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OPTIMIZATION OF NEWLY FABRICATED LACCASE BIOSENSOR BASED ON SINGLE-WALLED CARBON NANOTUBE FOR TYRAMINE DETECTION

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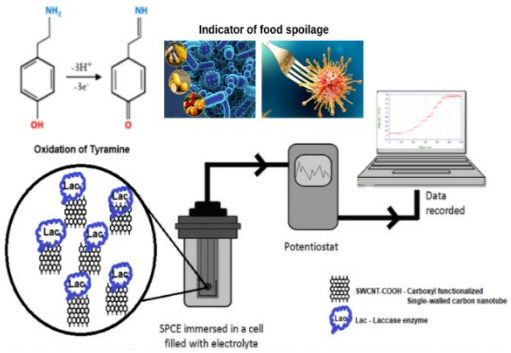
Graphical abstract



Abstract

Tyramine (TYR), also identified as 4-(2-aminoethyl) phenol, is an organic compound. Its elevated presence indicates prolonged food storage, leading to spoilage and potentially impacting human well-being. To address this concern, a biosensor was designed on a single-walled carbon nanotube carboxyl-functionalized screen-printed carbon electrode (COOH-SWCNT-SPCE). Laccase (LAC)-based electrochemical biosensors were effectively created using a simple and innovative technique involving enzyme LAC being drop cast. Electrochemical Impedance Spectroscopy (EIS), Cyclic Voltammetry (CV), and Differential Pulse Voltammetry (DPV) were utilized to assess the properties and electrochemical behavior of the modified SPCEs. Under optimal experimental conditions, the LAC/COOH-SWCNT-SPCE biosensor exhibited favorable performance at scan rates of 50 mV s^{-1} (within the range of 10 to 500 mV s^{-1}), pH 8.0 (ranging from 7.0 to 10.0), 4 µL enzyme LAC (varying from 2 to 10 µL), and 1.0 mg mL^{-1} SWCNTs (ranging from 0.2 to 3.0 mg mL^{-1}). Deposition potential and time were set at 0.5 V. The modified SPCEs demonstrated effective usage for TYR measurement, achieving a Correlation Coefficient (R^2) of 0.981 and a Limit of Detection (LOD) of 0.070 mM.

Keywords: Laccase, screen-printed carbon electrode, single-walled carbon nanotube, carboxyl functionalized, tyramine

Abstrak

Tyramine (TYR), juga dikenali sebagai 4-(2-aminoethyl) phenol, merupakan sebatian organik. Kehadirannya yang meningkat adalah petunjuk penyimpanan makanan yang berpanjangan menyebabkan kerosakan makanan, berpotensi memberi impak kepada kesihatan manusia. Bagi mengatasi kebimbangan ini, satu biosensor telah dibangunkan menggunakan elektrod karbon cetakan skrin yang telah difungsikan dengan karboksil pada tiub nanokarbon dinding tunggal (COOH-SWCNT-SPCE). Biosensor elektrokimia berasaskan Laccase (LAC) telah berjaya dibangunkan melalui teknik yang mudah dan inovatif dengan menggunakan enzim LAC yang dititiskan. Spektroskopi impedans elektrokimia (EIS), voltammetri kitaran (CV), dan voltammetri denyutan beza (DPV) telah digunakan untuk menilai sifat-sifat dan tingkah laku elektrokimia SPCE yang telah dimodifikasi. Dalam keadaan eksperimen yang optimum, biosensor LAC/COOH-SWCNT-SPCE menunjukkan prestasi yang baik pada kadar imbasan 50 mV s^{-1} (dalam julat 10 hingga 500 mV s^{-1}), pH 8.0 (dalam julat 7.0 hingga 10.0), enzim LAC 4 µL (dalam julat 2 hingga 10 µL), dan

SWCNT 1.0 mg mL^{-1} (dalam julat 0.2 hingga 3.0 mg mL^{-1}). Potensi dan masa pemendapan ditetapkan pada 0.5 V. SPCE yang telah dimodifikasi menunjukkan penggunaan yang berkesan untuk pengukuran TYR, mencapai pekali korelasi (R^2) sebanyak 0.981 dan had pengesanan (LOD) sebanyak 0.070 mM.

Kata kunci: Laccase, elektrod karbon bercetak skrin, nanotube karbon berdinding tunggal, karboksil berfungsi, tyramine

1.0 INTRODUCTION

Tyramine (TYR) is classified as a Biogenic Amine (BA), an organic compound present in plants, animals, and foods, especially those that have undergone fermentation. Elevated BA levels are indicative of food spoiled due to prolonged storage. This trait and its influence on psychotropic and vasoactive properties raise concerns for individuals utilizing monoamine oxidase inhibitors like pain relievers, stress and depression medications, and treatments for Parkinson's disease [23, 19]. As a result, TYR significantly contributes to assessing food safety and human well-being through diverse methodologies, encompassing High-Performance Liquid Chromatography (HPLC) [4, 19], capillary electrophoresis [4, 12], and electrochemistry [17, 30]. Predominant techniques for phenolic compound determination, including TYR, encompass spectrophotometric and chromatographic methodologies. However, these approaches possess limitations, such as the demand for skilled labor, lengthy procedures, and costly equipment and reagents.

Recently, the focus has shifted to bioanalytical tools, notably biosensors, offering benefits over traditional techniques, marked by their capacity for precise selection and detection, heightened sensitivity, swift assay times, and cost-effectiveness [11]. Enzymes hold a pivotal role as adaptable, efficient, and specialized catalysts within mild chemical processes [6]. Research within the biosensor field has concentrated on identifying phenolic compounds, employing enzymes like tyrosinase (tyro) [3, 28] and horseradish peroxidase [7]. Reza *et al*. engineered a biosensor utilizing tyro on an reduced graphene oxide/chitosan (rGO/Chit) platform [27] for electrochemically detecting bisphenol A. The reduced graphene oxide (GO)/ gold nanoparticles (AuNPs) film has also been evaluated for tyro immobilization to detect phenol [20]. Investigating phenolic derivatives, an electrochemical biosensor was created employing graphene-polyaniline alongside a tyro-modified GCE [10]. Notwithstanding, these enzymes carry specific limitations. Laccase (LAC) or polyphenol oxidase (EC 1.10.3.2) is a blue-copper oxidoreductase that drives the oxidation of phenolic substances while simultaneously converting molecular oxygen into water [22]. Moreover, due to its sole co-substrate reliance on molecular oxygen and its sole byproduct being water, the enzyme garners recognition for its environmental friendliness. Furthermore, enzyme conjugation with carbon nanotubes can elevate enzyme activity and stability, as evidenced in this study employing carboxyl-functionalized single-walled carbon nanotubes (COOH-SWCNT).

Nanomaterials like SWCNTs exhibit exceptional qualities like flexibility, electrical conductivity, thermal stability, and chemical robustness, making them promising for electrode applications. Further introduction of SWCNT with carboxyl groups (-COOH) via acid treatment can effectively mitigate aggregation concerns of dispersibility in solutions [13]. The primary focus of this study's biosensor optimization revolves around the LAC enzyme. The interaction between immobilized LAC and the coupling reaction between 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS) were investigated, which was influenced by the detection of TYR through Cyclic Voltammetry (CV) and Differential Pulse Voltammetry (DPV). To improve the analytical capabilities of the proposed biosensor, the Screen-Printed Carbon Electrode (SPCE) was modified by introducing COOH-SWCNTs via an acid treatment method.

2.0 METHODOLOGY

2.1 Apparatus

TYR, potassium hexacyanoferrate (III) reagent plus®, LAC sourced from Trametes versicolor (EC 1.10.3.2) at a concentration of ≥0.5 unit mg^{-1} and Single-Walled Carbon Nanotubes (SWCNTs) (98% carbon basis) were procured from Sigma Aldrich based in St. Louis, MO, USA. Potassium chloride (KCl) was supplied by Friedmann Schmidt, while 1-ethyl-3-(3-dimethylaminopropyl) EDC was offered by G-Bioscience, USA. All the compounds used in this study are of analytical grade and have not undergone further purification. A Milli-Q system, located in Bedford, MA, USA, was employed to purify water to prepare aqueous solutions.

2.2 Chemicals and Instrumentations

A Mettler Toledo weight balance and pH meter were used to measure pH, with calibration performed using pH 4, 7, and 10 buffers. The precipitate was separated

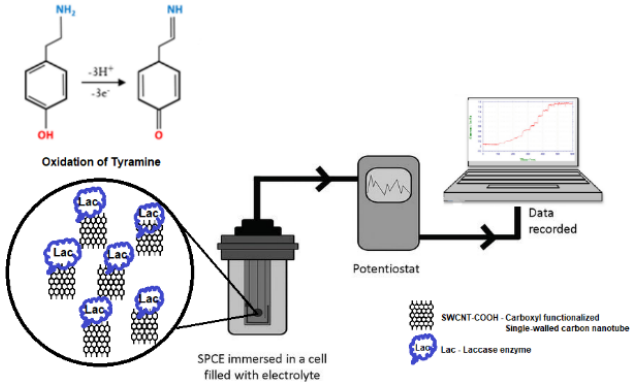
using Beckman Allegra 64R ultracentrifuges, and solution ultrasonic treatment utilized an Elmasonic EASY 120 H Elma Ultrasonic device. For electrochemical measurements, NOVA 2.1.4 software was employed for data analysis alongside a potentiostat from Ecochemie, Utrecht, The Netherlands.

2.3 Electrodes and Electrochemical Cell

The electrochemical experiments utilized an SPCE provided by Biogenes Technology Sdn Bhd (Bangi, Malaysia). This SPCE featured a 5 mm diameter carbon electrode, along with carbon counter electrodes and silver chloride (AgCl). Characterization of the biosensor using CV as an electrochemical technique, with a scan rate of 0.05 V s^{-1}, ranging from -0.6 to 1.0 V, and DPV spanning from 0.0 to 1.0 V. DPV employed a scan rate of 0.05 V s^{-1} and a potential of 0.5 V. These parameters remained consistent throughout the study unless otherwise specified. An Electrochemical Impedance Spectroscopy (EIS) solution containing the ferricyanide and Ferrocyanide Complex $(Fe(CN)_6)^{3-/4-}$ was employed to conduct the test measurement. A Phosphate Buffer (PB) solution with a pH of 7.0 was employed in voltammetry measurements and experiment optimization.

2.4 Biosensor Fabrication

In general, the surface of the pre-treated SPCE was coated by drop-casting a solution containing 0.5 mg mL^{-1} of COOH-SWCNT, followed by air-drying for 30 minutes at room temperature. Next, a solution containing 10 µL of 0.5 M EDC/NHS was applied to the dried COOH-SWCNT-SPCE to activate the COOH groups. Subsequently, 4 µL of LAC enzyme (400 mg mL^{-1}) was introduced onto the prepared COOH-SWCNT-SPCE surface. To eliminate loosely bound or unattached enzymes, the electrode was rinsed with PB solution. Three separate analyses were conducted for each fabrication layer. The process for detecting TYR using the LAC/COOH-SWCNT-SPCE is illustrated in Scheme 1.



Scheme 1 Schematic illustration of the detection procedure for the determination of TYR using the LAC/COOH-SWNCT/SPCE

2.5 Optimization Procedure

To optimize the modified electrode, the experiment was conducted to investigate the influence of several factors. These factors included the pH of the electrolyte (ranging from pH 7.0 to 10.0), the concentration of COOH-SWCNT (varying from 0.2 to 3.0 mg mL^{-1}), and the quantity of the enzyme (ranging from 2 to 10 µL). Our optimization process followed a One-Factor-At-a-Time (OFAT) approach, where all the other parameters were kept constant while varying only one parameter at a time. The measurement was repeated three times to consider error bars in the analysis.

To assess the electrochemical response of the biosensor, the DPV was employed at a potential of 0.50 V. This analysis was performed in a PB solution (50 mM PB solution) containing TYR at a concentration of 0.05 M. The resulting peak current (I_p) was measured.

For the immobilization of the enzyme, a stock solution of LAC at a concentration of 2 mg mL^{-1} was prepared. Consequently, this stock solution was diluted to 400 mg mL^{-1}. The six different LAC enzyme concentrations (≥ 0.5 unit mg^{-1}), namely 2, 4, 6, 8, and 10 µL were used in this experiment. After applying EDC/NHS, the modified electrode was promptly immobilized by the LAC enzyme onto COOH-SWCNT-SPCE.

3.0 RESULTS AND DISCUSSION

3.1 Cyclic Voltammetry Measurements

The CV experiments were performed to evaluate the redox response against electrolyte of the three levels of electrode modification: unmodified SPCE (a) the bare SPCE, SPCE fabricated with SWCNT-COOH (b) SWCNT-COOH/SPCE, and SPCE fabricated with LAC immobilized on COOH-SWCNT (c) LAC-COOH-SWCNT-SPCE. These electrodes were immersed in electrolyte 0.05 M $(Fe(CN)_6)^{3-/4-}$ with 100 mM KCl, as illustrated in Figure 1 (a). The anodic peak current (I_{pa}) exhibited an increment from 52.67 µA for bare SPCE to 120.54 µA and 143.32 µA for COOH-SWCNT-SPCE and LAC/COOH-SWCNT-SPCE, respectively. This increase in I_p indicates the successful fabrication and development of the electrode, enabling electron transfer between the electrode and the redox probe. The E_{pa}/E_{pc} values were 0.37V/-0.24V, 0.27V/-0.35V, and 0.29V/-0.31V for bare SPCE, COOH-SWCNT-SPCE, and LAC/COOH-SWCNT-SPCE, respectively.

Peak separation assists in determining the number of transported electrons following Nernstian behavior. The electroactive area (A) of the SPCE was calculated using the Randles-Sevcik equation (formula 1). Here, I_a represents the I_{pa}, A signifies the electroactive area, C stands for the concentration of electrolyte solution in mol cm^{-3}, D denotes the diffusion coefficient (7.6×10^{-6} cm^2 s^{-1}), n indicates the number of electrons in the redox. Meanwhile, v symbolizes the volts per second [8].

$$I_{pa} = (2.69 \times 10^5)\, n^{3/2} A C D^{1/2} v^{1/2}. \quad (1)$$

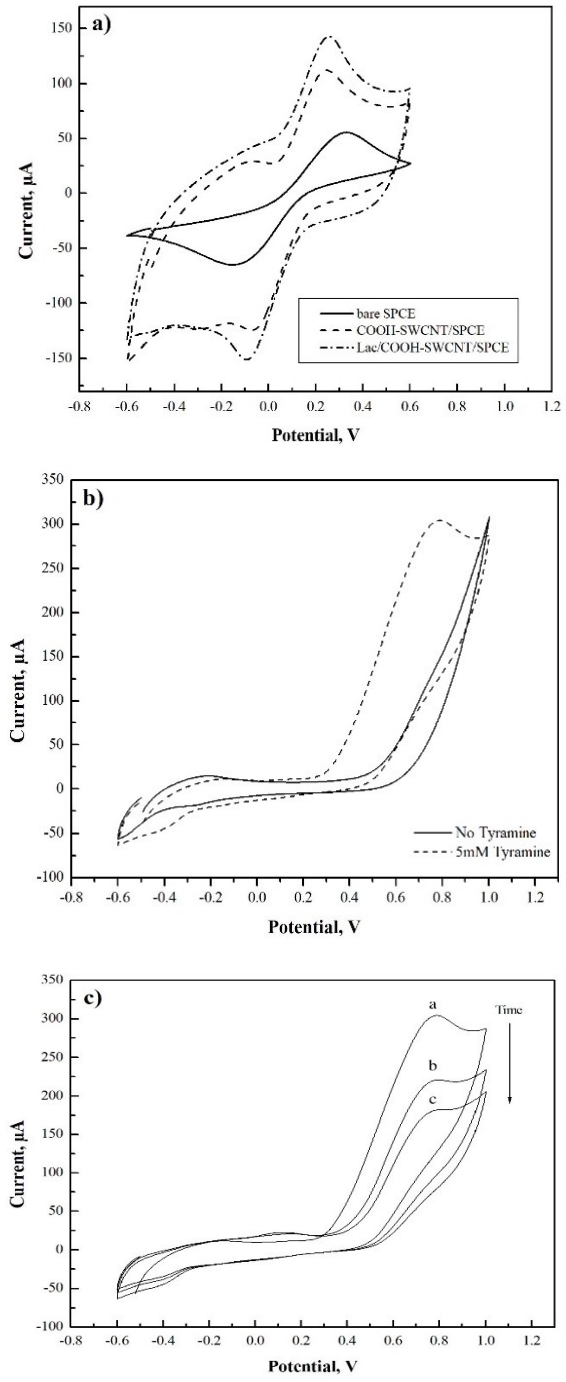


Figure 1 (a) Cyclic voltammetry for bare SPCE (curve 1), COOH-SWCNT-SPCE (curve 2) and LAC/COOH-SWCNT-SPCE (curve 3) in 0.05 M $(Fe(CN)_6)^{3-/4-}$ with 100 mM KCl (b) Cyclic voltammetry for LAC/COOH-SWCNT-SPCE with absence of TYR (curve 1) and in the presences (curve 2) and (c) Cyclic voltammetry for LAC/COOH-SWCNT-SPCE with TYR present in 0.05 M PB solution of pH 8.0 at a scan rate of 100 mV s^{-1} for three consecutive cycles

Incorporating COOH-SWCNT and LAC onto the modified electrode yielded elevated electron transfer rates and expanded active surface area on the LAC/COOH-SWCNT-SPCE electrode [5], signifying the successful modification at each electrode layer. Figure 1(b) depicts the CVs of LAC/COOH-SWCNT-SPCE in the absence and presence of TYR. CVs were obtained from -0.6 to 1.0 V with a scan rate of 50 mV s^{-1} for TYR analysis in a 0.05 M PB solution at pH 8.0. Meanwhile, in the presence of TYR, LAC/COOH-SWCNT-SPCE exhibited a higher surface area compared to the absence of TYR due to the oxidation peak of TYR's hydroxyl group to a carbonyl group, occurring at 0.67 V with Ip = 311 µA. In contrast, the solid line lacked peaks as TYR was absent in the buffer solution, failing to interact with the electrode surface, resulting in no observed current. The successful fabrication of SPCE electrode modification via COOH-SWCNT and LAC is indicated by the absence of oxidation peaks for the bare SPCE. Previously, a similar peak potential (E_p) for TYR oxidation was also discovered [9].

Figure 1(c) displays that the CV was scanned three times to investigate the stability of LAC/COOH-SWCNT-SPCE. The stability of the electrode was demonstrated by the anodic E_p, which was at 0.67 V for all peaks. I_p decreased for all adjusted electrodes from curve a to curve b, dropping from 319 I_p (curve a), 225 I_p (curve b), and 176 I_p (curve c), with percentage reductions of 41% and 27% from a to b and from b to c, respectively. After three scans, the results reveal the decreased I_p, which indicates the lower availability of the enzyme LAC to react with TYR in the solution after numerous scans and is most likely related to the bulky surface of the TYR electrode.

3.2 EIS Measurement

EIS serves as the electrochemical method employed to analyze the associations between voltage and current within an electrochemical cell resistant to alternating current. Impedance measurements yield two forms of impedance: the actual part (Z') and the imaginary segment (Z''). These aspects allow the study of the capacitance and resistance elements present in the electrochemical system [16]. Thus, the impedance behaviors were also investigated during the fabrication process in this experiment, as illustrated in Figure 2. Figure 2 portrays the Nyquist plot evaluation of (a) bare SPCE, (b) COOH-SWCNT-SPCE, and (c) LAC/COOH-SWCNT-SPCE in a 0.05 M $(Fe(CN)_6)^{3-/4-}$ solution with 100 mM KCl.

Figure 2 (curve a) displays the bare SPCE with $R_{ct} \approx$ 4316, revealing a well-defined semicircle, which is attributed to the good electron transfer capacity of the bare SPCE before modification with nanomaterial and enzymes. However, a large semicircle diameter increase was observed, demonstrating the presence of COOH-SWCNT on SPCE. Figure 2 (curve b) illustrates a large semicircle, indicating a very high electron transfer resistance for COOH-SWCNT-SPCE. The subsequent formation of SWCNT film results in a significant decrease in R_{ct}, implying that high electrical conductivity SWCNTs could accelerate the electron transfer of the electrochemical probe [26].

Figure 2 (curve c) LAC-COOH-SWCNT-SPCE exhibits the second greater interfacial R_{ct} after (b) COOH-SWCNT-SPCE. This is related to the blocking behavior of the self-assembled layer of LAC conjugated with SWCNT on the surface of SPCE, represented by the increasing semicircle. On the electrode surface, a layer of LAC forms, posing charge transfer resistance and reducing current flow through the redox active species. In addition, after modification with the LAC enzyme, the immobilization coupling method, EDC/NHS, demonstrates a high R_{ct}. EDC/NHS activation typically increases the R_{ct}, according to Guler *et al*., in comparison to a bare electrode, as the EDC/NHC are unable to facilitate electron transfer [14]. It is also proven that the modification was successful, and when the EDC/NHS system is attached, the R_{ct} should typically increase.

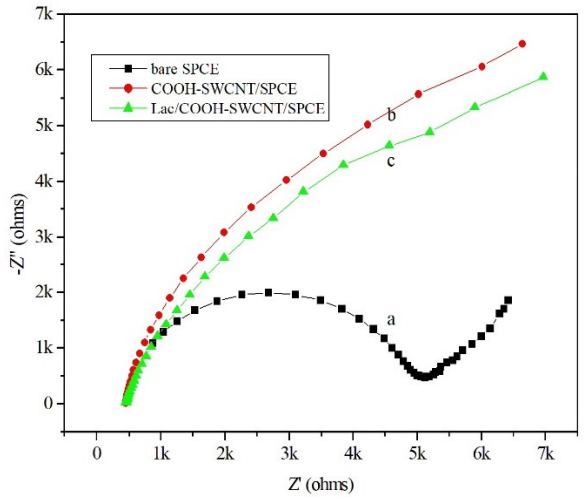


Figure 2 EIS of (curve a) bare SPCE, (curve b) COOH-SWCNT-SPCE, and (curve c) LAC/COOH-SWCNT-SPCE (0.05 M $(Fe(CN)6)^{3-/4-}$ solution containing 100 mM KCl

3.3 Optimization of Analytical Condition

The pH effect was investigated extensively in this study, covering a broad pH range spanning from 3.0 to 11.0. However, LAC's inactivation under highly acidic and alkaline conditions showed no peak at pH below 7.0 and beyond 9.0. Figure 3(a) illustrates the impact of pH within the range of pH 7.0 to 9.0 on the detection of TYR. The activity of LAC increased up to pH 8 and subsequently declined towards pH 9.0. The decreased biosensor response could be attributed to limited biocatalytic activity, leading to enzyme denaturation [25]. Consequently, pH 8.0 was chosen as the optimum pH for all electrochemical TYR analyses. Correspondingly, positive outcomes were observed for LAC from Cerrena unicolor, indicating an optimal pH of 8.0 [1]. LAC's activity was significantly hindered during immobilization in acidic conditions compared to neutral or alkaline conditions. Given the observation of minimal changes in enzyme activity at pH levels exceeding 7.0, it can be deduced that the immobilized LAC remains steady within neutral and alkaline pH ranges. The presence of protons in the environment at exceptionally low pH levels might modify the ionization status of particular amino acids. This, in turn, impacts the structure and operation of both unrestrained and immobilized enzymes [29].

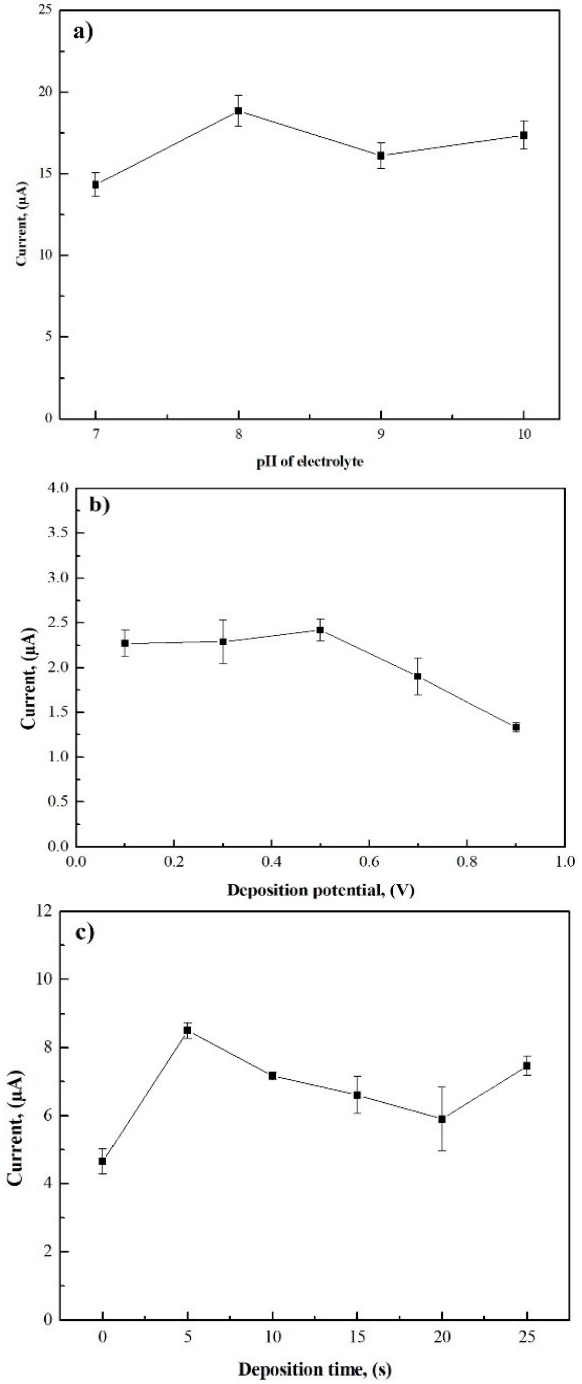


Figure 3 (a) The impact of pH level, (b) the applied potential during deposition, and (c) the duration of deposition at 50 on the LAC/COOH-SWCNT-SPCE response were investigated using DPV in a 0.05 M PB solution containing 0.05 M TYR at +0.05 V

Optimizing the deposition potential and deposition time is crucial in minimizing factors that can lead to inaccuracies in analytical results. The range of deposition potentials was explored from 0.1 V to 0.9 V, and observed noteworthy trends. Specifically, when the deposition potential reached 0.5 V, an initial increase in peak height was observed, followed by a gradual decrease from 0.5 V to 0.9 V. Interestingly, the highest I_p, indicating the most effective oxidation of the hydroxyl group (-OH) in TYR, occurred at a deposition potential of 0.5 V. Beyond this point, as the deposition potential exceeded 0.5 V, the current progressively decreased due to less efficient deposition of the hydroxyl group in TYR. Conversely, at reduction potentials below 0.5 V, the oxidation of the hydroxyl group demonstrated reduced efficiency, likely due to competition with hydrogen production, a common phenomenon at lower potentials [15]. Remarkably, the method demonstrated its optimal performance at a potential of +0.5 V, attributed to the effective oxidation of TYR. This phenomenon is illustrated in Figure 3(b).

Figure 3(c) depicts the optimization of the deposition time. The effect of the quantity of TYR deposition on the electrode on the Limit of Detection (LOD) and the overall time required for the DPV process is known as the deposition time. At the optimal deposition potential of 0.5 V, the I_p of anodic TYR stripping increased from 4.8 to 8.5 µA and reduced from 7.2, 6.8, and 6.0 µA (Figure 3 (b)). The I_p increased with deposition time initially; however, it declined significantly after 10 seconds, most likely owing to TYR saturation on the electrode surface. As a result, 5 seconds was chosen as the optimum deposition time for detecting TYR with excellent selectivity.

The effect of COOH-SWCNT concentration on the modified electrode is displayed in Figure 4(a). This optimization is significant, as it identifies an appropriate concentration of COOH-SWCNT on the modified electrode, with rising performance related to the increase of the surface area of the electrode in the presence of SWCNT. This allows the increase of enzyme immobilization and redox reaction capacity. When enzymes are added in the following step of fabrication, the high surface area of the electrode allows for increased immobilization of bioreceptor units compared to the bare SPCE [31]. The graph demonstrates an increasing pattern starting at 0.2 mg mL^{-1} and peaking at 1.0 mg mL^{-1}, then gradually decreasing to the lowest COOH-SWCNT concentration of 2.0 mg mL^{-1}. However, as the concentration of COOH-SWCNT increased, the biosensor response decreased. This is due to the nanocomposite thickness, preventing electric current from flowing across the electrode's surface [21]. As a result, the optimum concentration of COOH-SWCNT for biosensor construction of the COOH-SWCNT layer on SPCE was 1.0 mg mL^{-1}.

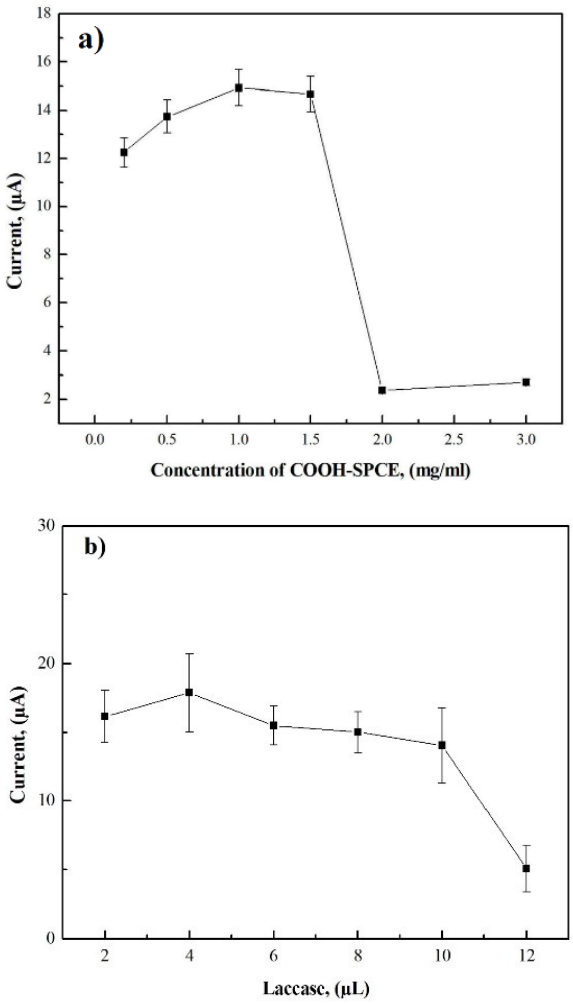


Figure 4 (a) The impact of COOH-SWCNT concentration and (b) the quantity of LAC on the LAC/COOH-SWCNT-SPCE response was assessed in a 50 mM PB solution containing 0.05 M TYR at +0.05 V

In Figure 4(b), the effect of increasing enzyme concentrations was investigated, ranging from 2, 4, and 6 µL up to a maximum of 12 µL. However, a slight reduction in biosensor response was observed at the 6 µL LAC concentration. This decline could be attributed to limited mass transfer due to an excessively high protein immobilization on the electrode [24]. Consequently, the optimal parameter for biosensor modification was an enzyme concentration of 4 µL.

The LAC/COOH-SWCNT-SPCE biosensor exhibited excellent performance under various conditions, including a scan rate of 50 mV s^{-1} (ranging from 10 to 500 mV s^{-1}), pH 8.0 (ranging from 7.0 to 10.0), 4 µL enzyme LAC (ranging from 2 to 10 µL), 1.0 mg mL^{-1} SWCNTs (ranging from 0.2 to 3.0 mg mL^{-1}), and a deposition potential and time of 0.5 V and 25 seconds, respectively.

The standard addition method in DPV techniques to confirm the E_p of TYR on the LAC/COOH-SWCNT-SPCE biosensor was employed. Figure 5 illustrates the gradually introduced TYR into a PB solution, ranging from 0.0 to 0.3 mM, with the PB solution set at 0.05 M and pH 8.0. At a potential of 0.53 V, an oxidation peak appeared, and the I_p increased with the TYR concentration. The calibration plot suggested a high correlation coefficient $R^2 = 0.981$ and a remarkably low LOD of 0.070 mM. The LAC/COOH-SWCNT-SPCE biosensor proved to be a proficient tool for TYR detection, exhibiting impressive sensitivity and an exceptionally low LOD.

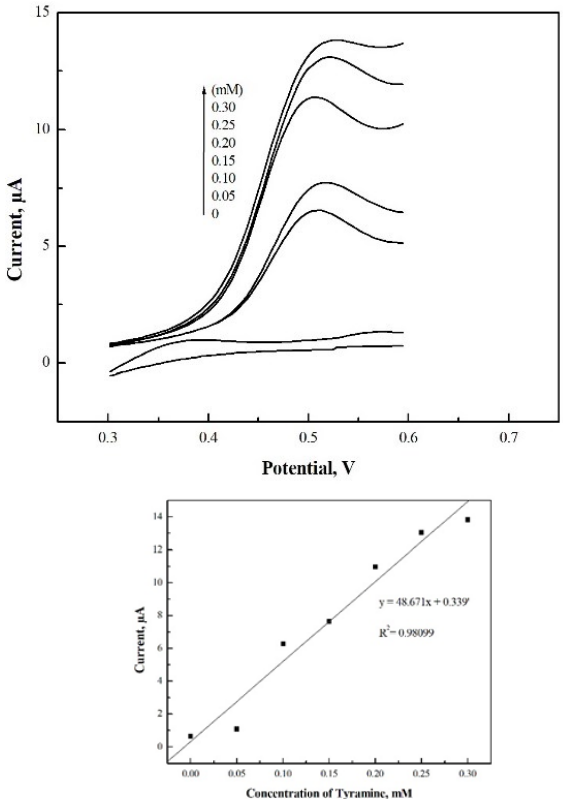


Figure 5 Differential Pulse Voltammetry (DPV) was conducted using LAC/COOH-SWCNT-SPCE in the presence of TYR (0.05 to 0.15 mM) within a 0.05 M PB solution at pH 8.0, employing a scan rate of 100 mV s^{-1}. A calibration graph illustrating the current response against TYR concentration was generated

4.0 CONCLUSION

This study successfully developed, described, and optimized a novel biosensor for TYR detection based on LAC/COOH-SWCNT-SPCE. The COOH-SWCNT established a favorable microenvironment for immobilizing the enzyme LAC, preserving its bioactivity. After thorough experimentation, the following parameters were identified as optimal for the LAC/COOH-SWCNT-SPCE biosensor: a scan rate of 50 mV s^{-1}, pH 8.0 in a (PB solution), 4 µL of enzyme LAC, 1.0 mg mL^{-1} of COOH-SWCNTs, a deposition potential of 0.5 V, and a deposition time of 25 seconds. Notably, the designed biosensor exhibited favorable performance with an R^2 of 0.981 and a low LOD of 0.070 mM. These encouraging outcomes underscore the electroactive nanomaterial capabilities of COOH-SWCNT and LAC for enhancing biosensor surface functionality in TYR detection. This biosensor could potentially find application in real food sample analysis for TYR.

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Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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