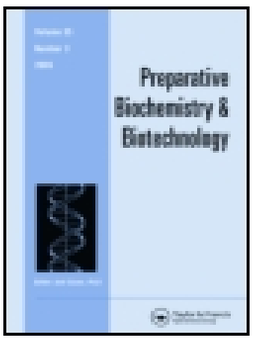

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A novel amperometric bienzymatic biosensor based on alcohol oxidase coupled PVC reaction cell and nanomaterials modified working electrode for rapid quantification of alcohol

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ABSTRACT

A new amperometric sensor has been fabricated for sensitive and rapid quantification of ethanol. The biosensor assembly was prepared by covalently immobilizing alcohol oxidase (AOX) from *Pichia pastoris* onto chemically modified surface of polyvinylchloride (PVC) beaker with glutaraldehyde as a coupling agent followed by immobilization of horseradish peroxidase (HRP), silver nanoparticles (AgNPs), chitosan (CHIT), carboxylated multi-walled carbon nanotubes (c-MWCNTs) and nafion (Nf) nanocomposite onto the surface of Au electrode (working electrode). Owing to properties such as chemical inertness, light weight, weather resistance, corrosion resistance, toughness and cost-effectiveness, PVC membrane has attracted a growing interest as a support for enzyme immobilization in the development of biosensors. The amperometric biosensor displayed optimum response within 8 s at pH 7.5 and 35°C temperature. A linear response to alcohol in the range of 0.01 mM–50 mM and 0.0001 μM as a minimum limit of detection was displayed by the proposed biosensor with excellent storage stability (190 days) at 4°C. The sensitivity of the sensor was found to be 155 μA $mM^{-1} cm^{-2}$. A good correlation ($R^2 = 0.99$) was found between alcohol level in commercial samples as evaluated by standard ethanol assay kit and the current biosensor which validates its performance.

KEYWORDS

Alcohol biosensor; alcohol oxidase; ethanol; HRP; polyvinyl chloride

Introduction

An accurate and rapid quantification of alcohol is enormously imperative in many diverse areas such as food industry, medicine, fermentation processes and clinical analysis of human body fluids (blood, urine, serum, breath, saliva) because of its toxicological and psychological effects.[1] It is also essential to have meticulous analytical methods for ethanol for controlling the quality of food, pulp, and different alcoholic beverages, e.g., beer, spirits, wine, and liquor[2] as well as tax regulation purposes. Several conventional methods for the determination of this analyte have been reported during the years, including redox titrations, spectroscopic techniques, refractrometry, chromatography and colorimetry.[2] These methods are time-consuming, relatively expensive, require trained operators and complex in performing; consequently, unconventional approaches are inevitable.[3] We considered that such shortcomings can be overcome by using enzyme based amperometric biosensors for the detection of alcohol as they offer brilliant specificity, sensitivity, efficient catalytic properties, and low cost of fabrication. The two major enzymes involved in catalytic reaction of ethanol sensing amperometric biosensor are alcohol dehydrogenase (ADH)[4] and alcohol oxidase.[5] In ADH based ethanol biosensors, oxidation of ethanol to acetaldehyde is catalyzed by the enzyme in the presence of NAD^+ and further NAD^+ is reduced to NADH. This NADH can be oxidized by releasing electrons and protons, which is monitored by amperometric detection.[6] The ADH based biosensors have restricted operational stability and the inconvenience to be reliant on the coenzyme NAD^+ which needs continuous recovery in the assay leading to enhancement in the overall cost of manufacture of these biosensors.[7] Alternatively, alcohol oxidase (EC 1.1.3.13) based alcohol biosensors are straightforward as they work by using only molecular oxygen to oxidize alcohol into the corresponding aldehyde with concomitant release of H_2O_2, without addition of any other co-factor.[8] Some key advantages credited to AOX based bio-recognition system include the presence of flavin-based co-factor firmly bound at the redox center of the enzyme and the irreversible catalytic oxidation of alcohols.[1] Various electrochemical alcohol biosensors based on immobilized alcohol oxidase have been reported.[9] In all these biosensors, AOX has been immobilized either on graphite powder/an epoxy resin bicomposite electrode,[10] conducting polymers of 3,4-ethylenedioxythiophene (PEDOT) and 4,7-dithien-2-yl-2,1,3-benzothiadiazole (TBTD),[11] graphite epoxy composite electrode (GECE),[12] multi-walled carbon nanotube (MWCNT) modified glassy carbon electrode [13] or membrane such as poly(vinyl alcohol) membrane,[14] eggshell membrane [15] and nafion membrane.[16,17] Although the biosensors employing electrodeposited layer are found to

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be ultrasensitive and fast responding when compared with membrane using sensors, however membrane immobilized enzyme showed better stability and reusability. PVC has drawn remarkable interest to develop an enzyme reaction beaker which acts as electrochemical cell for three electrode-based systems for analyte detection.[18–21] Taking account of the astounding properties allied with PVC such as easy availability, chemical inertness, corrosion resistance, weather resistant, light weight, toughness, high strength-to-weight ratio as well as cost-effectiveness,[19] we have reported the use of a non-fragile and conducting PVC beaker as a direct support for covalent immobilization of alcohol oxidase onto its surface. The exploitation of polyvinyl chloride (PVC) in bioanalytical techniques will open a new pathway in the field of biosensor fabrication and design, diagnostic kits, and allied biomedical instrumentation.

During the past years, owing to their excellent mechanical, electronic, thermal and optical properties, nanoparticles have been widely employed in the fabrication of next generation biosensors to improve important performance indexes such as sensitivity, stability, and repeatability. c-MWCNTs offer exceptionally high electroactive surface (due to its material properties such as electrical and thermal conductivity) for rapid exchange of electrons between the electrode and the enzyme.[22] AgNPs and carbon nanotubes display strong electrical properties and high surface to volume ratio which makes them capable of increasing the enzyme loading onto the surface of electrode for amplifying the sensor signal. Chitosan is a significant biopolymer, which has been gradually used for fabricating sensors owing to its high mechanical strength, easy availability, exceptional film-forming ability, non-toxicity, high permeability, low cost, and easy to modify with the help of chemicals.[23] Due to the hydrophilic nature of chitosan, it also facilitates the rate of electron transfer after its swelling in reaction mixture. Nafion is a sulfonated tetrafluorethylene copolymer that has been extensively utilized in the biosensor applications.[24] Biocompatibility, mechanical strength, excellent thermal stability, and antifouling properties of Nafion, makes its use advantageous in biosensor applications. Nafion is an effective solubilizing agent for carbon nanotubes (CNTs) that resulted in fabrication of CNT-based biosensors exhibiting both the antifouling properties of Nafion films as well as the efficient electrocatalytic action of CNT toward H_2O_2.[25] Immense potentiality of enzymes, carbon nanotubes, and nanoparticles has boosted scientific enthusiasm towards fabrication of alcohol biosensors with excellent sensitivity, which is an ever-lasting goal in the field of health area for commercial successes.

In the present work, we aimed to develop a novel amperometric alcohol biosensor that possesses properties such as reusability, rapid response, low cost, and good stability. The bienzymic sensor was constructed using alcohol oxidase and horseradish peroxidase. Alcohol oxidase was extracted and purified from yeast *Pichia pastoris* and used for the fabrication of an electrochemical biosensor by covalently immobilizing it on the chemically modified inner surface of PVC beaker (using glutaraldehyde as a coupling agent) rather onto the working electrode. A working electrode was prepared by incorporating HRP (EC 1.11.1.7), silver nanoparticles, c-MWCNTs, chitosan, and nafion onto the surface of Au electrode. In this bienzymatic alcohol biosensor, AOX catalyzed the oxidation reaction of alcohol and released H_2O_2, which was then bioelectrocatalytically reduced by HRP through DET mechanism to generate the current response. The novelty of this work lies in the combination of PVC immobilized alcohol oxidase and AgNPs based working electrode for alcohol determination in commercial samples. This encourages the opportunity to change the working electrode on decay, without wasting the immobilized enzyme, which makes it economical and advantageous. In addition, the working electrode can be re-used with another AOX immobilized PVC beaker if enzyme is denatured or leached out.

Experimental

Chemicals and reagents

Methylene chloride, benzamidine, tween 60, NaN_3, c-MWCNT (length 0.1–10 µM, OD 15–20 nm, ID 4–8 nm), polyethylene glycol, chitosan (CHIT), nafion 117 (Nf) (5% *w*/*v* in isopropanol), $AgNO_3$, sepharose 2B column were purchased from Merck. ABTS (2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)), methanol, trisodium citrate, glutaraldehyde (grade 1.25%), NH_4OH, $(NH_4)_2SO_4$, Tris-HCl, acetic acid, H_2SO_4, and HNO_3 were obtained from Himedia. HRP (0.5 U/mg) was purchased from SISCO Research Laboratory Pvt. Ltd., Mumbai. PVC (polyvinyl chloride) beaker of 20 mL capacity was bought from the local market. All other chemicals were of analytical reagent grade. Double distilled water (DDW) was used throughout the experiments.

Instruments and equipment

Cyclic voltammetry (CV) measurements were performed on a potentiostat/galvanostat (PARSTAT 4000) with a three-electrode system composed of an Ag/AgCl electrode as reference electrode, a platinum (Pt) wire as an auxiliary electrode and nanocomposite modified Au as working electrode. UV-Vis Spectrophotometer (UV 2450, Shimadzu Corporation, Japan), Fourier transform infrared spectrophotometer (FTIR) (Varian 7000), Temperature controlled water bath (SunRon), Digital pH meter (EUTECH), Refrigerator (LG), Microwave oven (LG), Rotatory incubator shaker (HICON), Magnetic stirrer (HICON), Refrigerated centrifuge (SIGMA), and Deep freezer (Voltas). The study of surface morphology was carried out with scanning electron microscope (SEM) at AIRF, Jawaharlal Nehru University, New-Delhi.

Microorganism

The lyophilized sample of yeast *Pichia pastoris* (MTCC 34) was purchased from Institute of Microbial Technology (IMTECH) based in Chandigarh, India.

Revival and growth of yeast

The yeast strain is revived by scraping some cells off the lyophilized sample and streak onto plates containing the rich medium yeast extract, peptone, dextrose (YPD). The growth was assessed by using a UV-VIS spectrophotometer by determining the optical density (OD) at 600 nm.

Extraction and purification of AOX from Pichia pastoris

The enzyme AOX was extracted and purified using method described by Gvodev et al.[26] with little modifications. After desirable growth, the cells were centrifuged at 12,000×*g* for 20 min and washed twice in 0.02 M phosphate-citrate buffer (pH 8). The cell suspension was supplemented with 0.02% NaN_3,[27] 0.05% Tween 60, 0.1 mM benzamidine and then lysed in the presence of 1.2% (*w*/*v*) methylene chloride.[28] Using 0.1 M NH_4OH, the pH of the cell-free preparation was adjusted to 8.0 and then $(NH_4)_2SO_4$ was added to the 30% mixture (pH 7.0). The mixture was centrifuged at 20,000×*g* for 30 min. The supernatant was collected and supplemented with $(NH_4)_2SO_4$ to 60% of saturation (pH 7.0) and kept undisturbed at 5°C for 2 hr. The fraction was again centrifuged at 20,000×*g* for 10 min and the resulting precipitate was dissolved in 0.01 M phosphate-citrate buffer (pH 8). The precipitate was separated from salts through ultrafiltration and then 6–12% (*w*/*v*) polyethylene glycol (6000 Da), 0.02% NaN_3 and 0.1 mM benzamidine were added to the mixture and stored for 3 days at 5°C. The crystals of alcohol oxidase (AOX) were obtained by centrifugation at 10,000×*g* for 10 min and then dissolved in 0.02 M phosphate-citrate buffer supplemented with 0.02% NaN_3 and 0.1 mM benzamidine. Purification of crude alcohol oxidase (AOX) was done using analytical centrifugation followed by gel filteration (column- Sepharose 2B) and SDS-PAGE.

Enzyme assay and protein estimation

The enzyme activity was measured by detecting the color produced from H_2O_2 in a coupled reaction using horseradish peroxidase and 2, 2'-azino-bis(3-ethylbenzthiazoline 6-sulfonate) (ABTS).[29] 0.1 mL of purified alcohol oxidase enzyme solution was mixed with 2.8 mL ABTS solution (pH 7.5), 0.01 mL horseradish peroxidase solution, 0.01 mL potassium phosphate buffer and 0.1 mL methanol solution. The reaction mixture was incubated for 40 min at 30 °C. The method involves two successive enzymatic reactions:

$$Methanol + O_2 \xrightarrow{Alcohol\ \ oxidase} Formaldehyde + H_2O_2$$

$$H_2O_2 + ABTS_{(Red.)} \xrightarrow{Horseradish\ \ peroxidase} 2\ H_2O + ABTS_{(Ox.)}$$

The AOX and peroxidase-based reaction involve the oxidation of methanol by AOX that produce formaldehyde and H_2O_2. Further horseradish peroxidase use ABTS as an electron donor and reduces H_2O_2 to H_2O, a green and soluble end-product was formed. The color produced was measured in a spectrophotometer at 420 nm. The reading was corrected for the blank, which was obtained with enzyme and all reagents were present, except methanol. The enzyme activity was followed by measuring of the increase of absorbance at $\lambda_{420\,nm}$ due to the formation of H_2O_2. One unit of enzyme activity was expressed as the quantity of enzyme required to produce 1 µM hydrogen peroxide per min under these assay conditions. The total protein content of the dissolved AOX was measured by Lowry method using bovine serum protein as standard protein.[30]

Covalent immobilization of AOX on the inner surface of PVC beaker

Alcohol oxidase extracted from *Pichia pastoris* was covalently immobilized onto the inner surface of PVC beaker (20 mL) by using the method described by Hooda et al.[19] During immobilization, first the beaker was treated with HNO_3 and H_2SO_4 mixture, which breakdown the vinyl polymers of plasticized PVC material to introduce nicks in the long polymer chain. Then the beaker was incubated with 1.25% (*w*/*v*) glutaraldehyde solution in 0.05 M sodium phosphate buffer (pH 7.0) for 10 hr at 32°C. The generated free ends of vinyl chain reacted with one aldehyde group of glutaraldehyde to form –C = CH– bond between the plasticized PVC sheet. The beaker was rinsed with distilled water twice to remove excess of glutaraldehyde attached to the surface. Hence, the surface of the PVC beaker was activated for the covalent immobilization of the enzyme. Then the inner surface of PVC beaker was incubated with 5 mL solution of dissolved alcohol oxidase in sodium phosphate buffer (50 mM, pH-8.0) at 4°C for 24 hr in the dark. The $-NH_2$ group on the surface of alcohol oxidase was attached covalently to the –CHO group of glutaraldehyde (already attached to the PVC surface) through Schiff-base formation. Then beaker surface was rinsed with buffer to eliminate the unbound enzyme. In this way, alcohol oxidase was covalently bonded on the inner surface of PVC beaker (Figure 1). The reaction beaker was stored in a 0.1 M sodium phosphate buffer (pH 7.0) at 4°C until use. To verify the immobilization of enzyme, SEM and FTIR studies of the surface of PVC beaker were carried out, before and after enzyme immobilization at AIRF, Jawaharlal Nehru University, New-Delhi.

Assay of immobilized alcohol oxidase

The assay of immobilized alcohol oxidase was carried out in the same PVC beaker in which it was immobilized. It was done in this beaker in the same manner as that described for its native/free form, except that 0.1 mL of the reaction buffer was used to replace free enzyme and stirred continuously during incubation. Then after incubation, 2.8 mL ABTS solution (pH 7.5), 0.01 mL horseradish peroxidase solution, 0.01 mL potassium phosphate buffer and 0.1 mL methanol solution were added to the reaction beaker and incubated at 30 °C for 40 min to develop the color. The color produced was measured in a spectrophotometer at $\lambda_{420\,nm}$. The beaker containing immobilized enzyme was washed with distilled water 2–3 times. The conjugation yield

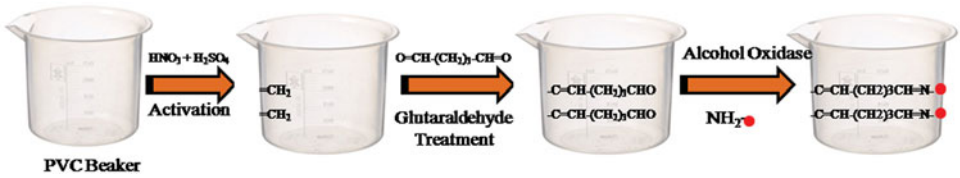

Figure 1. Schematic representation of covalent immobilization of alcohol oxidase onto the surface of a PVC beaker.

and % retention of enzyme activity after immobilization were determined by using formula:

$$\text{Conjugation yield} = \frac{\text{mg enzyme immobilized}}{\text{cm}^2 \text{ of PVC wall}}$$

$$\text{Retention (\%)} = \frac{\text{Specific activity of immobilized enzyme}}{\text{Specific activity of native/free enzyme}} 100$$

Synthesis of silver nanoparticles and their characterization

The silver nanoparticles were prepared by using the existing method reported by Fang et al.[31] with some modification. Firstly, 80 mL of 1×10^{-3} M $AgNO_3$ was heated to boiling. Then 1% trisodium citrate (8 mL) was added dropwise to this solution with vigorous stirring. The solution was then heated at boiling point until the color changed to pale brown. Finally, it was removed from the heating element and cooled at room temperature with continuous stirring. To elucidate their shape and size, transmission electron microscopy (TEM) of the resultant nanoparticles was done at AIRF, Jawaharlal Nehru University, New-Delhi.

Fabrication of working electrode

Prior to coating, the bare Au electrode (2 mm diameter) was polished using alumina slurry (diameter 0.10 µm) and then washed carefully with distilled water. Subsequently the Au electrode was ultrasonicated in ethanol and distilled water to eliminate adsorbed components. Then the electrode was again rinsed with deionized water two to three times and allowed to dry. c-MWCNTs (5 mg) were dissolved in 500 µl of 5% Nf and sonicated for 30 min to get a homogeneous suspension. 5 µl of c-MWCNT-Nf solution was added dropwise on the surface of Au electrode and allowed to dry at room temperature. CHIT (0.2 g) flakes were suspended in 20 mL of 1% acetic acid and then continuously stirred for 4 hr at room temperature awaiting complete dissolution. AgNPs (10 mg) were suspended into chitosan solution and kept on magnetic stirring for about 1 hr at room temperature and then sonication of the resulting solution was done for about 2 hr. The hybrid nanocomposite film of AgNPs/CHIT biopolymer matrix was electrodeposited on the surface of c-MWCNT/Nf/Au electrode by cyclic voltammetry (potential range from: −0.75 to 1.2 V) and allowed to dry in air before use. The resulting electrode AgNPs/CHIT/c-MWCNT/Nf/Au was washed with deionized water and dried.

To prepare enzyme electrode, AgNPs/CHIT/c-MWCNT/Nf/Au electrode was immersed into 1.5 mL of HRP solution (0.5 U/mL) and kept overnight at 4°C for immobilization. The resulting electrode HRP/AgNPs/CHIT/c-MWCNT/Nf/Au was rinsed 2–3 times with 0.1 M Tris-HCl buffer (pH 8.0) to depose the unbind enzyme and used as the working electrode. The bio-electrode was stored dry at 4°C in a refrigerator until use.

Assembly of alcohol biosensor and its electroanalytical measurements

An amperometric alcohol biosensor was developed by using enzyme alcohol oxidase immobilized PVC reaction beaker containing the three electrodes -Ag/AgCl electrode as reference electrode, Pt wire as auxiliary electrode, HRP/AgNPs/CHIT/c-MWCNT/Nf/Au as working electrode and being connected through potentiostat/electrochemical analyzer. Figure 2 represents the schematic presentation showing the assembly of fabricated alcohol biosensor. The electroanalytical measurements for alcohol were performed using a potentiostat/electrochemical analyzer. Cyclic voltammetric measurements were performed at 35 °C. The voltammogram was continuously recorded between: −0.70 V to +2.0 V at a scan rate of 50 mV/s. The optimum working conditions were studied for the current biosensor.

Optimization of experimental parameters

The proposed alcohol biosensor was evaluated in term of its analytical recovery, precision, linearity, accuracy, and minimum detection limit. To obtain optimum conditions for alcohol determination, the pH was varied between pH 4.0 to 8.0 at an interval of pH 0.5 using sodium acetate buffers (pH 4.0–5.0) and sodium phosphate buffer (pH 5.5–8) each at a final concentration of 0.1 M. In the same way, the optimum temperature and the time of reaction was determined by incubating the mixture at various temperature (5–70°C) and time (1–20 s). The effect of substrate amount on the response current was investigated by using different concentration of ethanol in the range 0.1–50 mM. The minimum detection limit and linear working range were calculated by correlating the values with standard graph. The storage stability of the present biosensor was investigated over a period of 2 months, when reaction beaker was stored in 0.1 M sodium phosphate buffer (pH 7.0) at 4°C. LOD was calculated as follows:

$$\text{LOD} = 3.3(\text{SD}/\text{S})$$

SD = standard deviation of the response
S = slope of the calibration curve

The interference caused by ascorbic acid, urea, glucose, and lactic acid on the response of biosensor was evaluated.

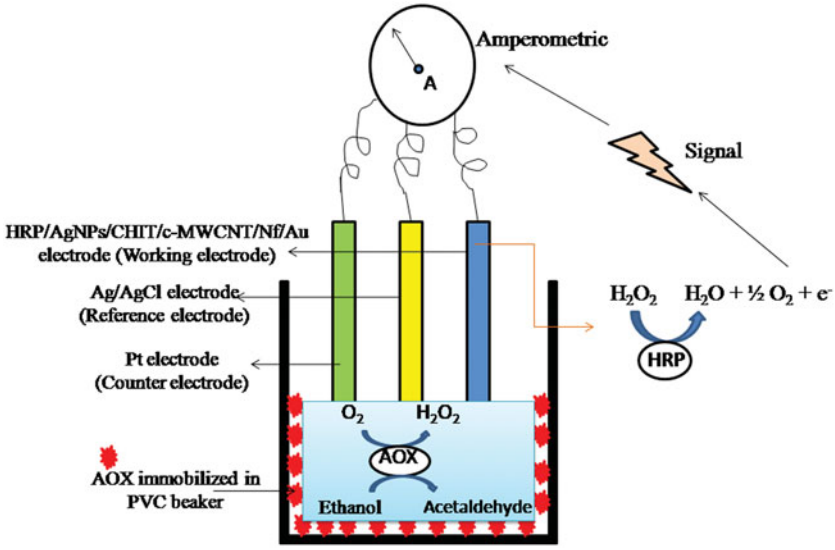

Figure 2. Schematic diagram showing assembly of the fabricated alcohol biosensor.

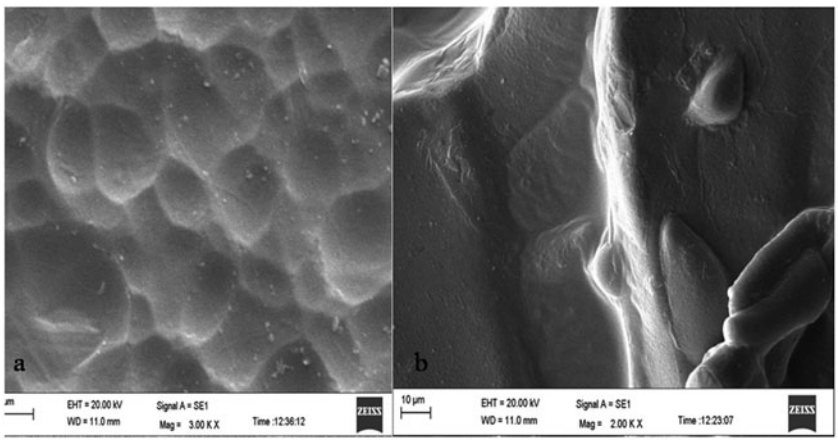

Figure 3. Scanning electron microscopic (SEM) images of (a) bare PVC surface (b) enzyme immobilized PVC surface.

Results and discussion

Purification of alcohol oxidase from Pichia pastoris

One symmetric peak was obtained during gel filteration of AOX on a column with Sepharose 2B and one symmetric peak was observed during analytical ultracentrifugation which indicates molecular homogeneity of the preparation. The evident purification efficiency was equivalent to 5.4×. The specific activity of alcohol oxidase was 60 units/mg. The purified enzyme showed a single band at 73 ± 5 kDa on SDS-PAGE.[32]

Evidences for immobilization of alcohol oxidase on the surface of PVC beaker

AOX was immobilized covalently onto the inner surface of a PVC beaker with a conjugation yield of 0.38 mg/cm^2 and 66.23% retention of its initial activity.

The SEM of the bare surface of beaker showed a uniform polymeric layer of PVC (Figure 3a). The SEM of chemically modified surface of PVC beaker with immobilized enzyme displayed globular beaded structures (Figure 3b). This transformation in surface morphology of PVC beaker suggests that the enzyme molecules are uniformly packed in the PVC membrane.

The FTIR spectra of bare PVC sheet (Figure 4) displayed characteristic regions of PVC sheet: (i) The peak in the range from 600–700 cm^{-1} represents C–Cl stretching region (ii) small peaks in the range of 900–1200 cm^{-1} which are signature peaks of C–C stretching, and (iii) absorption peaks in the range of 1500–2970 cm^{-1} are assigned to numerous CH bending vibrations. Following the immobilization of enzyme onto PVC surface, the FTIR spectra exhibited a new peak at 1724 cm^{-1} and small peaks in the range of 3000 to 3500 cm^{-1} corresponds to carbonyl stretch and amide bond, respectively. These studies assured that the enzyme was immobilized onto PVC surface.

Characterization of silver nanoparticles

UV/Vis spectra recorded in UV-Vis spectrophotometer (Figure 5a) reveals the formation of AgNPs by showing surface plasmon absorption maxima at 425 nm. TEM of AgNPs, as shown in Figure 5b revealed that AgNPs were very fine with diameter in the range of 20–50nm and more or less spherical in shape.

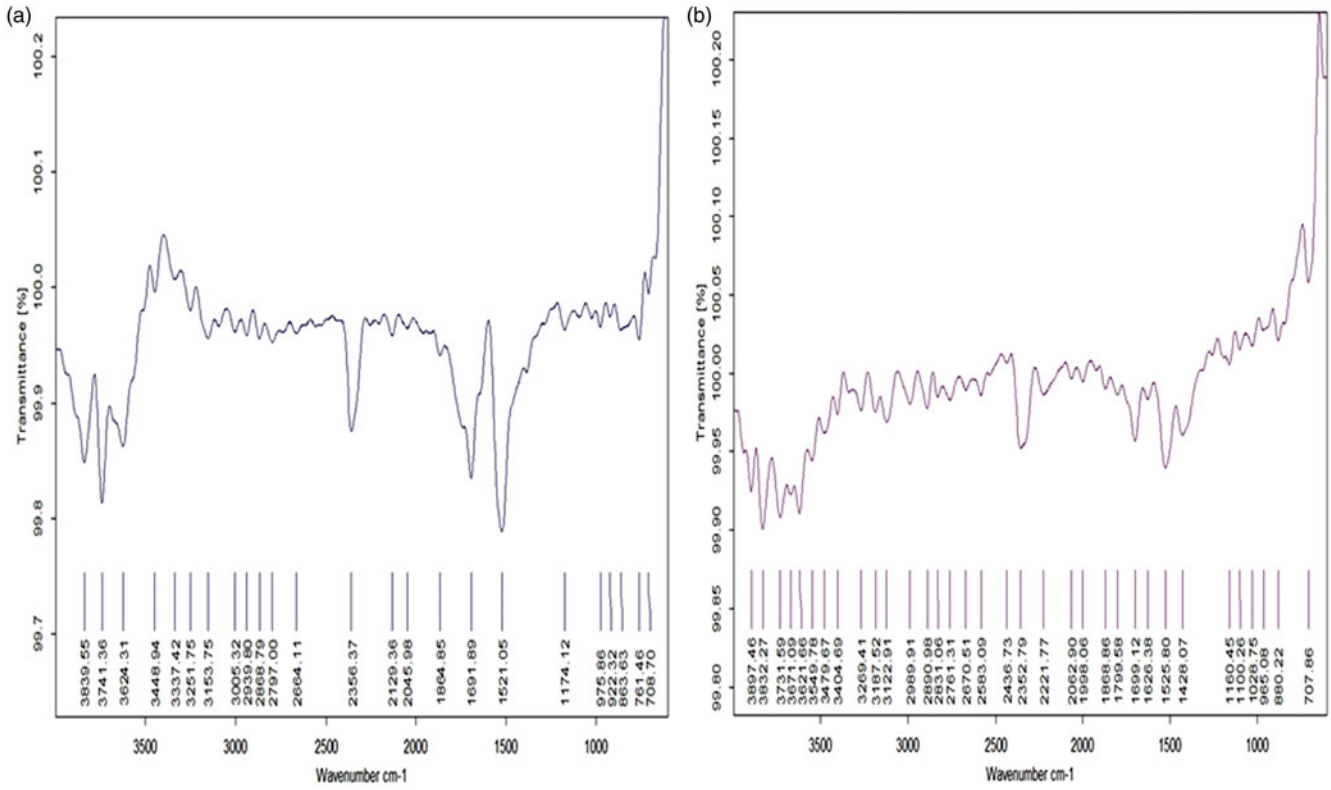

Figure 4. FTIR spectra of (a) bare PVC surface (b) enzyme immobilized PVC surface.

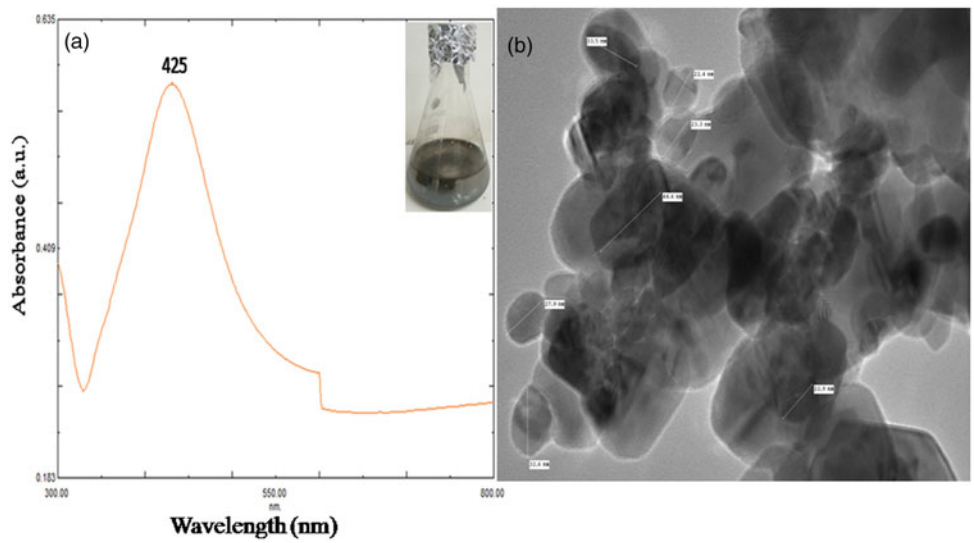

Figure 5. (a) UV-Visible spectrum of synthesized silver nanoparticles (b) TEM image of synthesized silver nanoparticles.

Characterization of HRP immobilized working electrode

The electrochemical impedance spectroscopy (EIS) was performed to provide the useful information to validate the impedence changes of the electrode surface due to formation of different layers during the fabrication process. It was employed to confirm the immobilization of HRP onto the surface of Au electrode. The linear part at lower frequencies match with the Warberg diffusion process while the diameter of the semicircle at higher frequencies in the impedence spectrum corresponds to the electron transfer resistance (R_{CT}) which depends on the insulating and dielectric features at the electrode interface.[33] The Nyquist plot (Figure 6) exhibits EIS with bare Au electrode, c-MWCNT/Nf/AuE, CHIT/AgNPs/c-MWCNT/Nf/AuE, HRP/CHIT/AgNPs/c-MWCNT/Nf/AuE in 6 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ displaying R_{CT} values as 0.6 kΩ, 1.6 kΩ, 1.76 kΩ, 2.76 kΩ, respectively (the average of three replications). CHIT/AgNPs/c-MWCNT/Nf/AuE showed decrease in R_{CT} value as compared to the bare Au electrode, implying that attachment of highly conductive c-MWCNT film and AgNPs onto

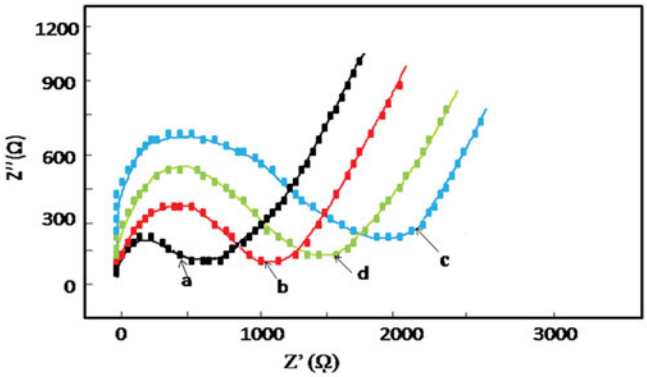

Figure 6. The Nyquist plot showing electrochemical impedence spectra (EIS) of (a) bare Au electrode (b) c-MWCNT/Nf/Au electrode (c) CHIT/AgNPs/c-MWCNT/Nf/Au electrode (d) HRP/CHIT/AgNPs/c-MWCNT/Nf/Au electrode.

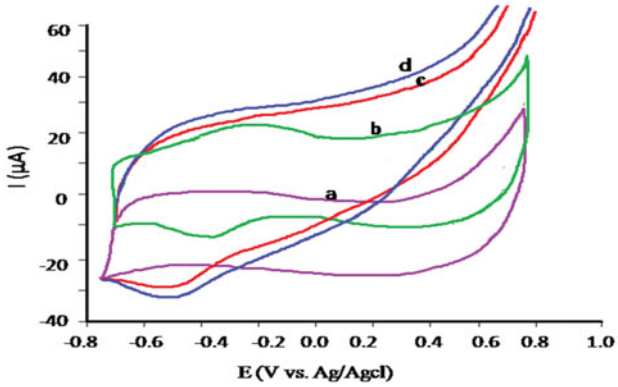

Figure 7. Cyclic voltammograms of (a) bare Au electrode (b) HRP/AgNPs/CHIT/c-MWCNT/Nf/Au electrode (c) complete assembly of biosensor without substrate (d) complete assembly of biosensor with substrate.

the electrode accelerating the charge transfer efficiency. After immobilization of HRP, the increase in R_{CT} value of HRP/CHIT/AgNPs/c-MWCNT/Nf/AuE can be accredited to the binding of enzyme (HRP) molecules, which are poor electrical conductors at low frequencies and resist the electron transfer. These results assured the immobilization of HRP onto the surface of CHIT/AgNPs/c-MWCNT/Nf modified Au electrode.

Response characteristics of AOX and HRP based alcohol biosensor

An amperometric alcohol biosensor was fabricated by covalently immobilizing alcohol oxidase onto PVC surface and HRP onto the CHIT/AgNPs/c-MWCNT/Nf modified Au electrode. To evaluate the redox potentials of the fabricated biosensor, cyclic voltammetry was done as shown in Figure 7. There were no clear peaks for bare Au electrode (curve a). The working electrode HRP/AgNPs/CHIT/c-MWCNT/Nf/Au prepared by immobilizing AgNPs-CHIT and HRP on the c-MWCNT/Nf film showed a pair of redox peaks at −0.24 V and −0.35 V, which highlights the characteristic potential for direct electrochemistry of HRP[34] (curve b). Subsequent immobilization of AOX onto the surface of PVC beaker was done to generate complete assembly of the biosensor. The generated assembly (curve c) displayed redox peaks comparable to curve b but with enhanced peak currents. The formal potential of the redox couple at the bioelectrode assembly was −0.29 V, calculated from the average value of anodic and cathodic peak potentials $[(E_{pa}+E_{pc})/2]$. This potential was nearly conforming to the earlier reports for HRP (Fe^{III}/Fe^{II}) on direct electrochemistry.[35] With addition of ethanol to the fabricated biosensor, the magnitude of cathodic peak current was found to be considerably higher relative to the anodic peak current (curve d) which further confirmed the redox potential of HRP. Alcohol oxidase (immobilized on to PVC beaker) catalyze the oxidation of alcohol to generate H_2O_2 which is then biocatalytically reduced by the HRP (present on the working electrode) resulting in the formation of HRP (Ox). At potential of −0.35 V, HRP (Ox) electrocatalytically reduced to HRP (Red) on the electrode surface through DET mechanism and revived the reaction for the next cycle as shown in equation below.

$$CH_3CH_2OH + O_2 \xrightarrow{AOX} CH_3CHO + H_2O_2$$

$$HRP\ (Red) + H_2O_2 \longrightarrow HRP\ (Ox) + 2H_2O$$

$$HRP\ (Ox) + 2\ e^- + 2H^+ \xrightarrow{Electrode} HRP\ (Red)$$

Optimization of alcohol biosensor

The optimum bioelectrode response was identified at pH 7.5 (Figure 8a) and then confirmed to the pH optimum for the biocatalytic reaction of AOX.[36] The stability of AOX declines with the deviation from the physiological pH.[37] The optimum incubation temperature of the proposed biosensor was 35°C (Figure 8b) and the sensor showed optimum response within 8 s (Figure 8c), which is shorter than previously reported sensors as compared in Table 1.[12,13,15–17,38,39] It was observed that the magnitude of cathodic current increased linearly with increasing concentration of ethanol in the range of 0.01 mM–50 mM, the response was steady after 50 mM of ethanol (Figure 8d). Under optimum conditions, the wide working range of the biosensor 0.01 mM–50 mM, is better than earlier biosensors (0.02–1.7 mM, 10–750 μM, 2.5–25 μM, 60–0.80 mM, 5–3000 μM, 8–42 μM).[11–13,15–17] The LOD of the present biosensor (0.0001 μM), was better than earlier reported biosensors (2.32 μM, 5 μM, 10 μM, 29.7 μM, 9.9 μM).[13,16,17,38,39]

Stability and interference studies of the biosensor

20 successive measurements were taken to investigate the operational stability of the constructed biosensor with 50 mM of alcohol (for which the highest response was observed in the linear range) for the duration of 6 hr. The results indicated that there was steady current response till the last measurement and the biosensor still retained 90% of its initial activity (Figure 9a). The response of the constructed biosensor to various concentrations of alcohol was examined at three bioelectrodes made by similar method to

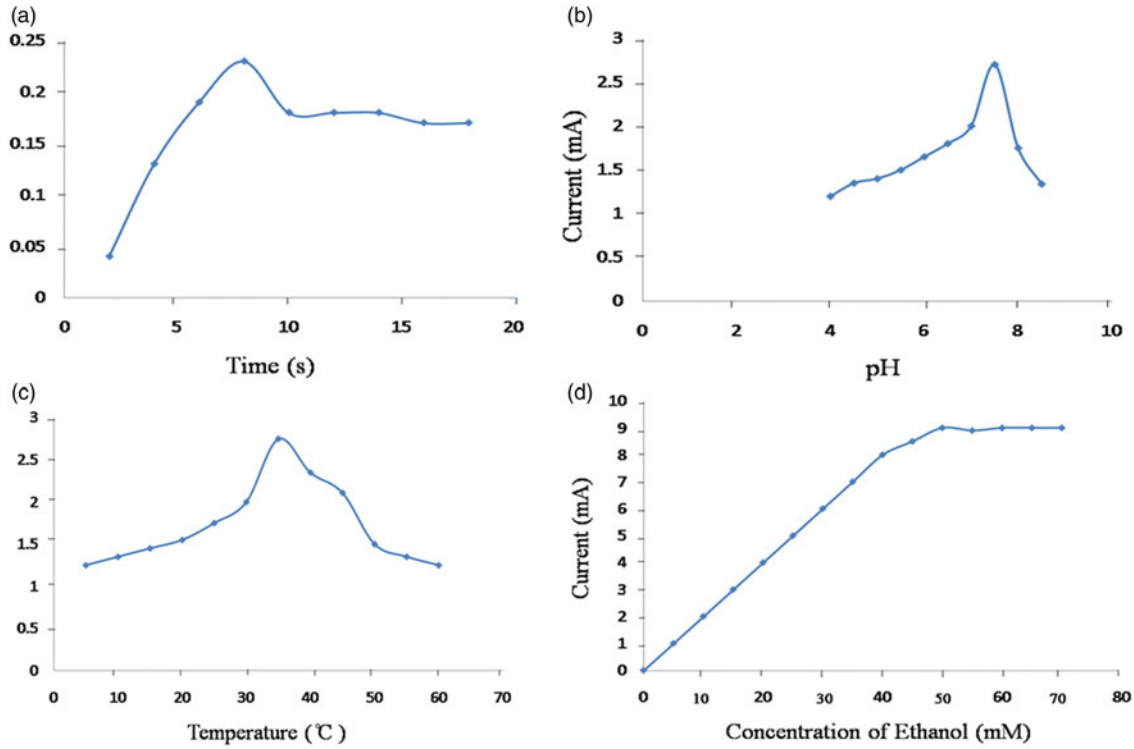

Figure 8. (a) Influence of time on the current response of biosensor (b) Influence of pH on the current response of biosensor (c)) Influence of temperature on the current response of biosensor (d) Influence of ethanol concentrations on the current response of biosensor.

Table 1. Comparison of various amperometric AOX based alcohol biosensors on basis of their performances.

Sr. No.	Transducer	Enzyme immobilization method	Minimum detection limit	Linearity	Response time	References
1.	Graphite powder/an epoxy resin/AOX bicomposite electrode	Entrapment	–	0.5–29 mg/mL	–	[10]
2.	PEDOT/TBTD/AOX electrode	Crosslinking	203.70 nA/mM	0.02–1.7 mM	–	[11]
3.	Graphite epoxy composite electrode (GECE)	Entrapment	–	2.5 μM–25 μM	70 s	[12]
4.	Nafion/HRP-SWCNT/MWCNT-ZnO electrode	Crosslinking	10 μM	10 μM–750 μM	10 s	[17]
5.	Carbon film/PNR/ AOX electrode	Crosslinking	29.7 μM	–	–	[38]
6.	Gas-diffusion working electrode	Crosslinking	9.9 μM	0.10 μM–30 mM	69 s	[39]
7.	Eggshell membrane electrode	Entrapment	30 μM	60 μM–0.80 mM	60 s	[15]
8.	Multi-walled carbon nanotube (MWCNT) modified glassy carbon electrode	Layer-by-layer technique	2.32 μM	5 μM–3000 μM	20 s	[13]
9.	Au/MWCNT/Nf/AOX/PEI bioelectrodes	Crosslinking	5 μM	8 μM–42 μM	55 s	[16]
10.	AOX onto PVC surface and HRP/AgNPs/CHIT/c-MWCNT/Nf/Au electrode	Covalent bonding	0.0001 μM	0.01 mM–50 mM	8 s	Present work

AOX: Alcohol oxidase; HRP: horseradish peroxidase; MWCNT: multiwall carbon nanotube; Nf: Nafion; PEI: polyethylenimine; PEDOT: 3, 4-ethylenedio xythiophene; SWCNT: single wall carbon nanotube; TBTD: 4, 7-dithien-2-yl-2, 1, 3-benzothiadiazole..

predict the reproducibility. The acceptable reproducibility with a relative standard deviation (RSD) of ~1% for the constructed biosensor was shown in results, which confirmed the reliable repeatability of the presented biosensor. The response measurements were carried out at a regular interval of 5 days to examine the storage stability of the biosensor and it was found that the fabricated biosensor maintained ~90% of the original response even after 5 weeks when stored in 0.02 M sodium phosphate buffer (pH 7.0) at 4°C (Figure 9b). The biosensor lost 50% of its initial activity after its regular use of 100 times over a period of 6 months. The half life of the biosensor was estimated to be ~180 days under the above storage conditions. The selectivity of the constructed sensor was studied towards alcohol. The interference caused by some probable interfering agents present in real samples such as glucose, ascorbic acid, uric acid, lactic acid and urea was studied in 50 mM phosphate buffer saline (pH 7.5) at −0.35 V. These interferents were exposed separately with the analyte in 1:1 ratio to examine the biosensor response (Figure 10). For each interferent, the selectivity co-efficient (SC) was found to be ~1, estimated as follows:

$$SC = I_{c+i}/I_c$$

where I_c and I_{c+i} are response for alcohol in the absence and presence of each interferents respectively. The result showed that practically there was no significant effect of these interferents. The proposed biosensor has tolerable anti-interferent ability.

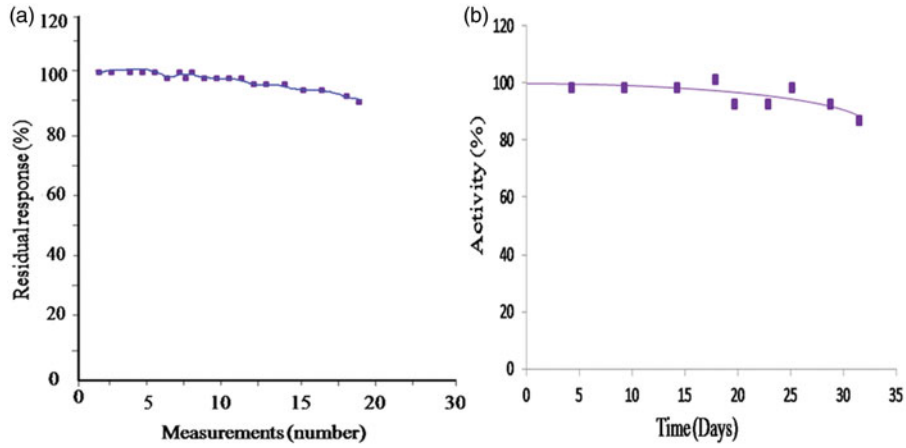

Figure 9. Stability of the fabricated biosensor (a) Operational stability and (b) Storage stability.

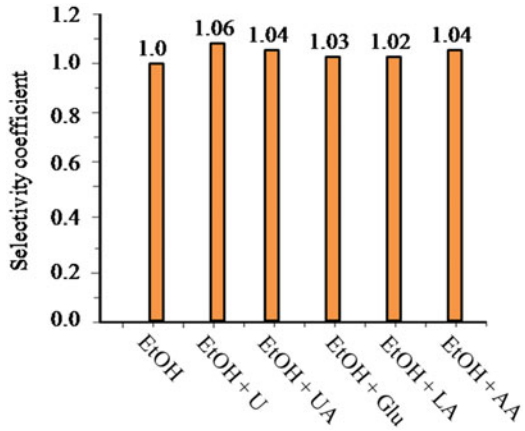

Figure 10. Interference studies with biosensor (EtOH: Ethanol, U: urea, UA: uric acid, Glu: glucose, LA: lactic acid, AA: ascorbic acid).

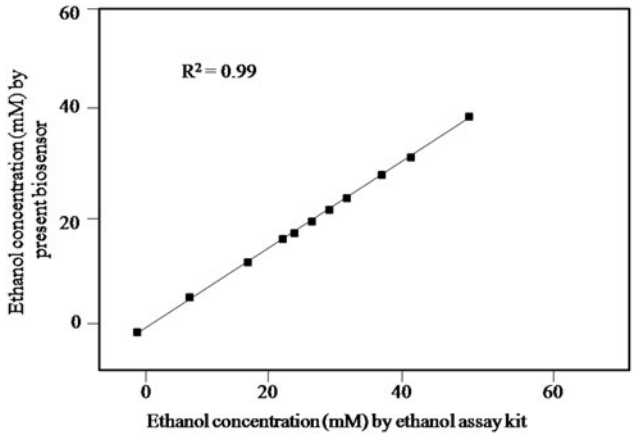

Figure 11. Correlation between ethanol level measured by ethanol assay kit (x-axis) and the present method (y-axis) employing the alcohol biosensor based on AOX and HRP.

Analysis of commercial alcohol samples

The four different beer samples (I, II, III, and IV) were taken and ethanol content (mg/mL) was determined at −0.35 V using the current biosensor by the multiple standard addition method (without prior treatment of samples). Ethanol content was obtained as 0.35 ± 0.015, 0.53 ± 0.016, 0.52 ± 0.018, and 0.51 ± 0.021 for the samples I, II, III, and IV respectively. Ethanol assay kit was also used to measure the concentration of ethanol in the same samples and results were 0.37 ± 0.017, 0.54 ± 0.021, 0.55 ± 0.022, and 0.50 ± 0.013 for the samples I, II, III, and IV, respectively. The data measured by the present sensor was compared with those obtained by standard ethanol assay kit and it was found that there were no significant differences between the results obtained by separate methods. A good correlation of 0.99 was observed (Figure 11) and hence the results authenticate the reliability of the existing alcohol biosensor for the quantitative and sensitive analysis of industrial samples.

Conclusions

A novel and sensitive amperometric alcohol biosensor fabricated by immobilizing a mutimeric alcohol oxidase onto PVC surface and HRP on the surface of AgNPs/CHIT/c-MWCNT/Nf/Au electrode (working electrode) has been reported for the first time. It was confirmed that the use of MWCNTs and AgNPs facilitates the direct transfer of electrons between the HRP and the electrode. The immobilized AOX onto the surface of PVC beaker retains good electrocatalytic activity at physiological pH. PVC has emerged as an idyllic support and its application in the construction of present electrochemical cell not only allowed its repeated use but also resulted in its improved analytical performance and storage stability. As compared to many previously reported alcohol biosensors, the values on storage and operational stability and minimum detection limit in the linear response range obtained by using the fabricated biosensor towards the substrate alcohol were improved. The existing biosensor responds within 8 s at pH 7.5 with incubation temperature of 35°C. The finding confirmed the potential applications of the constructed biosensor in alcohol industries as it exhibits good correlation with the standard analytical methods.

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