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Amperometric biosensor based on coupling aminated laccase to functionalized carbon nanotubes for phenolics detection

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ABSTRACT

A biosensor for phenolic compounds based on a chemically modified laccase from *Coriolus hirsuta* immobilized on functionalized screen-printed carbon electrodes (SPCEs) was achieved. Different enzyme modifications and immobilization strategies were analyzed. The electrochemical response of the immobilized laccase on SPCEs modified with carboxyl functionalized multi-walled carbon nanotubes (COOH–MWCNT) was the highest when laccase was aminated prior to the adsorption onto the working electrode. The developed laccase biosensor sensitivity toward different phenolic compounds was assessed to determine the biosensor response with several phenolic compounds. The highest response was obtained for ABTS with a saturation value of $I_{max} = 27.94$ μA. The electrocatalytic efficiency (I_{max}/K_m^{app}) was the highest for ABTS (5588 μA μM^{-1}) followed by syringaldazine (3014 μA.μM^{-1}). The sensors were considerably stable, whereby 99.5, 82 and 77% of the catalytic response using catechol as substrate was retained after 4, 8 and 10 successive cycles of reuse respectively, with response time average of 5 s for 12 cycles. No loss of activity was observed after 20 days of storage.

1. Introduction

Phenolic compounds are extensively utilized in pharmaceuticals, pesticides, dyes, cosmetics, plastic, photography, rubber, paint, tanning industries. Furthermore, most of them are tremendously dangerous to animals and humans because of their reduced biological deprivation [1]. Consequently, the determination of phenolics has become of significance. Numerous methods have been utilized to detect and quantify phenolic compounds like chromatography [2], spectrophotometry [3], electrochemistry [4], and chemiluminescence [5]. The electrochemical technique has attracted among them a widespread consideration due to its low cost, high sensitivity, rapid response, and simple preparation [6–9]. So, greatly efforts have been gained on the electrochemical recognition of phenolic compounds at innovative chemically adapted electrodes [10]. Progress of carbon nanomaterials has significantly contributed to improved performance of electrochemical sensors. In particular high exterior area, exceptional electrical conductivity, and uncomplicated surface adaptation [11–13] are characteristics that permit them to be utilized in electrochemical devices like fuel cells, batteries, super capacitors, (bio-) sensors, solar cells, and electro chromic devices [14].

Screen printed electrodes (SPE) provide an economic, and facile analytical tool for environmental pollution control [15,16]. Introduction of nanomaterials improve the properties of the SPE due to an enhanced sensitivity, a higher loading with selective biomolecules and a fast response [17,18]. Nanomaterials, especially carbon nanotubes (CNT), have received a great interest in the field of biosensors due to their optimal sensitivity, conductivity, wide potential range (by increasing the electrode surface area), and susceptibility to modify with functional groups for enzyme immobilization [19–21]. Carboxylic groups and amino groups are the most common functional groups used for CNT functionalization [22,23].

Enzyme immobilization using CNT for biosensor and biofuel cell fabrication is a promising biotechnological application, as it offers unique properties comprising enhanced electronic properties, high surface area ratio, and fast electrode kinetics [24,25]. In order to explore the enzyme-MWCNT electrode complex potency, optimal methods for enzyme immobilization are required [26]. So, covalent and non-covalent linkages have been reported for various enzymes immobilization. Covalent binding provides an excellent, durable attachment, but affects the enzyme conformational structure. By comparison, non-covalent attachment of enzymes to CNTs is limited by the gradual loss of the immobilized enzyme during CNT-enzyme complex reusability [25].

Investigations on phenol oxidizing enzymes using biosensors offer for technological purposes intriguing methods for the estimation of clinically and environmentally significant phenolic composites [27]. Among the enzymes used for detection of phenolic compounds, laccases

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provide special advantages over other oxidoreductase enzymes through their high stability, their broad substrate spectrum, and their high turnover number [9,28]. Laccases were considered as candidates for total phenol determination since laccases oxidize various phenolic compounds like *o*-, *p*-, and *m*-diphenols, polyphenols, amino phenols in presence of low molecular weight mediators [26,29,30]. Laccase (E.C. 1.10.3.2, *p*-benzenediol: oxygen oxidoreductase), is a multicopper blue oxidase containing four copper atoms at its active site, which are necessary for the catalytic activity [15,16,31]. Recently, laccase-based amperometric biosensors were described for application in food and pharmaceutical industries and also for environmental and clinical analysis to measure phenols [32], polyphenols [33], catechols [34], catechin [35], morphine [36], catecholamines [37], pyrocatechol [38], hydroquinone [39] and many other compounds [28].

Enzyme immobilization is a critical step in biosensors development [20,28]. Enzymatic kinetics (parameters like K_m and V_{max}) can be affected by various immobilization processes and consequently, a deviation from the free enzyme performance can be observed [40]. In this view, the immobilization process is crucial step in sensor optimization [41]. Various immobilization methods of laccase onto several electrode materials and arrangements have been applied previously for biosensors construction [30]. *Coriolus hirsuta* laccase (*Ch*L) has been applied for different analytical purposes by a variety of immobilization techniques, such as by entrapment with gelatin and nafion for clinical analysis to measure dopamine [42], or by covalent binding on a Diethylaminoethyl cellulose (DEAE)-cellulose column to analyze catechols in the extract of tea leaves in the food industry [34]. Through cross-linking using bovine serum albumin (BSA) and glutaraldehyde (Glu), *Coriolus hirsuta* laccase was applied to detect *p*-chlorophenol, guaiacol, chloro guaiacol for environmental control [43]. Furthermore, *Ch*L was adsorbed with tyrosinase on solid graphite in a bienzyme sensor for catechol [44] and was *co*-immobilized with pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH) in polyvinyl alcohol (PVA) for pharmaceutical analysis [36]. Enzyme amination has been applied to maximize the percentage of the multipoint covalent binding between enzyme/support configurations and to adapt the enzyme properties. A chemical route using carbodiimides has been the main strategy to accomplish protein surface amination [45], in combination with highly active nucleophiles, to modify the carboxyl groups in proteins and enzymes, but still the development of more efficient procedures for stabilization of the enzyme/support configuration is greatly needed [46,47].

The present study aimed to select the proper way for laccase immobilization onto the SPCE/MWCNT-COOH. For that we used two main forms of laccase *Ch*L and aminated *Coriolus hirsuta* laccase (*Ch*La)) and different immobilization strategies ranging between adsorption, electrostatic interaction, polymer entrapment, cross linking, and covalent binding. To our knowledge, this is the first report for using the *Ch*La in development of biosensors. In this paper, we developed a new electrochemical biosensor using *Ch*La immobilized to a layer of carboxyl functionalized multi-walled carbon nanotubes on SPCE for field monitoring of phenolic compounds. The performance of the developed laccase biosensor was compared to phenol sensors prepared using different immobilization techniques.

2. Material and methods

2.1. Chemicals

2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,6-dimethoxy phenol (DMP), (N,N-)dimethyl formamide (DMF), N-(3-dimethylaminopropyl)-N′-ethyl carbodiimide hydrochloride (EDC), ethylene diamine (EDA), glutaraldehyde (Glu) (25%), hydroquinone, 2,4-dihydroxy benzaldehyde, poly (2-hydroxyethyl methacrylate) (PHEMA), syringaldazine (SGZ), and polyethylenimine (PEI) were obtained from Sigma-Aldrich (Deisenhofen, Germany). 2-Aminophenol, 3-aminophenol, and N-hydroxy sulfo succinimide sodium salt (NHS) from Fluka (Germany), 3,4-dihydroxy benzaldehyde, and bovine serum albumin (BSA) was obtained from Roth (Karlsruhe, Germany), catechol from Riedel (Germany), 2,5-dihydroxy benzaldehyde from Alfa Aesar, poly ethylene glycol diglycidyl ether 400 (PEGDE) from Polyscience, and poly vinyl alcohol (PVA) 05/20 (Mwt 22,000 Da) from Serva were used. All other reagents used were of analytical grade. Pure water was obtained using a Millipore Q water purification apparatus with resistivity over 18MΩ cm (Millipore, Eschborn, Germany).

2.2. Enzyme

Coriolus hirsuta (*Trametes hirsuta*) laccase (*Ch*L) from the basidiomycete strain *T. hirsuta* 56 was obtained from the Moscow State University of Engineering Ecology culture collection. Aliquots were stored within small vials in a lyophilized form with a laccase concentration of 84.97 mg/mL. Laccase solutions were prepared using 50 mM potassium phosphate buffer pH 7.0 and kept at 4 °C.

The enzyme activity was assayed spectrophotometrically with ABTS (0.58 mM) as substrate in 100 mM citrate buffer at pH 4.5 and 25 °C in triplicate. The change in absorbance at 436 nm was monitored for 1 min and the laccase activity was calculated according to the following equation:

$$\text{Laccase activity}\left(\frac{\text{U}}{\text{mL}}\right) = \frac{\Delta\text{A} \times \text{V}_\text{t} \times 10^6}{\Delta\text{t} \times \text{l} \times \varepsilon \times \text{V}_\text{s} \times 1000}$$

where ΔA is the change in absorbance, Δt is the time of incubation (min), ε is the extinction coefficient of ABTS (ε_{436} = 29,300 M^{-1} cm^{-1}), l is the cuvette diameter (1 cm), V_t is the total assay volume, and V_s is the enzyme sample volume. One unit of laccase is defined as the ability to oxidize 1 μmol of ABTS per min [23,48,49]. The protein content in the enzyme preparations was determined from the absorbance at 280 and 260 nm wavelengths according to the Warburg and Christian formula to estimate protein concentration with the advantage of possible nucleic acid contamination correction because any protein contamination will have maximum absorption at 280 nm. Measurements were taken at both 260 nm and 280 nm where the absorbance at 260 nm accounts for nucleic acid interference as follows [50]:

$$\text{Protein}\left(\frac{\text{mg}}{\text{mL}}\right) = (1.55 \times \text{A}_{280}) - (0.76 \times \text{A}_{260})$$

2.3. Laccase amination

*Ch*L was aminated according to the previously published procedure (Othman et al., 2016). Briefly, 5.0 mL of purified enzyme solution (2 mg/mL) was mixed with 5.0 mL of 1.0 M EDA solution and 50 mM EDC at pH 4.75 for 1.5 h prior to dialysis using snake skin dialysis tubing with molecular weight cutoff of 10 kDa (Thermo Scientific) in distilled water for 24 h at 4 °C. The specific activity of aminated enzyme was determined to be 83.7 units/mg. The *Ch*La was stored at 4 °C until use with prior activity determination.

2.4. Preparation of the biosensors

For preparation of the biosensors, *Ch*L or *Ch*La were immobilized on screen-printed carbon electrodes modified with carboxyl functionalized multi-walled carbon nanotubes COOH–MWCNT/SPCE (type110CNT, DropSens, Oviedo, Spain). These electrodes consist of a carbon counter electrode, a silver reference electrode, and a COOH-MWCNT/carbon working electrode. The amount of enzyme used in the individual experiments was normalized in order to compare electrodes with similar enzyme loading.

The enzyme was immobilized by various chemo-physical and chemical techniques: (a) *Adsorption;* 7 μL (3.5 U) of *Ch*L or *Ch*La were dropped on the working electrode; (b) *PEI/AuNP/Lac entrapment:* 6 μL of diluted *Ch*L was added to 1 mL of PEI coated AuNP, the volume was completed to 1.2 mL with 0.1 M citrate phosphate buffer (CPB), pH 4.2, in order to have 24 U/mL in the total reaction mixture [51]. The mixture was stirred at room temperature for 1 h, and was then centrifuged for 20 min at 14000 rpm. The precipitated layer was washed with de-ionized H_2O three times, and then 5 μL of the suspension was dropped to the surface of working electrode, and left to dry under vacuum for 1 h; (c) *Lac/PVA entrapment (I, Stepwise):* 7 μL (3.5 U) of diluted *Ch*L or *Ch*La was dropped onto the working electrode surface, allowed to dry at room temperature, then 7 μL of 5% PVA was dropped over the previously dried enzyme layer. After that, the electrode was exposed to UV radiation for 30 min to crosslink the polymer; (d) *Lac/PVA entrapment (II, One step):* From a mixture containing the diluted *Ch*L or *Ch*La in CPB, pH 5.0 and PVA (5%), 7 μL (3.5 U) was dropped over the working electrode surface and then the electrode was exposed to UV radiation for 30 min; (e) *EDC/NHS/Lac covalent binding (I, Stepwise):*10 μL of a mixture containing 50 mM EDC/50 mM NHS in 0.1 M CPB, pH 5.0 was dropped on the working electrode, left to dry (2 h) at room temperature (25 °C), then 7 μL (3.5 U) of diluted *Ch*L or *Ch*La were dropped over the previous dried layer; (f) *EDC/NHS/Lac covalent binding (II, One step):* 7 μL of a mixture containing 50 mM EDC/50 mM NHS in 0.1 M CPB pH 5.0, and 3.5 U of diluted *Ch*La was dropped onto the working electrode surface, then left to dry at room temperature (25 °C); (g) *EDC/Lac covalent binding (one step):* 7 μL of a mixture containing 50 mM EDC in 0.1 M CPB, pH 5.0, and 3.5 U of diluted *Ch*La were dropped onto the surface of the working electrode then left to dry at room temperature (25 °C); (h) *Lac/PHEMA cross-linking (II, One step):*7 μL (3.5 U) from a mixture containing 10 μL of diluted *Ch*La and 10 μL of PHEMA polymer mixture (5 mg of PHEMA in 2.5 mL DMF, and 2.5 mL d.H_2O) were dropped over the working electrode surface, then it was exposed to UV radiation for 60 min; (i) *Lac/(PHEMA-Lac) cross-linking (I, Stepwise):* The *Ch*La was adsorbed to the electrode surface firstly, then the mixture of PHEMA and *Ch*La was applied with the same previous procedure; (j) *(Lac-BSA)/Glu cross-linking:* The diluted *Ch*La was mixed with BSA solution (25 mg/mL), and then 7 μL (3.5 U) of this mixture was deposited onto the SPCE/MWCNT electrode surface and allowed to dry at room temperature for 2 h. After that, 10 μL of Glu solution (5%) was dropped on the surface of the SPCE/MWCNT/Lac/BSA electrode surface to complete immobilization of laccase by the cross-linking process; (k) *Lac/Glu cross-linking:* 7 μL (3.5 U) of diluted *Ch*La in 0.1 M CPB, pH 5.0 was deposited onto the SPCE/MWCNT electrode surface and was allowed to dry at room temperature for 2 h. Later, 5 μL of the aqueous solution of Glu (1%) was cast onto the SPCE/MWCNT/Lac electrode surface followed by a drying process at room temperature for 3 h [52]; (l) *Lac/PEGDGE cross-linking:*7 μL (3.5) of diluted *Ch*La in 0.1 M CPB, pH 5.0 was dropped over the working electrode, then left to dry at room temperature for 2 h. 5 μL of the aqueous solution of PEGDGE (0.5%) was added to the surface of the working electrode and left to dry at open air [53].

After all immobilization procedures and complete dryness, the biosensors were washed gently with 2 mL of 50 mM potassium phosphate buffer (PPB) pH 7 to remove the non-immobilized laccase, and were stored at 4 °C in a humid box until use. All experiments were performed in triplicate.

2.5. Electrochemical measurements

The electrochemical experiments were done using a homebuilt electrochemical cell with a total volume of 4.5 mL placed in a Faraday cage in connection with a CHI-750A electrochemical workstation (CH Instruments, Austin, TX, USA) using CHI software version 2.05. A DropSens specific connector (ref. CAC) was used to connect the screen-printed electrode and the electro-analytical setup depicted in Fig. 1. All electrochemical measurements were performed at room temperature. The supporting electrolyte in which the desired compounds were mixed was a 0.05 M citrate-phosphate buffer (CPB), pH 5.0. The amperometric measurements were carried out in air-saturated solution at a constant working potential of −100 mV under stirring conditions, unless stated otherwise. The current response was measured after addition of aliquots of the respective substrates. All experiments were performed in triplicate, and the data were expressed as mean values.

3. Results and discussion

3.1. Laccase immobilization on COOH–MWCNT/SPCE

The maximum current response (I_{max}) and the apparent Michaelis–Menten constant (K_m^{app}) using ABTS as substrate for the working electrode modified with laccases under different immobilization conditions were calculated (Table 1). K_m^{app} was used as an indicator of the affinity of laccase for its substrate, whereby a decreased K_m^{app} supports an increased affinity. I_{max} was interpreted as a measure for the catalytic activity.

Electrostatic adsorption of *Ch*La was observed as the best technique for laccase immobilization onto the COOH–MWCNT/SPCE working electrode, giving the highest I_{max} value of 27.94 μA, a K_m^{app} value of 0.005 μM and a I_{max}/K_m^{app} ratio of 5588 μA/μM among all tested immobilization techniques. The response of the sensor with adsorbed *Ch*L was low in comparison to the sensor with the *Ch*La. The I_{max} value for *Ch*L/COOH–MWCNT/SPCE was only 7.38 μA, yielding a I_{max}/K_m^{app} ratio of 48.87 μA/μM and a K_m^{app} value of 0.151 μM. An effective immobilization was thus obtained of the modified laccase via electrostatic adsorption. In this regard, the sensitivity of 831 nA/μM for the *Ch*La/COOH–MWCNT/SPCE was superior relative to the electrode with adsorbed non- modified laccase of 150 nA/μM (for *Ch*L/COOH–MWCNT/SPCE) (Table 1- a). In the present case, it is suggested that the introduced amino groups significantly enhance the attachment by charge interaction to the carboxylated surface. Freire et al. [43] stated that adsorption of unmodified laccase was found to be stabilized by the laccase exterior hydrophobic region and the CNT wall. The π–π stacking between walls of the CNT and the laccase aromatic residues on its surface were also suggested to contribute to the increased sensitivity.

The lowest sensitivity (18 nA/μM), I_{max} value (2.05 μA) and I_{max}/K_m^{app} ratio (17.81 μA/μM) were obtained through laccase entrapment using PEI/AuNP (forming *Ch*L/PEI/AuNP/COOH–MWCNT/SPCE) (Table 1-b). PVA is a broadly used entrapment material, due to its robust chemical and mechanical properties [54]. Here, we applied PVA as an additional cover layer over the pre-bound laccase (Table 1-c) and alternatively (d) as copolymerized with Lac on the COOH–MWCNT/SPCE. In both cases, PVA polymerization was stimulated using photopolymerization. Results obtained indicated a preference for the second strategy (d), whereby with *Ch*La a sensitivity of 410 nA and a I_{max}/K_m^{app} ratio of 1859 μA/μM was obtained. In contrast, Lac/PVA (stepwise) had an increased I_{max}/K_m^{app} ratio (2886 μA/μM) with *Ch*L but was less sensitive (297 nA) due to its lowered K_m^{app} value.

EDC is an effective coupling agent used for peptide bond formation between the amino groups in laccase and surface carboxyl groups on the oxidized CNT. This chemical coupling typically diminishes the risk of enzyme leakage from the support and, subsequently, improves its stability [55]. In this study, covalent coupling of laccase onto COOH–MWCNT/SPCE was performed by three different strategies (Table 1- e, f, g) where (e) a mixture of EDC and NHS was dropped on the working electrode and was left to dry prior laccase addition; (f) EDC/NHS and *Ch*La were mixed in a single step and were then dropped on the electrode surface, and (g) EDC and *Ch*La were mixed and were dropped on the surface of COOH–MWCNT/SPCE in absence of NHS. The results obtained indicated that the application of EDC and ChLa mixture without NHS (trial g) was the best coupling strategy for covalent binding. An increased sensitivity (403 nA/μM), I_{max}/K_m^{app} ratio of 424.22 μA/μM and a decreased K_m^{app} value (0.003 μM) was achieved compared to the other

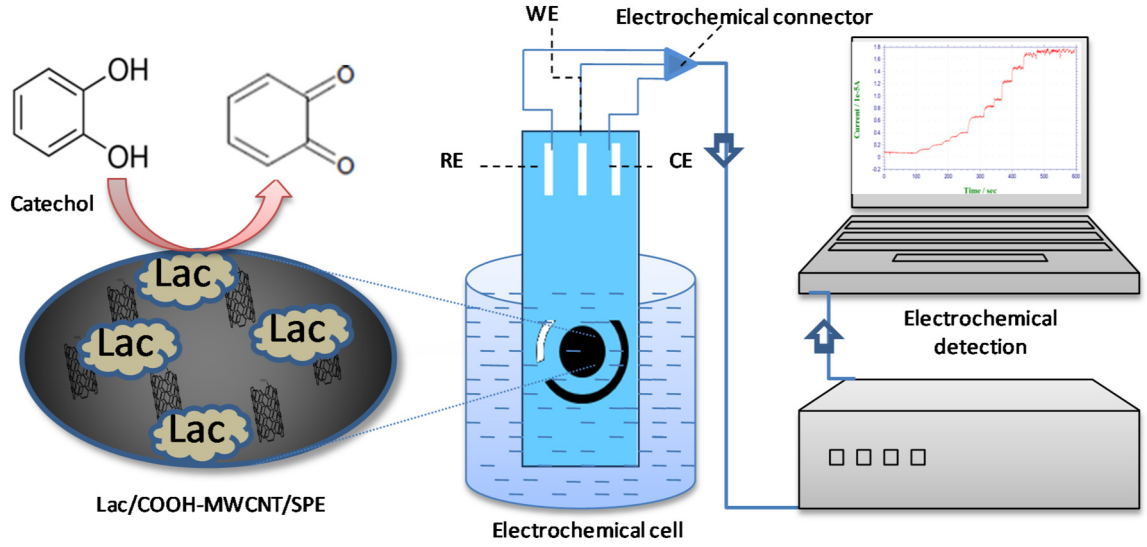


Fig. 1. Schematic illustration of the detection procedure for the determination of phenols using the disposable SPCE/MWCNT-COOH/Lac.

two covalent coupling strategies followed by trial (f) with sensitivity value of 140 nA/μM (Table 1- e, f, g).

PHEMA is a biocompatible, non-hazardous synthetic crosslinking polymer with sufficient mechanical strength for the majority of different applications in biotechnology field. An additional advantage is the hydroxyl functional groups, which can operate as sites for bioactive species attachment or for subsequent derivatization to different polymer series [56]. The K_m^{app} values obtained in this study from crosslinking using PHEMA reveals that simple mixing of the cross-linker with the laccase monolayer was better than adding two successive layers of laccase one of them before dropping laccase/crosslinker mixture, where the K_m^{app} and I_{max}/K_m^{app} ratio for *Ch*La–PHEMA/COOH–MWCNT/SPCE were 0.077 μM and 95.09 μA/μM, and for *Ch*La–PHEMA/*Ch*La/COOH–MWCNT/SPCE were 0.118 μM and 65.96 μA/μM, respectively (Table 1-h, i).

Glu is a widely used cross-linking agent, which in lower concentrations preserves surface immobilized enzyme and prevents enzyme leakage (Alarcón et al., 2010). In the present study, the amperometric current responses of *Ch*La-BSA/Glu/COOH–MWCNT/SPCE (trial j) and *Ch*La/Glu/COOH–MWCNT/SPCE (trial k) were assessed to determine the effect of Glu and BSA on laccase immobilization and hence on the developed electrodes. *Ch*La/Glu/COOH–MWCNT/SPCE (Table 1-k) showed a ten-fold increase in sensitivity (591 nA/μM) relative to *Ch*La-BSA/Glu/COOH–MWCNT/SPCE (59 nA/μM) (Table 1-j). As is seen from Table (1-k), the I_{max} value for the *Ch*La/Glu/COOH–MWCNT/SPCE biosensor (21.62 μA) was three-fold higher than for *Ch*La-BSA/Glu/COOH–MWCNT/SPCE. The obtained results indicate that the Glu cross-linking does not introduce a large diffusion resistance which consequently provides high phenolic catalytic oxidation activity for the coupled laccase.

PEGDGE is used in the development of biomaterials as a bi-functional cross-linker for hydroxyl, amine and carboxyl functional polymers. The optimal solubility of PEGDGE makes it a suitable component in enzyme immobilization to develop microelectrode biosensors. In aqueous solution, it undergoes hydrolysis and ring cleavage, resulting in hydroxyl group formation. PEGDGE binds with proteins either covalently or non-covalently. Results from application of PEGDGE as a cross-linker (Table 1-l) showed a high sensitivity (743 nA/μM) and a high catalytic activity, where K_m^{app} and I_{max} values are 0.009 μM and 19.45 μA, respectively and a considerable I_{max}/K_m^{app} ratio of 2161.1 μA/μM.

The results of the different immobilization procedures summarized in Table 1 shows that the noncovalent immobilization (electrostatic adsorption) of *Ch*La on COOH–MWCNT/SPCE is more promising compared to other coupling methods, which may be mainly due to its preservation of the laccase conformation. The most effective way for laccase immobilization onto the COOH–MWCNT/SPCE is obtained through electrostatic adsorption of chemically aminated laccase *Ch*La, which resulted in the highest I_{max}/K_m^{app} ratio (5588 μA/μM) and the highest sensitivity (831 nA/μM) of the procedures studied. EDA was applied to increase

Table 1
Effect of different laccase immobilization techniques on the bioelectrocatalytic performance of laccase-MWNTs-SPCE.

No.	Type of immobilization		Purified laccase (*Ch*L)					Aminated laccase (*Ch*La)				
			Sensitivity (nA.μM^{-1})	I_{max} (μA)	Error (μA)	K_m^{app} (μM)	I_{max} / K_m^{app} (μA.μM^{-1})	*Sensitivity* (nA.μM^{-1})	I_{max} (μA)	Error (μA)	K_m^{app} (μM)	I_{max} / K_m^{app} (μA.μM^{-1})
a	Electrostatic interaction	Adsorption	150	7.38	0.86	0.151	48.87	831	27.94	4.19	0.005	5588.0
b	Entrapment	PEI/AuNP/Lac	18	2.05	0.27	0.115	17.81	n.d.	n.d.	n.d.	n.d.	n.d.
c		Lac/PVA	297	8.66	1.45	0.003	2886.0	255	7.605	0.40	0.009	845.00
d		Lac/PVA(mix)	230	8.29	0.96	0.008	1036.5	410	18.59	1.96	0.010	1859.0
e	Chemically covalent	EDC/NHS/Lac	131	10.66	2.96	0.054	197.41	117	12.34	4.16	0.104	118.65
f	binding	EDC/NHS/Lac (mix)	n.d.	n.d.	n.d.	n.d.	n.d.	140	14.55	4.07	0.063	230.95
g		EDC/Lac (mix)	n.d.	n.d.	n.d.	n.d.	n.d.	403	13.58	0.73	0.032	424.22
h	Cross linking	Lac/PHEMA (mix)	n.d.	n.d.	n.d.	n.d.	n.d.	41	7.322	0.49	0.077	95.09
i		Lac/(PHEMA-Lac)	n.d.	n.d.	n.d.	n.d.	n.d.	75	7.783	2.44	0.118	65.96
j		(Lacc-BSA)/Glu	n.d.	n.d.	n.d.	n.d.	n.d.	59	2.91	1.13	0.043	67.67
k		Lac/Glu	n.d.	n.d.	n.d.	n.d.	n.d.	591	21.62	2.79	0.012	1801.7
l		Lac/PEGDGE	n.d.	n.d.	n.d.	n.d.	n.d.	743	19.45	3.93	0.009	2161.1

n.d.: not determined.

the number of primary amino groups on the surface of *M. thermophila* laccase that improved immobilization efficiency through activating the reaction between the generated surface amino groups and the oxidized CNT [23]. CNTs are ideal matrices for biomolecules deposition due to their unique electronic, mechanical, and thermal properties, in addition to their large surface area, high-aspect ratio, and tunable specificity for the adsorption of enzymes [57]. Interestingly, the COOH–MWCNT is a MWCNT that was previously oxidized to introduce carboxylic groups on their defect sites to ensure their efficient adsorption to the enzyme molecule. González-Domínguez et al. [55] stated that a considerable portion of COOH–MWCNT structure sticks out in a radial fashion that could endows the final composite with an increased specific surface area and improved mechanical strength. These properties were interpreted to effectively enhance electron transfer between electrodes and proteins [23,58]. In turn, the *Ch*La/COOH–MWCNT/SPCE working electrode (trial a) was used throughout this study, due to its high sensitivity and low K_m^{app} value.

3.2. pH optimum and stability

Laccases exhibit different optimum pH values based on the employed substrate and its redox potential [59]. Laccases from fungal sources are generally stable at slightly acidic pH, even though pH stability varies noticeably depending on the enzyme source [60]. Patrick et al. [61] declared that the pH value affected laccase activity toward different substrates through two contrasting effects: 1) The difference in redox potential between the laccase type 1 copper center and reducing substrate (phenolic compound), where the rate of electron transfer is preferential at an elevated pH for phenolic substrates. 2) Hydroxide anion binding to the laccase type 2/type 3 copper centers, which hinders the O_2 binding (the terminal electron acceptor), and consequently restrains the activity at high pH values due to the increase in OH^- ions [62].

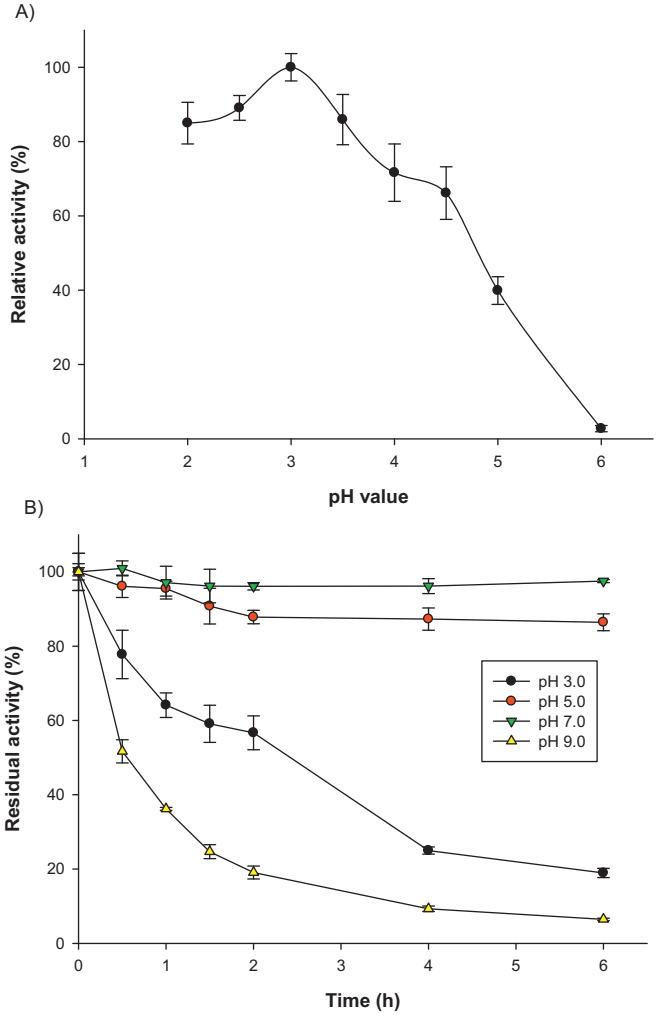


Fig. 2. (a) Optimum pH values for ABTS oxidation by *Ch*La, and (b) pH stability. Reaction mixture contained: Substrate (ABTS, 0.547 μmole); *Ch*La enzyme, 1.713 μg protein; total volume, 1 mL. Values are means of three replications ± standard deviation.

The effect of pH value on *Ch*La activity and its pH stability were investigated using free laccase preparations as a model to mimic the prepared biosensor (*Ch*La/COOH–MWCNT/SPCE), to assess the pH dependency, the enzyme stability, and the biosensor operating conditions. The effect of pH value on *Ch*La activity was investigated using ABTS as a substrate in 0.1 M Robinson buffer at different pH values. Fig. 2a shows the pH profile for *Ch*La in a homogeneous solution with a pH optimum at pH 3.0. This is in accordance with the results obtained by Schückel et al. [63] and Othman et al. [31], which showed that the optimum laccase activities from *Marasmius* sp. and *Pleurotus ostreatus* ARC280 were recorded at pH 3.0 using ABTS as substrate.

The effect of pH on *Ch*La stability was determined by incubating equal amounts of enzyme for 6 h at 30 °C in 0.1 M Robinson buffer at pH 3.0, 5.0, 7.0, and 9.0. The residual activity was measured using ABTS (0.58 mM) in 100 mM citrate buffer, pH 4.5 at room temperature (25 °C). The results showed that the *Ch*La is highly stable at neutral pH value (pH 7.0), where 97.1 and 96.2% of the initial activity were retained after 1 and 6 h, respectively. This stability test was also conducted at pH 5.0, but only 95.5 and 86.4% of the activity were retained after 1 and 6 h, respectively. The residual activity was further diminished to be 64.1, 18.6% and 36.2, 6.5% for pH 3.0 and 9.0 after 1 and 6 h, respectively (Fig. 2b). In agreement to our results, Luna et al. [64] reported that *P. ostreatus* laccase showed an increased stability at pH 7, with 42.1% of its initial activity after 15 h. In this connection, Guo et al. [65] found that, *Trametes* sp. HS-03 LacI, LacII, and LacIII isozymes at pH values higher than 7.0 lost their activity very rapidly. In contrast, the *P. ostreatus* ARC280 purified laccase was extremely stable at an alkaline range and retained 92.86 and 100.00% of its initial activity after 5 h at pH 9.0 and 10, respectively [31].

3.3. Characterization of ChLa/COOH–MWCNT/SPCE biosensor

3.3.1. Effect of successive ABTS and sodium azide injections

At constant potential (−100 mV), amperometry of the *Ch*La/COOH–MWCNT/SPCE biosensor (immobilized through electrostatic adsorption) was performed by standard additions of ABTS solution was done starting by 2.22 μM ABTS solution to a final concentration of 421.52 μM (five times 2.5 μL aliquots of 4 mM solution, followed by 2.5 μL, 10 μL, 20 μL, and 60 μL of 20 mM solution into 4.5 mL cell volume). The second step was to follow the inhibition of current response by adding sodium azide (2.22 μM - 421.52 μM) with the same concentrations sequence to the reaction cell containing the ABTS (421.52 μM) solution as described by Gupta et al. [66]. The reaction cell illustrated in Fig. 1 shows the reaction follow, where ABTS was added to 0.05 M of CPB (pH 5.0) solution present inside the cell. Gradual addition of ABTS (2.22 μM - 421.52 μM), then sodium azide was added to the mixture containing 421.52 μM ABTS.

Fig. 3 depicts the current response of the *Ch*La/COOH–MWCNT/SPCE biosensor polarized at −100 mV with subsequent additions of ABTS followed by sodium azide concentrations to the air saturated electrochemical cell and under constant stirring. The current responses obtained at *Ch*La/COOH–MWCNT/SPCE were determined against the substrate and inhibitor concentrations, respectively. The addition of sodium azide as an inhibitor under air-saturated conditions resulted in a fast decline of the current (Fig. 3). Binding of azide to the laccase trinuclear copper site caused the inhibition of laccase catalytic activity, which previously had been shown via magnetic susceptibility and spectroscopic studies [66]. In absence of oxygen, under nitrogen spurge

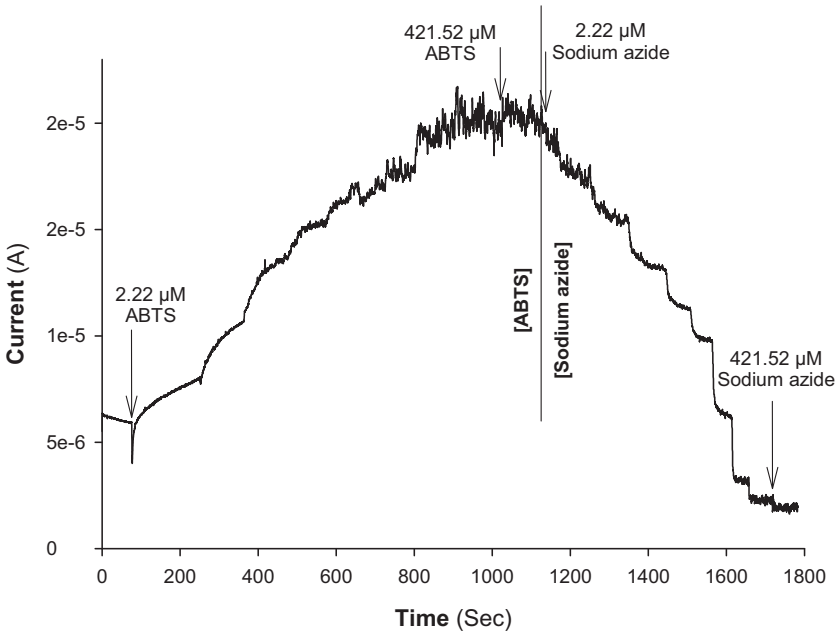


Fig. 3. Amperometric response of the *Ch*La biosensor (*Ch*La/COOH–MWCNT/SPCE) at −100 mV to additions of five times 2.5 μL of 4 mM ABTS-solution, and 2.5 μL, 10 μL, 20 μL, and 60 μL of 20 mM solution into 4.5 mL cell volume contains 0.05 M of CPB, (pH 5.0), followed by same sequence previous concentrations of sodium azide, under stirring and air saturated conditions.

condition (results not shown), the biosensor did not give catalytic current signals.

3.3.2. Substrate specificity of ChLa/COOH–MWCNT/SPCE biosensor

The laccase reaction in the biosensing process includes the oxidation of the enzyme molecule by an oxidizing agent using molecular oxygen under ambient conditions. *Re*-reduction process happens at the enzyme molecules to its native form by electrons received from the phenolic compound. After that, the phenoxy radical or quinine produced from the enzymatic reaction is re-reduced at the surface of the electrode, which results in a proportional current to the concentration of phenolic compound [67,68]. The responses from amperometric measurements with some of different phenolic compounds were determined at the optimal conditions for ABTS oxidation. The apparent Michaelis–Menten constants (K_m^{app}), maximal currents (I_{max}), and sensitivities were calculated through plotting the produced current peaks versus tested phenolic concentrations then applying the Michaelis–Menten electrochemical equation as follow:

$$I = I_{max}\,[S]/[S] + K^{app}{}_m$$

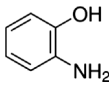
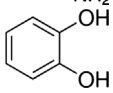
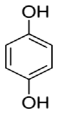
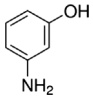
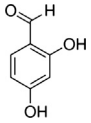
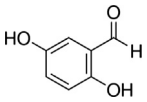
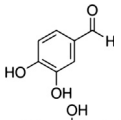
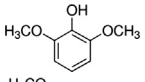
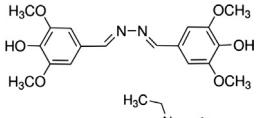
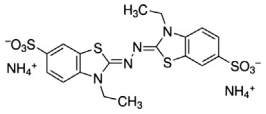
where *S* is the phenolic compound concentration, I_{max} the maximum value of current.

From the obtained results, it is clear that *Ch*La is able to oxidize a wide variety of substrates effectively with different extents of sensitivity (Table 2). The highest obtained I_{max} was 27.94 μA for ABTS. The catalytic efficiency was also high, i.e. $I_{max}/K_m^{app}$is 5588 μA.μM^{-1} with a high affinity for the employed substrate ($K_m^{app}$is 0.005 μM ABTS). Similar affinity values were obtained for syringaldazine with a I_{max}/K_m^{app} ratio of 3014 μA.μM^{-1}. The sensitivity of the developed *Ch*La/COOH–MWCNT/SPCE biosensor toward different *ortho* and *para,* and *meta* substituted phenols indicates the effect of the position of substituents. The current response order of *Ch*La/COOH–MWCNT/SPCE biosensor toward tested substrates was found to be as follows: ABTS > catechol > hydroquinone > 2,5-dihydroxybenzaldehyde > 3,4-dihydroxybenzaldehyde > 2,6-dimethoxy phenol > 3-aminophenol > syringaldazine > 2-aminophenol > 2,4-dihydroxybenzaldehyde. The presence of the –OH group in 2,5-dihydroxybenzaldehyde and 3,4-dihydroxybenzaldehyde in a *meta* position resulted in a very similar I_{max} (17.765, 17.68 μA) and K_m^{app} (0.041, 0.046 μM) values for both substrates, respectively. On the other hand, the change of the –OH group position in 2,4-dihydroxybenzaldehyde affected the I_{max} response value negatively to be 0.109 μA and I_{max}/K_m^{app} ratio of 13.9 instead of 434.19 and 386.96 μA.μM^{-1} in the case of 2,5-dihydroxybenzaldehyde and 3,4-dihydroxybenzaldehyde, respectively. The presence of two –OH groups at the *ortho* and *para* positions in catechol and hydroquinone affected the K_m^{app} values for both substrates to give 0.055 and 0.22 μM respectively, in spite of the similar I_{max} values (19.5 μA) for both substrates. Occurrence of the amino group in the *meta* position in 3-aminophenol instead of the *para* position in 2-aminophenol caused a great reduction in substrate specificity (high K_m^{app} value) to be 0.451 instead of 0.019 μM for 3-aminophenol and 2-aminophenol, respectively. 2,6-dimethoxy phenol, a well-known laccase substrate, resulted in a relatively high I_{max} value (17.655 μA) and a low K_m^{app} value (0.037 μM), that reflected its relative specificity.

In comparison to previous study by Haghighi et al., [67] using graphite electrodes modified with *T. versicolor* laccase, the response from *Ch*La/COOH–MWCNT/SPCE biosensor in our study is superior in its kinetic parameters. The previous gave I_{max} values of 10.185, 10.003, 9.314 μA for catechol, 2-aminophenol and hydroquinone respectively, whereas in this study respective I_{max} values of 19.525, 14.425 and 19,500 μA were obtained. In this regard, the K_m^{app} values from our study were lowered (for the *Ch*La/COOH–MWCNT/SPCE biosensor 55, 19 and 22 μM) with the substrates listed above than for those reported previously with graphite electrode modified with *T. versicolor* laccase (109, 31, and 33.1 μM) [67], reflecting a higher affinity between the developed laccase biosensor and tested substrates. Similarly, the *Ch*La/COOH–MWCNT/SPCE biosensor had higher affinity toward syringaldazine, catechol and 3,4-dihydroxybenzaldehyde (K_m^{app} value of 5, 55 and 46 μM) than recorded for graphite electrode modified with *Cerrena unicolor* laccase (K_m^{app} value of 280, 246 and 167 μM) [69].

With the intention to evaluate the performance of the developed biosensor, its analytical distinctiveness was contrasted to that reported for diverse amperometric biosensors functionalized in phenols detection (Table 3). As could be observed, the sensitivity of the aminated Lac/cMWCNTs/SPE developed biosensor was more than five magnitude orders superior to sensitivity value of the same purified enzyme

Table 2
Evaluation results from fitting of amperometric signals of the *Ch*La/COOH–MWCNT/SPCE biosensor to the Michaelis–Menten equation for some phenolic compounds.

Compound	Chemical structure	I_{max} (μA)	Error (μA)	K_m^{app} (μM)	I_{max} / K_m^{app} (μA. μM^{-1})	Linear dynamic range (μM)
2-Aminophenol		14.425	1.521	0.019	744.71	0.096–0.5
Catechol		19.525	0.759	0.055	357.15	0.0–0.102
Hydroquinone		19.50	1.595	0.022	877.98	0.011–0.088
3-Aminophenol		15.135	2.867	0.451	33.56	0.029–0.586
2,4-Dihydroxybenzaldehyde		0.109	0.047	0.008	13.90	n.d.
2,5-Dihydroxybenzaldehyde		17.765	1.717	0.041	434.19	0.006–0.073
3,4-Dihydroxybenzaldehyde		17.68	0.26	0.046	386.96	0.005–0.137
2,6-Dimethoxy phenol		17.655	0.712	0.037	475.30	0.006–0.068
Syringaldazine		15.12	2.046	0.005	3014.65	0.002–0.039
ABTS		27.94	4.19	0.005	5588.00	0.002–0.061

(Purified Lac/cMWCNTs/SPE) but not aminated one, indicating the effectiveness of enzyme amination on the immobilization process. The same attitude could be observed also with other cited amperometric laccase biosensors in Table 3, indicating the superiority of the developed biosensor over reported previous electrodes. This performance was attributed to coupling of carboxylated MWCNT which offer high available surface area and excellent electrical conductivity, and aminated laccase in a proper way conserving the structure of laccase and suitable for its catalytic action.

3.3.3. Response time and reusability of the developed ChLa/COOH–MWCNT/SPCE biosensor

The operational stability of the developed *Ch*La/COOH–MWCNT/SPCE biosensor through *Ch*La immobilization onto the COOH–MWCNT/SPCE via electrostatic adsorption was determined by reaction of the modified electrodes with different catechol concentrations (0.0–0.954 mM) in 4.5 mL of 50 mM CPB (pH 5.0). Ten cycles of measurements were carried out under constant stirring at room temperature (25 °C). During each cycle, aliquots of catechol stock solution

Table 3
Some of amperometric laccase biosensors in terms of immobilization type, phenol analyte, and performance characteristics.

Biosensor	Immobilization type	Analyte	Linear range	Sensitivity	Ref.
Lac/MWCNTs/CS	Entrapment	Gallic acid	0.79–2.1 μM	3.450 mA/mM	[70]
Lac/CNTs/CS/GC	Entrapment	Catechol	1.2–30 μM	0.66 μM	[71]
Lac/graphite	Adsorption	ABTS	1–10 μM	38.63 nA/μM	[72]
		DMP	0.1–2 μM	202.09 nA/μM	
		Catechol	1–20 μM	24.4 nA/μM	
		Syringaldazine	20–100 μM	2.5 nA/μM	
Lac/Fe_3O_4 NPs/cMWCNTs/PANI/Au	Covalent coupling	Phenolic content/guaiacol	0.1–500 μM	0.18 μM	[73]
Lac/CuNPs/Cs/cMWCNTs/PANI/Au	Covalent binding & adsorption	Polyphenol/guaiacol	1–500 μM	0.694 μA/μM cm^{-2}	[74]
Lac/SPE/thionine–carbon black nanocomposite	Adsorption	Bisphenol A	0.5–50 μM	5.0 ± 0.1 nA/μM	[75]
Lac/nafion/Au/SPE	Adsorption	Gallic acid	2–7 μM	1.1 nA/μM	[76]
Purified Lac/cMWCNTs/SPE	Adsorption	ABTS		150 nA.μM^{-1}	This work
Aminated Lac/cMWCNTs/SPE			0.002–0.061 μM	831 nA.μM^{-1}	

Lac, Laccase; cMWCNTs, Carboxylated multiwalled carbon nanotubes; GC, glassy carbon; NP, Nanoparticle; PANI, Polyaniline; DMP, 2,6-dimethoxyphenol; SPE, Screen-printed electrodes.

(20 mM) were added to the reaction cell at 1.0 min intervals in order to get a gradual increase in catechol concentration and the generated current was measured. At the end of each cycle, the reaction was stopped, the reaction mixture was removed, and the electrode was cleaned by the same buffer, and then was re-inserted in a fresh buffer solution to start a new cycle. Amperometric response curves were recorded for consecutive cycles of successive additions of catechol in 50 mM CPB at pH 5.0. Results obtained indicate that the *Ch*La/COOH–MWCNT/SPCE biosensor had a quick response following catechol addition as a substrate. The response time for catechol (10 μM) solution was 4.75 ± 0.76 s to reach 99.61% of the maximum current response. Results indicated that *Ch*La/COOH–MWCNT/SPCE biosensor can be reused for 10 successive sensing cycles with a little loss of its catalytic function (Fig. 4), and 99.5, 82 and 77% retention of its catalytic response was obtained after 4, 8 and 10 successive cycles of reuse, respectively.

3.3.4. Storage stability of the developed ChLa/COOH–MWCNT/SPCE biosensor

The storage stability of the *Ch*La/COOH–MWCNT/SPCE biosensor against storage time was studied at 4 °C. At specific time intervals (1, 3, 7, 10, 15, and 20 days after preparation), a set of three modified electrodes were assayed to determine the residual bio-sensing activity under the standard assay conditions. The current response of *Ch*La/COOH–MWCNT/SPCE biosensor toward ABTS oxidation was shown in Fig. 5. Results obtained indicated a high stability of the *Ch*La/COOH–MWCNT/SPCE biosensor over a period of 20 days to be near 100% of its initial response. In agreement to our results, Li et al. [17] reported that the Lac/Si/MWCNT/SPCE biosensor retained 91.0% and 86.0% of the initial response after 10 and 30 days, respectively in a solution of 60.0 μM dopamine, using *T. versicolor* laccase in biosensor construction. The resulted good stability is mainly due to the effective protection of Lac due to the biocompatible microenvironment provided by MWCNT.

4. Conclusions

A disposable *Ch*La/COOH–MWCNT/SPCE biosensor has been developed for detection of phenolic compounds by immobilizing *Ch*La on COOH–MWCNT/SPCE. This is of importance for recognition and control of the environmental pollution on the basis of quick and low cost test systems for hazardous compounds, applicable for point of interest (field) measurements in portable devices. The resulting biosensor shows a good sensitivity advantage toward different phenolic compounds, which is of interest for screening polluted water. The developed biosensor sensitivity is based on cyclic enzymatic oxidation of phenol under consumption of oxygen and reduction of product and thus is critically dependent on suppressed direct electron transfer which guaranteed by the immobilization procedure. In addition, the unique electronic, chemical and mechanical properties of the carbon nanotubes (CNTs) facilitate fast regeneration of the phenol at the electrode at favorable low potential. The proposed enzyme immobilization strategy using *Ch*La shows a stable laccase biosensor without using chemical coupling agents that affect the immobilized enzyme conformation.

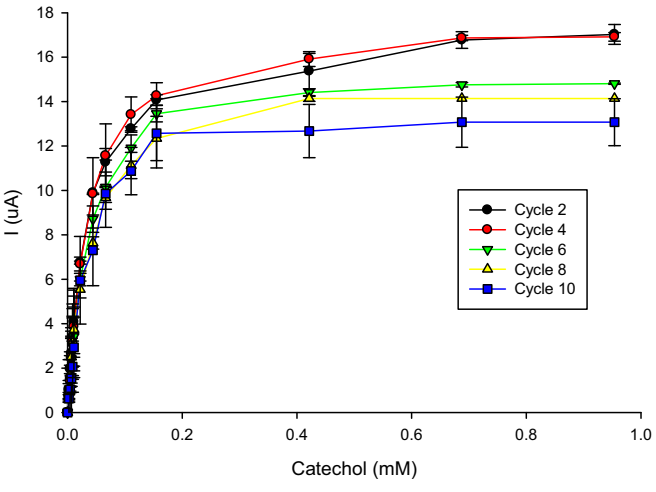


Fig. 4. Reusability of *Ch*La/COOH–MWCNT/SPCE developed biosensor. Ten consecutive oxidative cycles were performed using different catechol concentrations. Measurement conditions: 50 mM CPB (pH 5.0); applied potential, −100 mV.

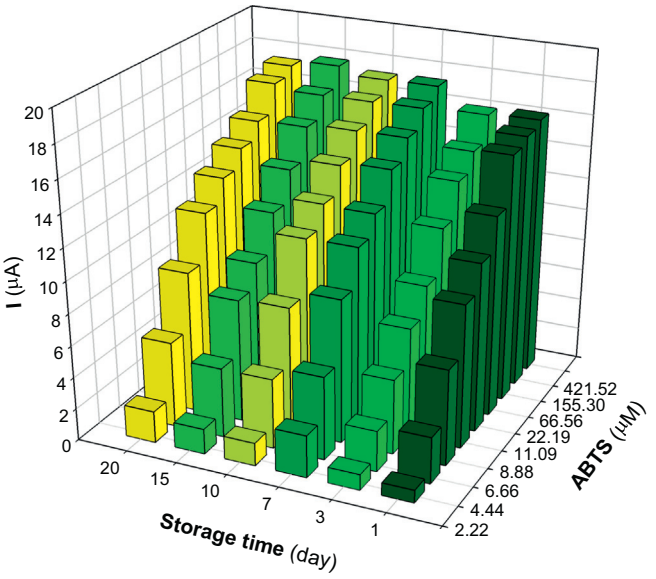


Fig. 5. Storage stability of *Ch*La/COOH–MWCNT/SPCE biosensor. ABTS was used as a model substrate with the following concentrations: 2.22, 4.44, 6.66, 8.88, 11.09, 22.19, 66.56, 155.30, and 421.52 μM, respectively. Three different electrodes were assessed and the presented data are the mean with standard deviation <5%.

CRediT authorship contribution statement

Abdelmageed M. Othman: Methodology, Investigation, Writing - original draft, Visualization, Formal analysis, Project administration, Funding acquisition.**Ulla Wollenberger:**Conceptualization, Software, Validation, Resources, Writing - review & editing, Supervision, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2020.03.049.

References

[1] H.-F. Wang, Y.-Y. Wu, X.-P. Yan, Room-temperature phosphorescent discrimination of catechol from resorcinol and hydroquinone based on sodium tripolyphosphate capped Mn-doped ZnS quantum dots, Anal. Chem. 85 (2013) 1920–1925, https://doi.org/10.1021/ac303374s.

[2] M. Becerra-Herrera, M. Sánchez-Astudillo, R. Beltrán, A. Sayago, Determination of phenolic compounds in olive oil: new method based on liquid–liquid micro extraction and ultra-high performance liquid chromatography-triple–quadrupole mass spectrometry, LWT - Food Sci. Technol. 57 (2014) 49–57, https://doi.org/10.1016/j.lwt.2014.01.016.

[3] M. Hasani, M. Mohammadi, M. Shariati-Rad, H. Abdollahi, H-point curve isolation method for determination of catechol in complex unknown mixtures, Spectrochim. Acta A Mol. Biomol. Spectrosc. 96 (2012) 563–568, https://doi.org/10.1016/j.saa.2012.07.024.

[4] P.A. Raymundo-Pereira, A.M. Campos, C.D. Mendonça, M.L. Calegaro, S.A.S. Machado, O.N. Oliveira, Printex 6L carbon nanoballs used in electrochemical sensors for simultaneous detection of emerging pollutants hydroquinone and Paracetamol, Sens. Actuators B Chem. 252 (2017) 165–174, https://doi.org/10.1016/j.snb.2017.05.121.

[5] M. Su, P. Chen, Y. Dong, H. Sun, Chemiluminescence of graphene quantum dots induced by acidic potassium permanganate and its application to quenchometric flow-injection assays of hydroquinone in water, J. Lumin. 177 (2016) 204–208, https://doi.org/10.1016/j.jlumin.2016.04.051.

[6] S. Erogul, S.Z. Bas, M. Ozmen, S. Yildiz, A new electrochemical sensor based on Fe3O4 functionalized graphene oxide-gold nanoparticle composite film for simultaneous determination of catechol and hydroquinone, Electrochim. Acta 186 (2015) 302–313, https://doi.org/10.1016/j.electacta.2015.10.174.

[7] L. Ge, S.-P. Li, G. Lisak, Advanced sensing technologies of phenolic compounds for pharmaceutical and biomedical analysis, J. Pharm. Biomed. Anal. 179 (2020), 112913. https://doi.org/10.1016/j.jpba.2019.112913.

[8] C. Salvo-Comino, C. Garcia-Hernandez, C. Garcia-Cabezon, M.L. Rodriguez-Mendez, Promoting laccase sensing activity for catechol detection using LBL assemblies of chitosan/ionic liquid/phthalocyanine as immobilization surfaces, Bioelectrochemistry 132 (2020), 107407. https://doi.org/10.1016/j.bioelechem.2019.107407.

[9] H. Zhang, A.G. Hay, Magnetic biochar derived from biosolids via hydrothermal carbonization: enzyme immobilization, immobilized-enzyme kinetics, environmental toxicity, J. Hazard. Mater. 384 (2020), 121272. https://doi.org/10.1016/j.jhazmat.2019.121272.

[10] Y. Liu, N. Song, Z. Ma, K. Zhou, Z. Gan, Y. Gao, S. Tang, C. Chen, Synthesis of a poly(N-methylthionine)/reduced graphene oxide nanocomposite for the detection of hydroquinone, Mater. Chem. Phys. 223 (2019) 548–556, https://doi.org/10.1016/j.matchemphys.2018.11.045.

[11] C. Lee, X. Wei, J.W. Kysar, J. Hone, Measurement of the elastic properties and intrinsic strength of monolayer graphene, Science 321 (2008) 385–388, https://doi.org/10.1126/science.1157996.

[12] M. Miculescu, V.K. Thakur, F. Miculescu, S.I. Voicu, Graphene-based polymer nanocomposite membranes: a review, Polym. Adv. Technol. 27 (2016) 844–859, https://doi.org/10.1002/pat.3751.

[13] Z. Cai, H. Xiong, Z. Zhu, H. Huang, L. Li, Y. Huang, X. Yu, Electrochemical synthesis of graphene/polypyrrole nanotube composites for multifunctional applications, Synth. Met. 227 (2017) 100–105, https://doi.org/10.1016/j.synthmet.2017.03.012.

[14] C. Chen, Z. Gan, K. Zhou, Z. Ma, Y. Liu, Y. Gao, Catalytic polymerization of N-methylthionine at electrochemically reduced graphene oxide electrodes, Electrochim. Acta 283 (2018) 1649–1659, https://doi.org/10.1016/j.electacta.2018.07.051.

[15] M. Verrastro, N. Cicco, F. Crispo, A. Morone, M. Dinescu, M. Dumitru, F. Favati, D. Centonze, Amperometric biosensor based on Laccase immobilized onto a screen-printed electrode by matrix assisted pulsed laser evaporation, Talanta 154 (2016) 438–445, https://doi.org/10.1016/j.talanta.2016.03.072.

[16] L.G. Mohtar, P. Aranda, G.A. Messina, M.A. Nazaren, S.V. Pereira, J. Raba, F.A. Bertolino, Amperometric biosensor based on laccase immobilized onto a nanostructured screen-printed electrode for determination of polyphenols in propolis, Microchem. J. 144 (2019) 13–18, https://doi.org/10.1016/j.microc.2018.08.038.

[17] Y. Li, L. Zhang, M. Li, Z. Pan, D. Li, A disposable biosensor based on immobilization of laccase with silica spheres on the MWCNTs-doped screen-printed electrode, Chem Cent J 6 (2012) 2–8.

[18] Z. Taleat, A. Khoshroo, M. Mazloum-Ardakani, Screen-printed electrodes for biosensing: a review (2008–2013), Microchim. Acta 181 (2014) 865–891, https://doi.org/10.1007/s00604-014-1181-1.

[19] P.J. Lamas-Ardisana, P. Queipo, P. Fanjul-Bolado, A. Costa-García, Multiwalled carbon nanotube modified screen-printed electrodes for the detection of p-aminophenol: optimisation and application in alkaline phosphatase-based assays, Anal. Chim. Acta 615 (2008) 30–38, https://doi.org/10.1016/j.aca.2008.03.034.

[20] J. Yang, D. Li, J. Fu, F. Huang, Q. Wei, TiO2-CuCNFs based laccase biosensor for enhanced electrocatalysis in hydroquinone detection, J. Electroanal. Chem. 766 (2016) 16–23, https://doi.org/10.1016/j.jelechem.2016.01.030.

[21] P. Das, M. Das, S.R. Chinnadayyala, I.M. Singha, P. Goswami, Recent advances on developing 3rd generation enzyme electrode for biosensor applications, Biosens. Bioelectron. 79 (2016) 386–397, https://doi.org/10.1016/j.bios.2015.12.055.

[22] A.A. Babadi, S. Bagheri, S.B.A. Hamid, Progress on implantable biofuel cell: nanocarbon functionalization for enzyme immobilization enhancement, Biosens. Bioelectron. 79 (2016) 850–860, https://doi.org/10.1016/j.bios.2016.01.016.

[23] A.M. Othman, E. González-Domínguez, Á. Sanromán, M. Correa-Duarte, D. Moldes, Immobilization of laccase on functionalized multiwalled carbon nanotube membranes and application for dye decolorization, RSC Adv. 6 (2016) 114690–114697, https://doi.org/10.1039/C6RA18283F.

[24] C. Tortolini, M. Di Fusco, M. Frasconi, G. Favero, F. Mazzei, Laccase–polyazetidine prepolymer–MWCNT integrated system: biochemical properties and application to analytical determinations in real samples, Microchem. J. 96 (2010) 301–307, https://doi.org/10.1016/j.microc.2010.05.004.

[25] J.P. Bebiano, in: Universidade do Porto (Ed.), Immobilization of Laccase over Carbon Nanotubes for Biocatalysis Applications, 2015 , Master thesis https://repositorio-aberto.up.pt/handle/10216/82605, Accessed date: 23 May 2016.

[26] T. Wasilewski, W. Kamysz, J. Gębicki, Bioelectronic tongue: current status and perspectives, Biosens. Bioelectron. 150 (2020), 111923. https://doi.org/10.1016/j.bios.2019.111923.

[27] M.G. Peter, U. Wollenberger, Phenol-oxidizing enzymes: Mechanisms and applications in biosensors, in: Front. Biosensorics I, Birkhäuser Basel, 1997: pp. 63–82. doi:https://doi.org/10.1007/978-3-0348-9043-4_5.

[28] M.M. Rodríguez-Delgado, G.S. Alemán-Nava, J.M. Rodríguez-Delgado, G. Dieck-Assad, S.O. Martínez-Chapa, D. Barceló, R. Parra, Laccase-based biosensors for detection of phenolic compounds, TrAC Trends Anal. Chem. 74 (2015) 21–45, https://doi.org/10.1016/j.trac.2015.05.008.

[29] U. Wollenberger, Chapter 2 Third generation biosensors—Integrating recognition and transduction in electrochemical sensors, Compr. Anal. Chem, Elsevier 2005, pp. 65–130, https://doi.org/10.1016/S0166-526X(05)44002-7.

[30] A.N. Sekretaryova, A.V. Volkov, I.V. Zozoulenko, A.P.F. Turner, M.Y. Vagin, M. Eriksson, Total phenol analysis of weakly supported water using a laccase-based microband biosensor, Anal. Chim. Acta 907 (2016) 45–53, https://doi.org/10.1016/j.aca.2015.12.006.

[31] A.M. Othman, A.M. Elshafei, M.M. Hassan, B.M. Haroun, M.A. Elsayed, A.A. Farrag, Purification, biochemical characterization and applications of Pleurotus ostreatus ARC280 Laccase, Br. Microbiol. Res. J. 4 (2014) 1418.

[32] S. Timur, N. Pazarlıoğlu, R. Pilloton, A. Telefoncu, Thick film sensors based on laccases from different sources immobilized in polyaniline matrix, Sens. Actuators B Chem. 97 (2004) 132–136, https://doi.org/10.1016/j.snb.2003.07.018.

[33] S. Chawla, R. Rawal, S. Sharma, C.S. Pundir, An amperometric biosensor based on laccase immobilized onto nickel nanoparticles/carboxylated multiwalled carbon nanotubes/polyaniline modified gold electrode for determination of phenolic content in fruit juices, Biochem. Eng. J. 68 (2012) 76–84, https://doi.org/10.1016/j.bej.2012.07.008.

[34] A.L. Ghindilis, V.P. Gavrilova, A.I. Yaropolov, Laccase-based biosensor for determination of polyphenols: determination of catechols in tea, Biosens. Bioelectron. 7 (1992) 127–131, https://doi.org/10.1016/0956-5663(92)90017-H.

[35] Md.A. Rahman, H.-B. Noh, Y. Shim, Direct electrochemistry of Laccase immobilized on au nanoparticles encapsulated-dendrimer bonded conducting polymer: application for a catechin sensor, Anal. Chem. 80 (2008) 8020–8027, https://doi.org/10.1021/ac801033s.

[36] C.G. Bauer, A. Kühn, N. Gajovic, O. Skorobogatko, P.-J. Holt, N.C. Bruce, A. Makower, C.R. Lowe, F.W. Scheller, New enzyme sensors for morphine and codeine based on morphine dehydrogenase and laccase, Fresenius J. Anal. Chem. 364 (1999) 179–183, https://doi.org/10.1007/s002160051320.

[37] O.D. Leite, O. Fatibello-Filho, A. de M. Barbosa, Determination of catecholamines in pharmaceutical formulations using a biosensor modified with a crude extract of fungi laccase (Pleurotus ostreatus), J. Braz. Chem. Soc. 14 (2003) 297–303.

[38] J. Kulys, R. Vidziunaite, Amperometric biosensors based on recombinant laccases for phenols determination, Biosens. Bioelectron. 18 (2003) 319–325, https://doi.org/10.1016/S0956-5663(02)00172-0.

[39] F. Vianello, A. Cambria, S. Ragusa, M.T. Cambria, L. Zennaro, A. Rigo, A high sensitivity amperometric biosensor using a monomolecular layer of laccase as biorecognition element, Biosens. Bioelectron. 20 (2004) 315–321, https://doi.org/10.1016/j.bios.2004.01.022.

[40] A. Amine, F. Arduini, D. Moscone, G. Palleschi, Recent advances in biosensors based on enzyme inhibition, Biosens. Bioelectron. 76 (2016) 180–194, https://doi.org/10.1016/j.bios.2015.07.010.

[41] V. Lettera, C. Pezzella, P. Cicatiello, A. Piscitelli, V.G. Giacobelli, E. Galano, A. Amoresano, G. Sannia, Efficient immobilization of a fungal laccase and its exploitation in fruit juice clarification, Food Chem. 196 (2016) 1272–1278, https://doi.org/10.1016/j.foodchem.2015.10.074.

[42] F. Lisdat, W.O. Ho, U. Wollenberger, F.W. Scheller, T. Richter, U. Bilitewski, Recycling systems based on screen-printed electrodes, Electroanalysis 10 (1998) 803–807, https://doi.org/10.1002/(SICI)1521-4109(199809)10:12<803::AID-ELAN803>3.0.CO;2-3.

[43] R.S. Freire, N. Durán, L.T. Kubota, Electrochemical biosensor-based devices for continuous phenols monitoring in environmental matrices, J. Braz. Chem. Soc. 13 (2002) 456–462, https://doi.org/10.1590/S0103-50532002000400008.

[44] A.I. Yaropolov, A.N. Kharybin, J. Emnéus, G. Marko-Varga, L. Gorton, Flow-injection analysis of phenols at a graphite electrode modified with co-immobilised laccase and tyrosinase, Anal. Chim. Acta 308 (1995) 137–144.

[45] R.C. Rodrigues, O. Barbosa, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R. Fernandez-Lafuente, Amination of enzymes to improve biocatalyst performance: coupling genetic modification and physicochemical tools, RSC Adv. 4 (2014) 38350–38374, https://doi.org/10.1039/C4RA04625K.

[46] J.P. Riehm, H.A. Scheraga, Structural studies of ribonuclease. XXI. The reaction between ribonuclease and a water-soluble carbodiimide*, Biochemistry 5 (1966) 99–115, https://doi.org/10.1021/bi00865a014.

[47] D.G. Hoare, D.E. Koshland, A method for the quantitative modification and estimation of carboxylic acid groups in proteins, J. Biol. Chem. 242 (1967) 2447–2453.

[48] A.M. Othman, M.A. Elsayed, A.M. Elshafei, M.M. Hassan, Purification and biochemical characterization of two isolated laccase isoforms from Agaricus bisporus CU13 and their potency in dye decolorization, Int. J. Biol. Macromol. 113 (2018) 1142–1148, https://doi.org/10.1016/j.ijbiomac.2018.03.043.

[49] A. Othman, M. Elsayed, A. Elshafei, M. Hassan, Application of central composite design as a strategy to maximize the productivity of Agaricus bisporus CU13 Laccase and its application in dye decolorization, Biocatal. Agric. Biotechnol. (2018) https://doi.org/10.1016/j.bcab.2018.02.008.

[50] E. Layne, Spectrophotometric and turbidimetric methods for measuring proteins, Methods Enzymol, Elsevier 1957, pp. 447–454, https://doi.org/10.1016/S0076-6879(57)03413-8.

[51] D. Brondani, B. de Souza, B.S. Souza, A. Neves, I.C. Vieira, PEI-coated gold nanoparticles decorated with laccase: a new platform for direct electrochemistry of enzymes

and biosensingapplications, Biosens. Bioelectron. 42 (2013) 242–247, https://doi.org/10.1016/j.bios.2012.10.087.
[52] G. Alarcón, M. Guix, A. Ambrosi, M.T. Ramirez Silva, M.E. Palomar Pardave, A. Merkoçi, Stable and sensitive flow-through monitoring of phenol using a carbon nanotube based screen printed biosensor, Nanotechnology 21 (2010), 245502. https://doi.org/10.1088/0957-4484/21/24/245502.
[53] G. Safina, R. Ludwig, L. Gorton, A simple and sensitive method for lactose detection based on direct electron transfer between immobilised cellobiose dehydrogenase and screen-printed carbon electrodes, Electrochim. Acta 55 (2010) 7690–7695, https://doi.org/10.1016/j.electacta.2009.10.052.
[54] M. Fernández-Fernández, M.Á. Sanromán, D. Moldes, Recent developments and applications of immobilized laccase, Biotechnol. Adv. 31 (2013) 1808–1825, https://doi.org/10.1016/j.biotechadv.2012.02.013.
[55] E. González-Domínguez, M. Comesaña-Hermo, R. Mariño-Fernández, B. Rodríguez-González, R. Arenal, V. Salgueiriño, D. Moldes, A.M. Othman, M. Pérez-Lorenzo, M.A. Correa-Duarte, Hierarchical nanoplatforms for high-performance enzyme biocatalysis under denaturing conditions, ChemCatChem 8 (2016) 1264–1268, https://doi.org/10.1002/cctc.201501401.
[56] T. Danisman, S. Tan, Y. Kacar, A. Ergene, Covalent immobilization of invertase on microporous pHEMA–GMA membrane, Food Chem. 85 (2004) 461–466, https://doi.org/10.1016/j.foodchem.2003.07.015.
[57] F. Lu, L. Gu, M.J. Meziani, X. Wang, P.G. Luo, L.M. Veca, L. Cao, Y.-P. Sun, Advances in bioapplications of carbon nanotubes, Adv. Mater. 21 (2009) 139–152, https://doi.org/10.1002/adma.200801491.
[58] R.J. Lopez, S. Babanova, Y. Ulyanova, S. Singhal, P. Atanassov, Improved interfacial electron transfer in modified bilirubin oxidase biocathodes, ChemElectroChem 1 (2014) 241–248, https://doi.org/10.1002/celc.201300085.
[59] D.M. Soden, J. O'Callaghan, A.D.W. Dobson, Molecular cloning of a laccase isozyme gene from Pleurotus sajor-caju and expression in the heterologous Pichia pastoris host, Microbiol. Read. Engl. 148 (2002) 4003–4014, https://doi.org/10.1099/00221287-148-12-4003.
[60] P. Baldrian, Fungal laccases - occurrence and properties, FEMS Microbiol. Rev. 30 (2006) 215–242, https://doi.org/10.1111/j.1574-4976.2005.00010.x.
[61] F. Patrick, G. Mtui, A.M. Mshandete, G. Johansson, A. Kivaisi, Purification and characterization of a laccase from the basidiomycete Funalia trogii (Berk.) isolated in Tanzania, Afr. J. Biochem. Res. 3 (2009) 250–258.
[62] C. Muñoz, F. Guillén, A.T. Martínez, M.J. Martínez, Laccase isoenzymes of Pleurotus eryngii: characterization, catalytic properties, and participation in activation of molecular oxygen and Mn2+ oxidation, Appl. Environ. Microbiol. 63 (1997) 2166–2174.
[63] J. Schückel, A. Matura, K.-H. van Pée, One-copper laccase-related enzyme from Marasmius sp.: purification, characterization and bleaching of textile dyes, Enzym. Microb. Technol. 48 (2011) 278–284, https://doi.org/10.1016/j.enzmictec.2010.12.002.
[64] L. Luna, M. Marcos, G. Mendoza, C. Montalvo, O. Loera, H. Losada, J. Vargas, J. Cortes, Effect and enzyme activity of Pleurotus ostreatus on cell wall of barley straw, Livest. Res. Rural. Dev. (2013) 25.
[65] W. Guo, Z. Yao, C. Zhou, D. Li, H. Chen, Q. Shao, Z. Li, H. Feng, Purification and characterization of three laccase isozymes from the white rot fungus Trametes sp. HS-03, Afr. J. Biotechnol. 11 (2012) 7916–7922.
[66] G. Gupta, V. Rajendran, P. Atanassov, Laccase biosensor on monolayer-modified gold electrode, Electroanalysis 15 (2003) 1577–1583, https://doi.org/10.1002/elan.200302724.
[67] B. Haghighi, L. Gorton, T. Ruzgas, L.J. Jönsson, Characterization of graphite electrodes modified with laccase from Trametes versicolor and their use for bioelectrochemical monitoring of phenolic compounds in flow injection analysis, Anal. Chim. Acta 487 (2003) 3–14, https://doi.org/10.1016/S0003-2670(03)00077-1.
[68] F. Campanhã Vicentini, L.L.C. Garcia, L.C.S. Figueiredo-Filho, B.C. Janegitz, O. Fatibello-Filho, A biosensor based on gold nanoparticles, dihexadecylphosphate, and tyrosinase for the determination of catechol in natural water, Enzym. Microb. Technol. 84 (2016) 17–23, https://doi.org/10.1016/j.enzmictec.2015.12.004.
[69] A. Jaroszwilkolazka, T. Ruzgas, L. Gorton, Amperometric detection of mono- and diphenols at laccase-modified graphite electrode: correlation between sensitivity and substrate structure, Talanta 66 (2005) 1219–1224, https://doi.org/10.1016/j.talanta.2005.01.026.
[70] M. Diaconu, S.C. Litescu, G.L. Radu, Laccase–MWCNT–chitosan biosensor—a new tool for total polyphenolic content evaluation from in vitro cultivated plants, Sens. Actuators B Chem. 145 (2010) 800–806, https://doi.org/10.1016/j.snb.2010.01.064.
[71] Y. Liu, X. Qu, H. Guo, H. Chen, B. Liu, S. Dong, Facile preparation of amperometric laccase biosensor with multifunction based on the matrix of carbon nanotubes–chitosan composite, Biosens. Bioelectron. 21 (2006) 2195–2201, https://doi.org/10.1016/j.bios.2005.11.014.
[72] A. Jarosz-Wilkołazka, T. Ruzgas, L. Gorton, Amperometric detection of mono- and diphenols at Cerrena unicolor laccase-modified graphite electrode: correlation between sensitivity and substrate structure, Talanta 66 (2005) 1219–1224, https://doi.org/10.1016/j.talanta.2005.01.026.
[73] S. Chawla, R. Rawal, S. Sharma, C.S. Pundir, An amperometric biosensor based on laccase immobilized onto nickel nanoparticles/carboxylated multiwalled carbon nanotubes/polyaniline modified gold electrode for determination of phenolic content in fruit juices, Biochem. Eng. J. 68 (2012) 76–84, https://doi.org/10.1016/j.bej.2012.07.008.
[74] S. Chawla, R. Rawal, C.S. Pundir, Fabrication of polyphenol biosensor based on laccase immobilized on copper nanoparticles/chitosan/multiwalled carbon nanotubes/polyaniline-modified gold electrode, J. Biotechnol. 156 (2011) 39–45, https://doi.org/10.1016/j.jbiotec.2011.08.008.
[75] M. Portaccio, D. Di Tuoro, F. Arduini, D. Moscone, M. Cammarota, D.G. Mita, M. Lepore, Laccase biosensor based on screen-printed electrode modified with thionine–carbon black nanocomposite, for bisphenol A detection, Electrochim. Acta 109 (2013) 340–347, https://doi.org/10.1016/j.electacta.2013.07.129.
[76] S.C. Litescu, S.A.V. Eremia, A. Bertoli, L. Pistelli, G.-L. Radu, Laccase-Nafion based biosensor for the determination of polyphenolic secondary metabolites, Anal. Lett. 43 (2010) 1089–1099, https://doi.org/10.1080/00032710903518518.