{2+} at trace levels by differential pulse cathodic stripping voltammetry for the first time. The optimum conditions for the determination of Cu^{2+} were as follows: cell ratio in CPE: %5.0 (w/w); preconcentration time: 30 min; preconcentration solution pH: 5.5; supporting electrolyte: 0.05 M $NaNO_3$; and scan rate of 80 mV/s. The calibration plot of Cu^{2+} was shown ideally to be in a linear relationship between 5.0×10^{-7} and 1.0×10^{-5} M, and correlation factor of 0.9938. The detection limit was 5.4×10^{-8} M. Besides, our work shows a good reproducibility as compared to other studies. The developed microbial biosensor is reliable, simple to prepare, of low cost, precise and does not require extensive preliminary sample treatment.

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