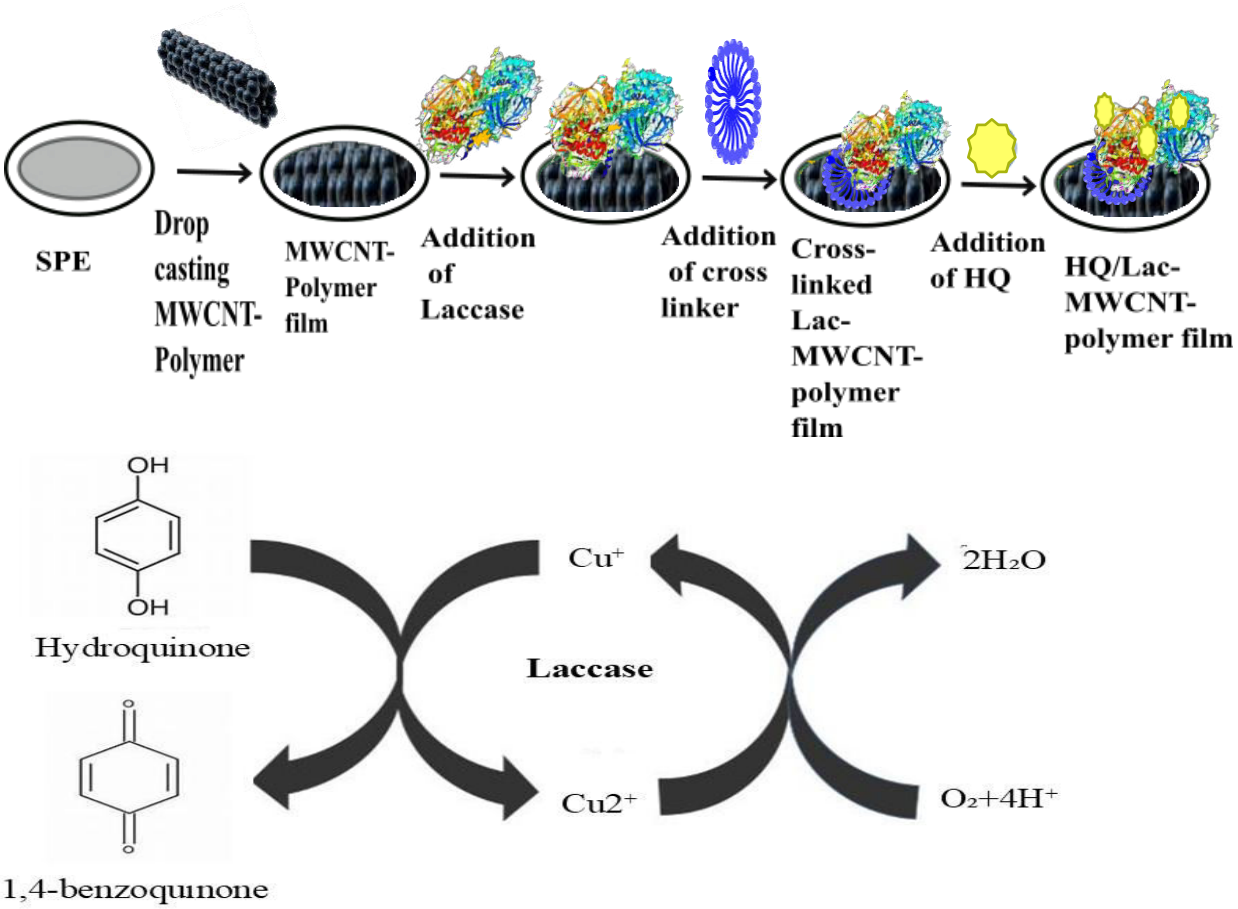
CHAPTER 2

NOVEL ELECTROCHEMICAL BIOSENSOR BASED ON IMMOBILIZED FUNGAL LACCASE FOR THE DETECTION OF HYDROQUINONE

Research highlights:

- Novel fabrication strategy was employed to design Laccase-based biosensor.
- Functionalized MWCNTs were incorporated into screen printed carbon electrode.
- Laccase molecules were bound on MWCNTs through amide bonds.
- The electrode showed linear range 50 to 1100 µM hydroquinone.
- Its lower and higher detection limits were 5 and 1700 µM respectively.

Graphical abstract:



2.1. Introduction:

Phenolic compounds are commonly found as chemical raw materials and by products in chemical industry (Lou et. al., 2018). They are broadly utilized in many industries like pharmaceuticals, pesticides, dyes, cosmetics, plastics, photography, rubber, paint, and tanning industries (Wang et. al., 2013). Some of them are hazardous being highly toxic and carcinogenic, besides they are non-degradable (Kulkarni et. al., 2013). Some phenolic compounds are common pollutants, produced by chemical industries, which can contaminate natural waters through the effluents (Abosadeh et. al., 2021). Hence, phenol has been identified as a priority pollutant by the US Environmental Protection Agency (EPA) (Kamel et. al., 2019). The acceptable limit set by EPA for phenol in surface water is less than 0.001 mg/L (Busca et. al., 2008) while the toxic level is in the range 9-25 mg/L (Kulkarni et. al., 2013).

One of the common phenolic compounds, hydroquinone (1,4-benzenediol) is commonly utilized in industries such as medicine, cosmetics, and pesticides (Tang et. al., 2014). Hydroquinone is generated during the initial phases of phenol oxidation, leading to a significant acceleration in the toxicity of phenol wastewater. Further, hydroquinone is highly toxic to all forms of life and its lower degradability when compared with the original phenol contaminants poses a hazard to the environment even at very low amounts (Enguita et. al., 2013). Brief exposure to higher concentrations of hydroquinone can cause headache, fatigue, tachycardia, kidney damage and cancer (Zhao et. al., 2006). Therefore, it is important to have a simple and highly efficient detection method for hydroquinone.

There are several traditional techniques available to detect phenols by various chromatographic techniques such as gas chromatography, liquid chromatography (Clement et. al., 1995), followed by spectroscopy and flame ionization detection (FID) for some derivatives of phenol (Robbins, R. J. 2003). Even though, these techniques provide accurate and ultimate results, they are complex and laborious involving skilled technicians and high throughput instrumentation. Sometimes the techniques encounter interference problems from other molecules in the samples and analysis in such cases requires sample purification. In most of the conventional methods, the analysis has to be carried out in centralized laboratories where sample collection, testing, report preparation and delivery consume time (Torre et al., 2002). Consequently, these methods do not leave scope for rapid and onsite testing analysis. Many a time, conventional methods suffer from poor analyte sensitivity. It is often observed that the biosensors are associated with the following

benefits: they are easy to use, quick in analysis, allow for onsite testing, have high sensitivity, and are reasonably priced (Torre et al., 2002).

Laccase (Lac) is a polyphenol oxidase enzyme that contains multiple copper ions and utilizes molecular oxygen to efficiently oxidize different phenolic compounds through a four-electron reduction of oxygen (O_2) to water molecule (Claus et al., 2004; Barton et al., 2001). This unique enzymatic capability allows laccase biosensors to directly catalyse the oxidation of hydroquinone to p-quinone without the need for solvents, enabling the quantitative determination of hydroquinone. Laccase has found extensive application in environmental wastewater treatment and the microbial conversion of natural products due to its extremely efficient biological catalysis. Furthermore, laccase-based biosensors are utilized in order to identify phenols in various contexts, including food analysis and environmental monitoring (Gamella et al., 2006; Torrecilla et al., 2007; Quan et al., 2004).

In recent years, enzyme electrode chips have gained considerable attention in biosensor research because of their capacity to direct electron transfer (DET). These chips are often used in conjunction with conductive carbon materials, particularly carbon nanotubes (CNTs), which exhibit optimal sensitivity, conductivity, a wide potential range (by increasing the electrode surface area), and the possibility for adding functional groups for the immobilization of enzymes (Lamas-Ardisana et al., 2008; Yang et al., 2016; Das et al., 2016). Functionalization of CNTs with carboxylic groups and amino groups is a frequently employed strategy (Babadi et al., 2016; Othman et al., 2016). Multi-walled carbon nanotubes (MWCNTs) have become preferred materials for sensor applications due to the properties of biocompatibility, electrochemical properties, quantum effects, surface area, high stability and adsorption characteristics which make them stand in good stead (Gupta et al., 2013; Qu et al 2013).

Screen-printed electrodes (SPE) are economical and can be used in the construction of analytical tools to monitor environmental pollution (Verrastro et al., 2016; Mohtar et al., 2018)

In the current study, a novel screen-printed carbon electrode (SPE) was fabricated by depositing functionalized MWCNTs and polymer mixture. Subsequently, laccase enzyme from *Trametes vesicolor* was bonded covalently to the film using a cross-linker. Laccase (Lac) acts on hydroquinone to produce 1, 4 benzoquinone. As shown in **Eqs. (2.1)** and **(2.2)** protons participate in the reduction process of laccase and combine with oxygen to form water.

$$HQ + Laccase\ (oxy) \rightarrow 1,4\ benzoquinone + Laccase\ (deoxy) + 2H^+ + 2e^- \quad (2.1)$$

$$Laccase\ (deoxy) + O_2 + 4H^+ \rightarrow Laccase\ (oxy) + 2H_2O \quad (2.2)$$

Electrons generated in this reaction are detected by the electrode.

2.2. Materials and Methods:

2.2.1. Reagents and Apparatus

Laccase from *Trametes versicolor*, (38429-1G) was purchased from Sigma Aldrich, India. MWCNTs (~ 10-20 nm & ~ 10 µM) were purchased from Ad nanotechnologies, India. Glutaraldehyde (03965) and polyvinyl alcohol (PVA - 0531500500) were bought from LOBA Chemie Pvt. Ltd., India. Na_2HPO_4 (sodium phosphate dibasic anhydrous - 1944143), NaH_2PO_4 (sodium phosphate monobasic anhydrous - 59443), Hydroquinone (25311), BSA (85171), KCl (84984) and Potassium ferricyanide (59558) were purchased from SRL Pvt. Ltd., India. Screen-printed carbon electrode was purchased from PalmSens BV, Netherlands.

All reagents, except for multi-walled carbon nanotubes (MWCNTs), purchased were of extra pure grade and used without further purification. Autoclaved Milli-Q water was employed throughout the experimental procedures.

2.2.2 Apparatus:

Cyclic voltammetry was carried out from -0.3 to + 0.5 V at 0.1 V/s scan rate on screen printed carbon electrode in 50 mM K_3 [Fe (CN_6)] with 0.1 M KCl in 50 mM phosphate buffer, pH 5.5. EmStat3$^+$ electrochemical workstation (PalmSens BV, Netherlands) was used to carry out CV measurements. Locally manufactured ultrasonicator bath was used to sonicate MWCNTs in PVA polymer to prepare a suspension. FEG-SEM (Field emission gun scanning electron microscope-JSM-7600F; Jeol, USA) was used to study the surface of the bare and coated electrodes.

2.2.3. Fabrication of Laccase/Glutaraldehyde/MWCNTs/PVA/SPE electrode:

In this study, MWCNT based fabrication chemistry was utilized to prepare the electrode. Screen printed carbon electrode used to develop the electrochemical sensor contains working, reference, and counter electrodes, all three on the same chip. Firstly, 1 mg of MWCNTs was suspended into 0.5 ml of PVA prepared at 1mg/mL concentration. The suspension was sonicated to homogenize for 3 hr in an ultrasonicator bath. Ice packs were kept

into the ultrasonicator bath to keep the suspension cool. To prepare the electrode, 4µl of the MWCNT solution in PVA was dropped onto SPE and air dried at room temperature (RT) for 20 minutes. Then, 25 µL of laccase (10 mg/mL) dissolved in pH 7.0, 50 mM phosphate buffer was layered on to it. The laccase solution was allowed to air dry at RT for around 30 min. afterwards, 2 µL of 2.5% glutaraldehyde was added on to the electrode surface to cross link to PVA. The solution was permitted to air dry at room temperature and the electrode was washed three times with phosphate buffer to remove excessive glutaraldehyde and again allowed it to air dry. The procedure was adopted from our earlier work with a glucose biosensor (Gupta et al., 2016). This was then followed by pre-treating it with 10 µl of 1% BSA for 10 min to block nonspecific binding (Ishikawa et al., 2009). The electrode was washed with Milli Q water several times. At a scan rate of 100 mV/s, the electrolyte solution of 50 mM K_3 [Fe $(CN)_6$] in 50 mM phosphate buffer (pH 7.0) was tested for change in the current in a 50 µL system. Then the electrode was washed with phosphate buffer, pH 7.0. The electrode was air dried at room temperature and stored at 4°C. The electrodes with and without laccase enzyme were compared for performance by running cyclic voltammetry from -0.3 to 0.5 V in 50 µl of 0.1 M KCl and 50 mM K_3 [Fe $(CN)_6$] prepared in 50 mM phosphate buffer, pH 5.5.

2.3. Results and Discussion:

2.3.1 Surface morphology studies:

A field emission gun scanning electron microscope (FEG-SEM) (Fig. 1) was employed to observe the structural properties of the electrodes bare SPE, SPE/MWCNTs, and SPE/MWCNTs/GA/Laccase. The electron micrographs given in **Fig. 2.1(a), 2.1(b) and 2.1(c)** clearly display distinct changes in the surface of the bioelectrode during every stage of its development. In the first step, the bare electrode in **Fig. 2.1(a),** was layered with the dispersion of MWCNTs displayed a filamentous layer as indicated in **Fig.** 2.**1(b).** Immobilization of laccase using GA revealed a porous structure on the electrode surface **Fig. 2.1(c)**.

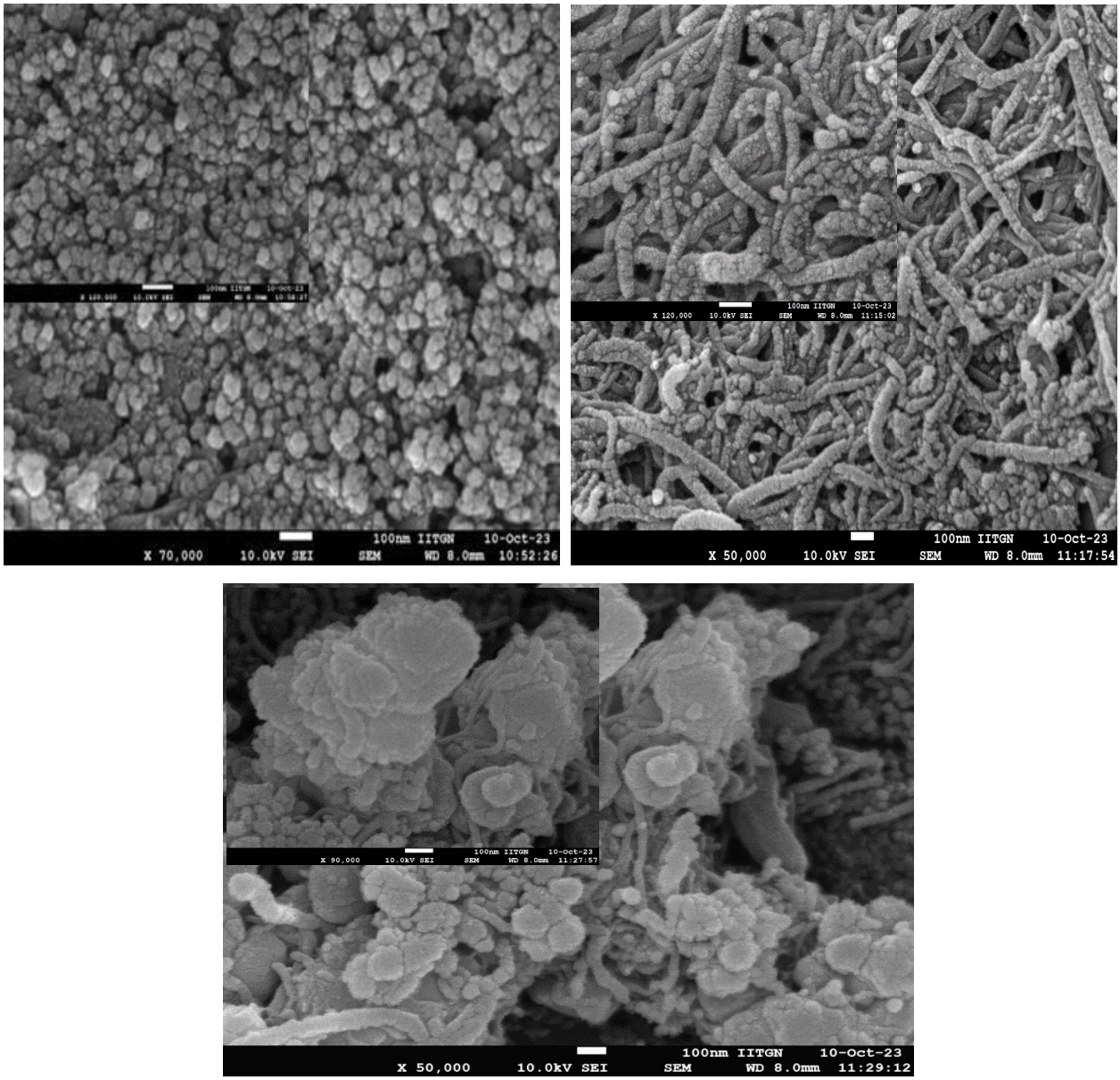


Fig. 2.1. FE-SEM micrographs of (a) SPE, (b) SPE/MWCNTs, (c) SPE/MWCNTs/GA/Laccase

2.3.2 Electrochemical behavior of Laccase/Glutaraldehyde/MWCNTs/PVA/SPE electrode:

The results of the cyclic voltammetry carried out on bare SPE and Laccase/Glutaraldehyde/MWCNTs/PVA/SPE in 50 mM $K_3[Fe(CN)_6]$, 0.1 M KCl in 50 mM phosphate buffer (pH 7.0) at 100mV/s scan rate are shown as voltammograms (CVs in) **Fig. 2.2(a)**. SPE showed a stronger signal and the current decreased after layering Laccase/Glutaraldehyde/MWCNTs/PVA mixture. It is well known that MWCNTs are good for the transportation of electrons but laccase, greatly hindered the electron mobility between electrolyte and electrode. The coated electrode Laccase/Glutaraldehyde/MWCNTs/PVA/SPE was then tested for varying scan rates between from 80 to 160 mV/s and the results are shown in **Fig. 2.2(b)**. The resultant voltammograms showed sharp redox peaks at all the scan rates tested. The values of anodic and cathodic peaks varied linearly with the scan rates. However, as the scan rate towards higher negative potentials. **Fig. 2.2(c)** displays a linear graph of scan rate against the increased anodic peaks moved towards higher positive potential and cathodic peaks moved anodic

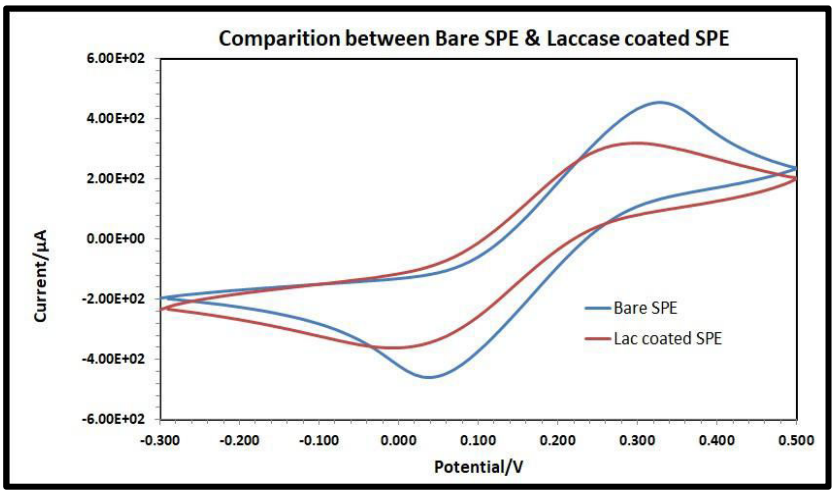


Fig. 2.2 (a). Cyclic voltammetry scans of bare SPE & SPE/MWCNTs/GA/Laccase coated electrode in 50 mM phosphate buffer, pH 7 with 50mM $K_3[Fe(CN)_6]$ and 0.1 M KCl at 100 mV/s scan rate.

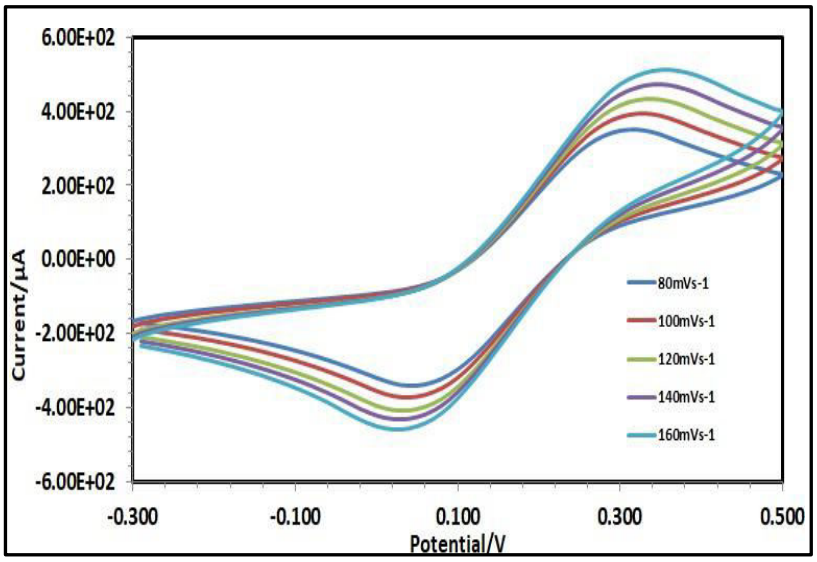


Fig. 2.2. (b). Cyclic voltammetry scans of SPE/MWCNTs/GA/Laccase coated electrode at different scan rates from 80 mV/s to 160 mV/s in 50 mM phosphate buffer, pH 7 with 50mM $K_3[Fe(CN)_6]$ and 0.1 M KCl.

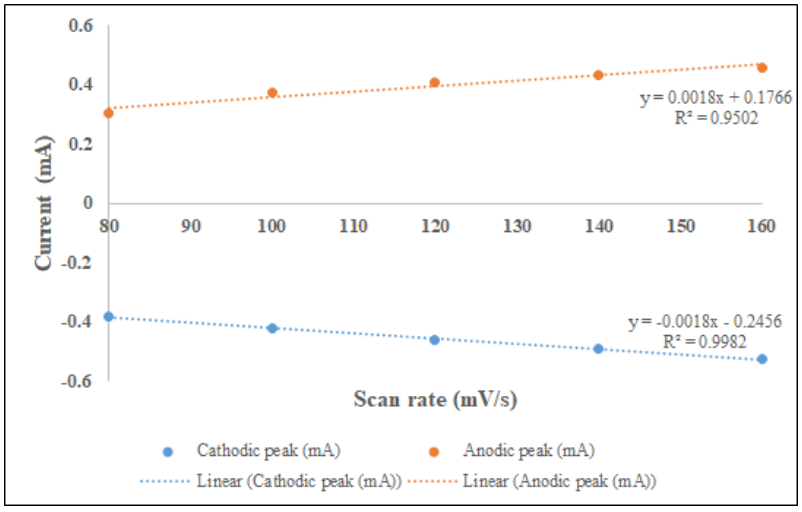


Fig. 2.2 (c). Plots of the corresponding anodic and cathodic peak current vs. scan rates in 50 mM phosphate buffer, pH 7 with 50mM $K_3[Fe(CN)_6]$ and 0.1 M KCl at 100 mV/s scan rate.

and cathodic peaks. The R^2 values of anodic and cathodic peaks were determined and found to be 0.95 and 0.99 respectively. Thus, cathodic peak showing better correlation was considered for further experimentation to check hydroquinone concentration. Moreover, the ratio of cathodic and anodic peak currents (Ipc/Ipa) approximated to 1. These results are in agreement with the observation that the electron transfer followed a surface-controlled process.

2.3.3 Effect of pH on Laccase/Glutaraldehyde/MWCNTs/PVA/SPE:

Cyclic voltammetry was carried out at a scan rate of 100 mV/s to examine the effect of pH on the laccase immobilized SPE in a solution of 50 mM $K_3[Fe(CN)_6]$ and 0.1 M KCl in 50 mM phosphate buffer solution in the pH range of 5.0 to 8.0 **(Fig. 2.3a)**. The concentration of hydroquinone was 1 mM in the experimental solution. The anodic and cathodic peak currents reduced when the pH rose from 5.0 to 8.0, which can be explained by a decrease in the number of positively charged moieties in the electrolyte.

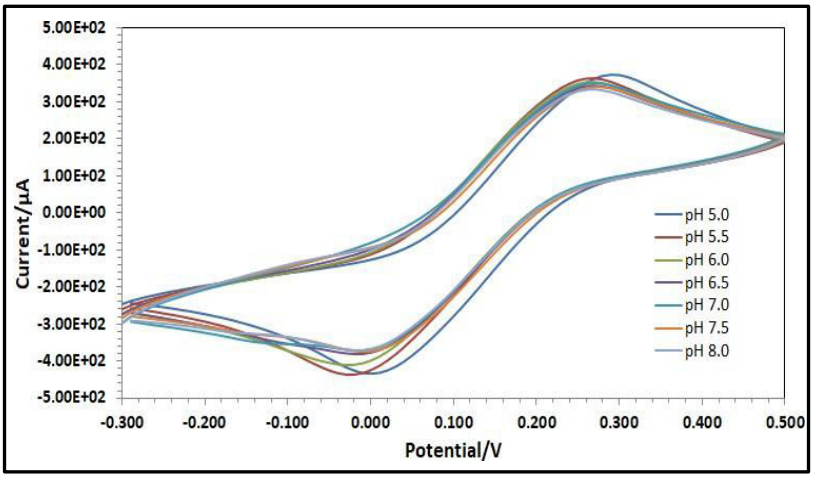


Fig. 2.3 (a). Cyclic voltammetry scans of SPE/MWCNTs/GA/Laccase coated electrode as function of pH in 50 mM phosphate buffer, with 50mM $K_3[Fe(CN)_6]$ and 0.1 M KCl at 100 mV/s scan rate.

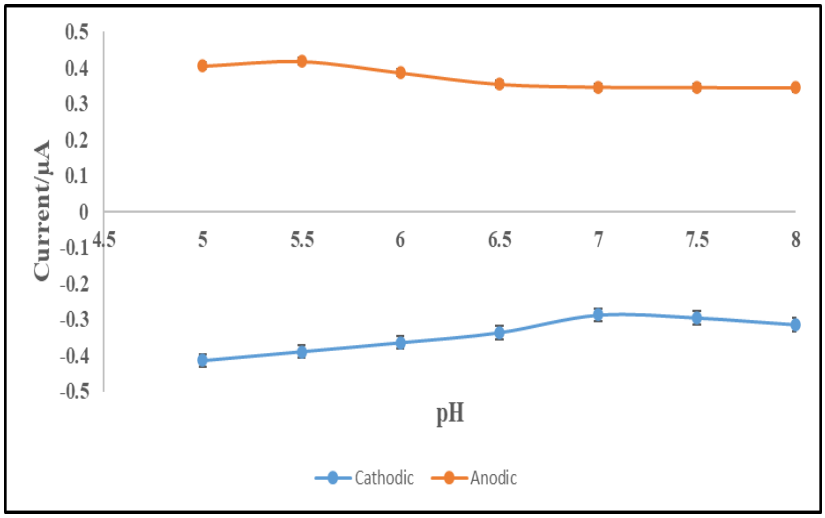


Fig. 2.3 (b). Effect of pH on the current density of anodic and cathodic peaks of SPE/MWCNT/GA/Laccase electrode in in 50 mM phosphate buffer with 50mM $K_3[Fe(CN)_6]$ and 0.1 M KCl containing 1000 µM hydroquinone at 100 mV/s scan rate.

Laccase immobilized electrode participates in the reduction of O_2 molecule to two water molecules. Also, it is evident from the graph **Fig. 2.3(b)** that change in the current with respect to change in the pH follows linearity at cathodic peak with an R^2 value of 0.9759. Hence, cathodic peak was considered as the measure of magnitude of the current at pH 5.5 and was considered as optimal value for all further experiments.

2.3.4 Reproducibility and storage of the electrode:

Three screen printed electrodes were coated simultaneously to obtain Laccase/Glutaraldehyde/MWCNTs/PVA/SPE. Current response of all the three electrodes was measured in 50 mM phosphate buffer (0.1 M KCl, 50 mM $K_3[Fe(CN)_6]$; pH 5.5). Margin of error for the sensor replication procedure was calculated by comparing both anodic and cathodic peak responses. **Fig. 2.4(a)** proves that the relative standard deviation (RSD) of cathodic peak is 0.56% and is reproducible. The sensor electrode's enzyme activity was measured every six days while it was kept dry at 4°C. Current response of the sensor was 85.89% compared to its original current response even after the lengthy storage of 30 days **Fig. 2.4(b)**.

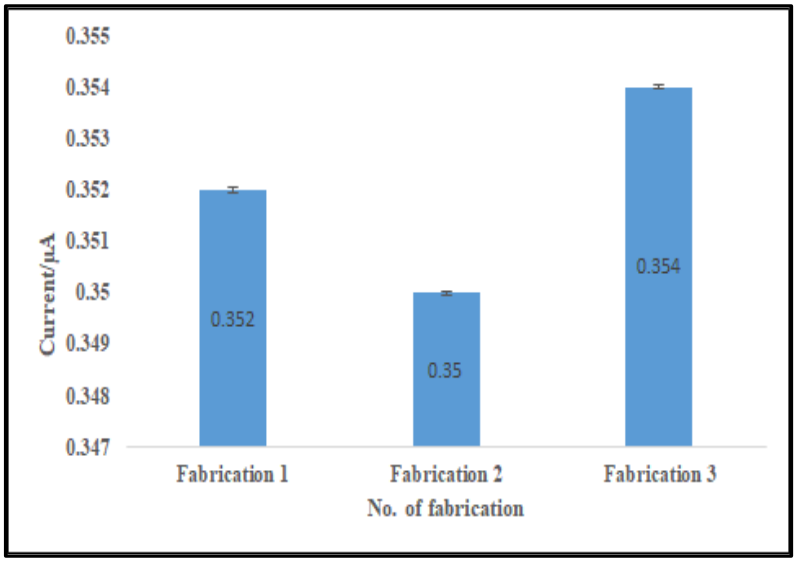


Fig. 2.4 (a). Cathodic peak currents of SPE/MWCNTs/GA/Laccase electrode in triplicates in 50 mM PB (50mM $K_3[Fe(CN)_6]$, 0.1M KCl, pH 5.5) at 100 mV/s scan rate(n=3)

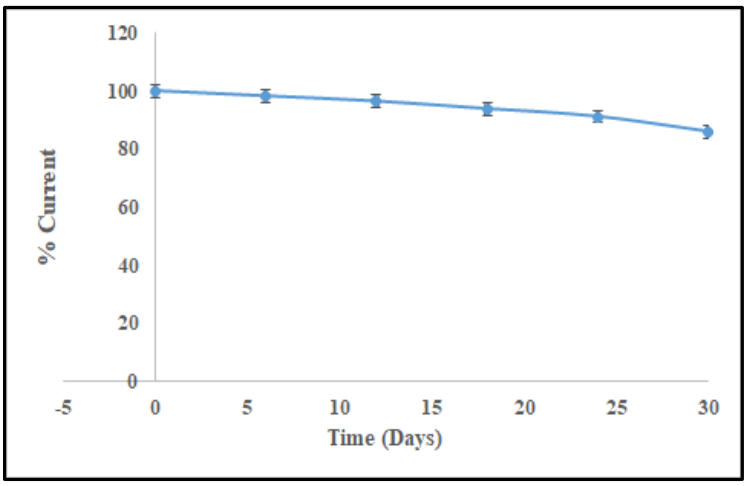


Fig. 2.4 (b). Storage stability of electrode monitored at 6 day intervals.

2.3.5 Cyclic voltammetric detection of hydroquinone:

Cyclic voltammetric response at cathodic sweep segment of the SPE/MWCNTs/GA/Laccase biosensor as a function of hydroquinone (HQ) concentration was recorded in 50mM $K_3[Fe(CN)_6]$, 0.1 M KCl in 50 mM phosphate buffer (pH 5.5) at the scan rate of 100 mV/s. It is evident from **Fig. 2.5** that as the concentration of HQ increases, cathodic peak current increases. This increase in magnitude of the current can be explained by the pH sensitive behavior of the biosensor. Protons were released during the enzymatic reaction by laccase, which in turn altered the pH of the reaction system. This change in pH ultimately led to change in magnitude of the current. The calibration curve for hydroquinone (HQ) detection exhibited a biphasic response across the tested concentration range (5–1200 µM), reflecting distinct phases of sensor behavior. At lower HQ concentrations (5–200 µM), the current increased linearly with concentration, indicating efficient electron transfer and diffusion-controlled kinetics due to ample active sites on the electrode surface. However, at intermediate concentrations (200–600 µM), deviations from linearity were observed, likely due to partial electrode surface saturation, accumulation of oxidation products (e.g., benzoquinone), mass transfer limitations, and ink leaching which collectively reduced sensitivity. Experimental factors, including uneven HQ distribution or electrochemical noise, may have further contributed to inconsistencies in this range (Monge-Romero, I. C., & Suárez-Herrera, M. F. 2013; Volgin, V.M. & Davydov, A.D. 2012).

The observed biphasic response in biosensor systems, underscores the interplay between diffusion, reaction kinetics, and surface phenomena. Despite these challenges, the sensor demonst

-rated a reliable linear response (R^2 = 0.974) at low HQ concentrations, with detection limits of 50–1100 µM, making it suitable for environmental and clinical HQ monitoring applications.

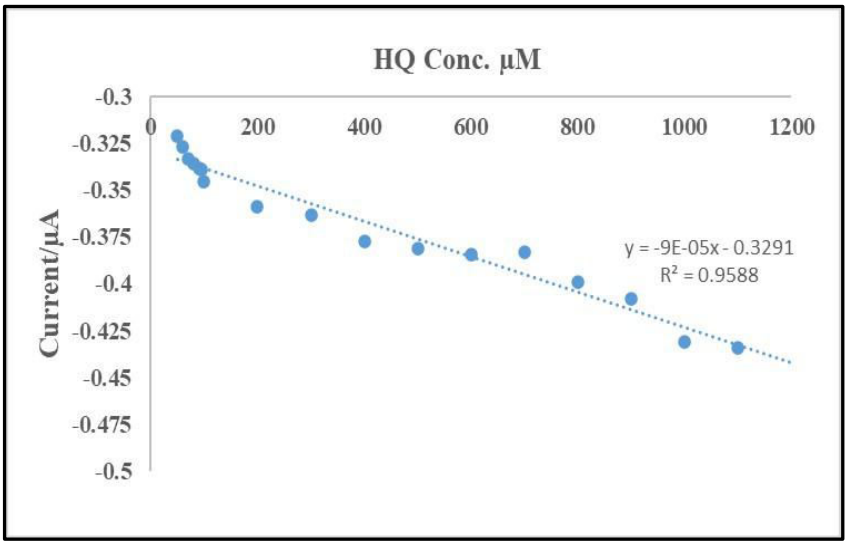


Fig. 2.5. Calibration curve of the current vs the concentration of hydroquinone in 50 mM phosphate buffer, pH 5.5 with 50mM $K_3[Fe(CN)_6]$ and 0.1 M KCl at 100 mV/s scan rate.

At intermediate hydroquinone (HQ) concentrations, deviations or irregularities in the current response can be attributed to several factors. The electrode surface may transition from an under-saturated to a partially saturated state, leading to variability in electron transfer processes. Mass transfer limitations could also play a role, as inconsistent diffusion of HQ molecules from the bulk solution to the electrode surface might result in unexpected stabilization or drops in current response. Additionally, competing kinetics, such as the balance between HQ oxidation and the accumulation of oxidized products like benzoquinone (BQ), could disrupt the reaction mechanism. Partial fouling of the electrode surface by BQ may temporarily hinder the oxidation process, creating gaps in the response. Electrochemical noise or instability, such as fluctuations in applied potential, solution homogeneity, or electrode surface conditions, can further contribute to inconsistencies, particularly in this concentration range. Lastly, experimental factors, including uneven HQ distribution, minor mixing inconsistencies, or variations in experimental conditions, may lead to irregular data points. These observations highlight the importance of optimizing the experimental setup and interpreting results cautiously within this critical transition zone to ensure accurate sensor performance. As seen in **Fig. 2.5**, change in the cathodic peak current magnitude with respect to the HQ concentration followed linear regression model with the R^2 value of 0.974. The lower and higher detection limits of HQ concentration were found to be 50 and 1100 µM, respectively.

The biphasic response observed in this study is a common phenomenon in biosensor systems and highlights the critical interplay between diffusion, reaction kinetics, and electrode surface phenomena. Overall, the high sensitivity and reliable linear response at low concentrations demonstrate the biosensor's suitability for detecting HQ in environmental and clinical applications.

2.3.6 Interference study:

Selectivity of a sensor is critical when it comes to practical application. To estimate the anti-interference of the biosensor, the impact of some possible interfering substances was examined under ideal conditions. Herein, the substances checked for interference were phenolic compounds (2-Nitro phenol), protein (BSA-bovine serum albumin), small molecules (glucose, cysteine, and L- lysine), $CuSO_4$ (copper sulphate), KCl (potassium chloride), DNSA (3,5- Dinitrosalicyclic acid), Uric Acid. Current response examined by the biosensor in solution with the substrate and the interferents in the ratio of 1:1. **Fig**. 2.**6**. The current either became low or negligible with the addition of every interferent used in the study.

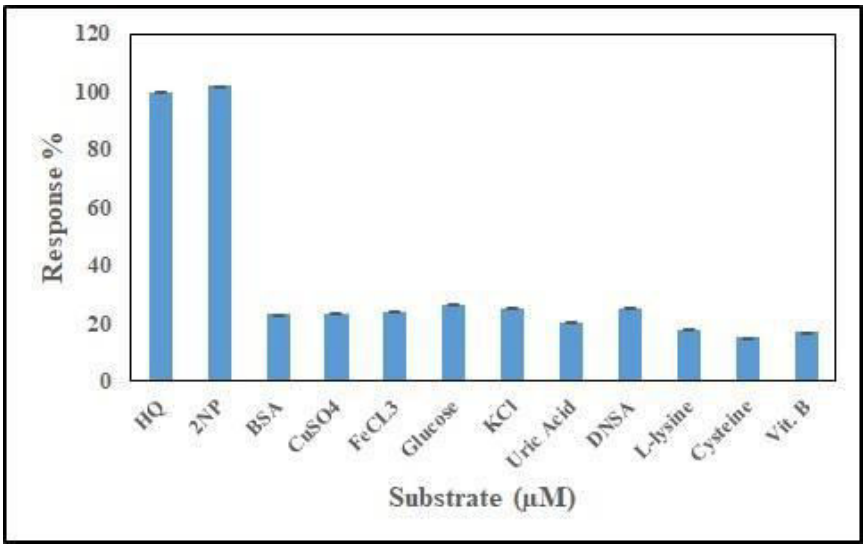


Fig. 2.6. Comparison of the response of SPE/MWCNTs/GA / in 50 mM phosphate buffer, pH 5.5 with 50mM $K_3[Fe(CN)_6]$ and 0.1 M KCl containing different interferent at 100 mV/s scan rate (n=3).

Interefrence often caused by various factors. Electroactive compounds like ascorbic acid, uric acid, and phenolic substances, which have similar redox potentials as the target analyte, can lead to competitive reactions and skewed results. Environmental factors such as pH fluctuations, temperature changes, and oxygen availability can disrupt enzyme activity and

sensor performance. Additionally, inhibitors such as heavy metals or chemical agents like azide and cyanide can directly block enzyme function.

2.3.7 Real sample detection:

To check the performance of the biosensor SPE/MWCNTs/Laccase/GA with real samples, the electrode was checked against sewage water, sewage water spiked with HQ, tap water, tap water spiked with HQ dissolved in a solution of 50mM PB (K_3 [Fe $(CN)_6$], 0.1 M KCl, pH 5.5). The scan rate for the samples was 100 mV/s. The results showed reliable current change at cathodic sweep segment of cyclic voltammogram. Recovery of the real sample was reported in the **Table 2.1** for the sewage water, sewage water spiked with HQ, tap water and tap water spiked with HQ respectively.

Sample	Added (µM)	Found (µM)	Recovery %
Tap water	0	0	0
Spiked tap water	1000	989	98.9
Sewage water	0	20	0.02
Spiked sewage water	1000	1015	101.5

Table 2.1. Hydroquinone detection of real samples by SPE/MWCNT/GA/Laccase electrode.

2.4 Conclusion:

In summary, SPE/MWCNTs/GA/LACASE biosensor was successfully fabricated approaching a novel chemistry for fabrication. Enzyme laccase was immobilized on the MWCNTs with glutaraldehyde covalent crosslinking. The electrode showed the linearity for hydroquinone (HQ) from 50 to 1100 µM with the detection limit of 5 to 1700 µM. The electrode was checked for its specificity in the presence of various interfering molecules. The biosensor showed good specificity for hydroquinone. The biosensor was checked against real samples for its practical application. The biosensor can be replicated with the SD of 0.56%. The biosensor is expected to serve as promising tool to detect hydroquinone. It will be interesting to immobilize other enzymes to develop biosensors for a variety of molecules with the same fabrication chemistry.