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Laccase Enzyme Electrode with Ferrocene-Monocarboxylic Acid Mediator for Determination of Phenol

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Laccase biosensor with ferrocene-based mediator for determination of phenol using voltammetry is discussed. Phenols are major constituents of several industrial effluents. Phenols were first isolated from coal tar and occur widely in nature particularly in protein-containing materials. Phenols are toxic to fish at levels above 2 µg/L and fatal in humans at 1 µg/L. Thus discharge of phenolic compounds into the environment is hazardous as they have the potential to contaminate superficial and underground water. Laccase oxidizes many different substrates including *ortho*- and *para*-substituted mono- and polyphenols, polyamines, lignins and aryl diamines as well as arscobate ion. Laccase is not a very strong oxidant and in some cases requires electron transfer mediators. Ferrocene monocarboxylic acid (Fc-COOH) was used as an electron transfer mediator for laccase. Thus a mixture of laccase enzyme and ferrocene monocarboxylic acid dissolved in phosphate buffer (pH 6.5), at a glassy carbon working electrode, was used as a biosensor for detection of phenol. The laccase biosensor was applied in wastewater samples collected from industrial effluents of coal-based power generation and coal mining plants. The results obtained show that the laccase electrode is suitable for determination of trace amounts of phenols at picomolar (pM) levels. Detection limit of 1.0 pM, linear range 0.5-100 pM and sensitivity of 0.2 µA/pM, were obtained. The coal-based effluent sample was found to contain an average of 47.7 pM of phenol.

Key Words: Laccase biosensor, Phenols, Ferrocene-based mediators, Voltammetry, Coal mining effluent.

INTRODUCTION

Phenols are a class of aromatic hydroxy compounds that have at least one hydroxyl group directly attached to the benzene ring[1]. They are major constituents of wastes from refineries and mineral ore mines and can be a source of serious health hazards if released into the environment[2]. Thus discharge of phenolic compounds into the environment is hazardous as they have the potential to contaminate superficial and underground water.

Phenols are toxic to fish at levels higher than 2 µg/L and fatal in humans[2] at 1 µg/L. Effluents from these industries have the potential of contaminating underground water and surface water sources. This makes it very important to monitor and control the level of phenolic pollutants in industrial effluents. The detection of mono-, di- and polyphenols is usually done by high performance liquid chromatography (HPLC) coupled to spectrometry[3]. However, spectrometric techniques are expensive, reagent- and time- consuming. Biosensors are an attractive alternative due to their unique characteristics such as selectivity, the relative low cost of fabrication and storage, potential of miniaturization and easy automation and rapid operation. Laccase is a copper containing oxidase that is widely distributed in fungi, higher plants, some bacteria and some insects[4]. Laccase is able to oxidise many different substrates including *ortho*- and *para*-substituted mono-, di- and poly- phenols with the concomitant reduction of oxygen to water ($O_2 + 4H^+ + 4e^- \rightleftharpoons 2H_2O$). Since the enzyme exhibits broad substrate specificity, its selective catalysis has been enhanced by addition of redox mediators[5]. Ferrocene derivatives and other electrochemically active species have been employed in diffusional mediated electron transfer. The mediator shuttles electrons between the electrode and enzyme active site. Penetration of mediator into the enzyme active sites of the protein matrix depends on the hydrophilic/hydrophobic properties of both the mediator and enzyme, the shape and size of the mediator and the electrostatic charge interactions of mediator and enzyme[6]. This study employed laccase with ferrocene monocarboxylic acid (Fc-COOH) mediator for the detection of phenols.

Cyclic voltammetry (CV) can be used to determine the electrochemical reversibility of a given system[7]. Thus CV has been used in this study to determine (i) the peak potential difference (ΔE_p) between anodic and cathodic scans, that is, $\Delta E_p = |E_p^A - E_p^C|$ where E_p^A and E_p^C are anodic and cathodic peaks, respectively and (ii) the current intensity ratio $|I_p^A/I_p^C|$ where I_p^A and I_p^C are anodic and cathodic currents, respectively. For a complete reversible system, it is expected that $\Delta E_p = 59/n$ mV and $I_p^A/I_p^C| = 1$. In addition, peak current, I_p is linearly proportional to the square root of scan rate, $|I_p| \alpha \nu^{1/2}$. For totally irreversible systems, no reverse scan peaks are observed whereas for quasi-reversible system, $|I_p|$ varies with $\nu^{1/2}$ but not necessarily in a linear manner. In addition, the peak potential difference, ΔE_p is > 59/n mV and increases with increasing scan rate (ν). The diffusional coefficient of the mediator is calculated using Randle-Seucik equation[7]. The Randle-Seucik equation is given as: $I_p = 2.969 \times 10^5 n^{3/4} C_o D^{1/2} A \nu^{1/2}$ where I_p is the peak current, n is the number of electrons, C_o the concentration of mediator at the electrode surface, D the diffusion coefficient, A the electrode surface area and ν the scan rate. Under specified

conditions, the quantities n, C_o, A and ν are known and therefore the diffusion coefficient D can be calculated from the slope of the plot, I_p *vs.* $\nu^{1/2}$.

Differential pulse voltammetry (DPV) is mainly used for quantitative analysis as it is more sensitive than CV[7]. Increasing the concentrations of the analyte may either increase or decrease peak current (I_p) intensities. The reason for using voltammetric techniques in this work were to: (i) study the redox properties of laccase enzyme, Fc-COOH mediator and phenol substrate with CV; (ii) apply the laccase biosensor in solution form (enzyme mixed with mediator) to carry out quantitative determination of phenols with DPV in real samples from coal mine effluent.

EXPERIMENTAL

Electrochemical measurements namely, CV and DPV were carried out on Bioanalytical System (BAS) Pty Ltd., (Indiana, USA) namely, BAS EC Epsilon electrochemical workstation. A 25 mL electrochemical cell (obtained from BAS) was used in a three-electrode configuration. Platinum disk electrode (surface area 0.0254 cm^2) or glassy carbon working disk electrode (surface area 0.1104 cm^2), platinum mesh counter electrode and Ag/AgCl (saturated) reference electrode, all purchased from BAS, were used. A magnetic stirrer (Schott, Germany) was used to stir solutions before recording electrochemical measurements.

The reagents used include; laccase (*Trametes versicolor*) purchased from Fluka (Switzerland), phenol (SAARCHEM, South Africa), ferrocene monocarboxylic acid (Fluka, Russia) mediator, potassium dihydogen orthophosphate (NT Laboratory Supplies, South Africa), sodium hydroxide (Aldrich, South Africa), alumina powder and microcloth pads purchased from BAS used for cleaning the electrodes and Millipore ultra pure water for preparing reagents.

Cyclic voltammetric scans were obtained (between 600 mV and -100 mV) for each of the following solutions in phosphate buffer (0.1 M, pH 6.5) separately; buffer blank, Fc-COOH (0.2 mM), laccase enzyme (4 mg), phenol (0.1 μM); a mixture of laccase and Fc-COOH in the absence and presence of phenol substrate.

The procedure for laccase biosensor analysis was as follows: laccase enzyme (4 mg) was added to 25 mL of phosphate buffer in the voltammetric cell, Fc-COOH mediator (0.2 mM) was added to the cell, the contents were thoroughly mixed using a magnetic stirrer and allowed to settle before taking any measurements. A potential scan using cyclic voltammetry was obtained between 600 mV to -100 mV in a cathodic scan *i.e.* positive to negative potentials as per polarographic notation given in the BAS operational manual[8]. (NB: Anodic scan is from negative to positive potentials). Phenol solution (substrate) was then added (0.1 μM) and the potential scan was repeated.

The parameters optimized were pH of the blank buffer and the concentration of the mediator. The effect of the pH of the blank was investigated by preparing the buffer at different pH values and using each buffer solution to prepare a mixture of enzyme, mediator and phenol. The amount of enzyme (4 mg), mediator (0.2 mM), phenol (0.1 μM) and buffer concentrations (0.1 M) were held constant as the pH was varied. The effective pH region of phosphate buffer[9] is between 5.8 and 8.2 and therefore the effect of pH was varied within this range.

Laccase enzyme (4 mg) was first added in the cell containing 20 mL of phosphate buffer, followed by ferrocene monocarboxylic acid mediator (0.2 mM) and then 0.1 μM phenol. The cell contents were mixed thoroughly with a magnetic stirrer. A potential scan of the solution was obtained. The experiment was repeated with different mediator concentrations up to 30 mM while both the amount of enzyme and phenol concentrations, were held constant.

Wastewater samples were collected from Morupule coal mine effluent (Sump) in Botswana. A given amount of the sample (10 mL) was mixed with the buffer solution and spiked with different concentrations of phenol standards (1.0 to 100 pM) in the cell containing laccase biosensor. DPV was used for determination of phenol in real samples.

RESULTS AND DISCUSSION

Redox studies using cyclic voltammetry

A full scan of phosphate buffer (0.1 M) blank between 1.000 V and -0.300 V at scan rate of 5 mV s^{-1}, gave a cathodic peak at 0.118 V with a current intensity of 1.19 μA. This peak is possibly due to reduction of the phosphorous in the phosphate group (PO_4^{3-}) from oxidation state +5 to +3 in PO_3^{3-}. The experiment was repeated with phenol (1.0 μM) in buffer blank (600 to -100 mV). The presence of phenol caused a peak shift from 0.118V to 0.131 V. The potential difference (ΔE_p) between blank and phenol was 0.013V. The observed peak shift is too insignificant to account for a new phenol peak and therefore may only be due to dilution effect or could be a result of interaction between the buffer and the phenol compound. The current intensity after adding phenol was 1.23 μA (difference in current = 0.04 μA). The difference in current, again, could be the result of blank-phenol interaction. This would then imply that phenol is neither reduced (during forward scan) nor oxidized (during the reverse scan). The possible explanation for this observation may be that under these current experimental conditions, it is not possible to reduce/oxidize phenol. This is supported by literature reports that direct electrochemical oxidation of phenols suffer a number of drawbacks[10]. These include a high overvoltage

during direct electrochemical oxidation of phenol hence the need to apply high anodic potential which would in turn open up the detection system to interfering reactions. Moreover, direct electrochemical oxidation of phenols is coupled with fouling reactions. Hence the use of enzyme based biosensors to overcome these obstacles has been reported[11-15]. Thus enzymes such as laccase[11,12], tyrosinase[13,14] and peroxidase[15] have successfully been integrated with electrochemical transducers in the electrochemical determination of phenols.

The above experiment was repeated with laccase enzyme alone in the buffer without phenol. Though a slight shift in potential peak of the blank was observed (ΔE_p = 0.001 V), the shift is too small to account for an independent peak. The absence of the enzyme peak could be explained by the fact that active site of an oxidoreductase is seldomly found on the surface of the enzyme[16]. Consequently, the electrochemistry of the enzyme is typically plagued by poor kinetics of heterogenous electron transfer between the enzyme and the electrode. Thus, it is assumed in this study that the absence of an enzyme peak could be explained by the heterogenous electron transfer phenomena. The problem of poor kinetics of the heterogeneous electron transfer has been circumvented by using redox mediators such as ferrocene derivatives. Other solutions to the problem of poor kinetics include either the use of surface promoters or site-directed mutagenesis, to alter the structure of the enzyme with the aim of improving the heterogenous electron transfer between the enzyme active site and the electrode surface[16].

Cyclic voltammetry of the mediator (Fc-COOH) alone in the same potential range (600-100 mV) as phenol and laccase, was done and the voltammograms are shown in Fig. 1 which illustrates a clear difference between the blank and the mediator scans. The forward (initial scan direction) was from positive to negative potential values (cathodic) while the reverse scan was anodic. The peak for buffer alone occurred at a potential of 118 mV with current of 1.19 µA. After adding the mediator, Fc-COOH (5 mM), a voltammogram with two peaks obtained in the forward scan, were observed. The first peak (**I**) appeared at 271 mV with current intensity of 1.80 µA while a second peak (**II**) appeared at 118 mV with current of 1.16 µA. The reverse scan also showed two peaks, the first one at 337.5 mV (**III**) with current of 1.7 µA and the second one at 352 mV (**IV**) with current of 2.5 µA. These observations confirm that the mediator unlike the laccase enzyme or phenol, showed electrochemical activity under the given experimental conditions. It is noted that during the forward scan, peak **II** at 118 mV most likely represents the buffer while peak **I** at 271 mV is that of the Fc-COOH resulting from reduction of the electrochemical centre (Fe^{3+} to Fe^{2+}) in the ferrocene compound[6].

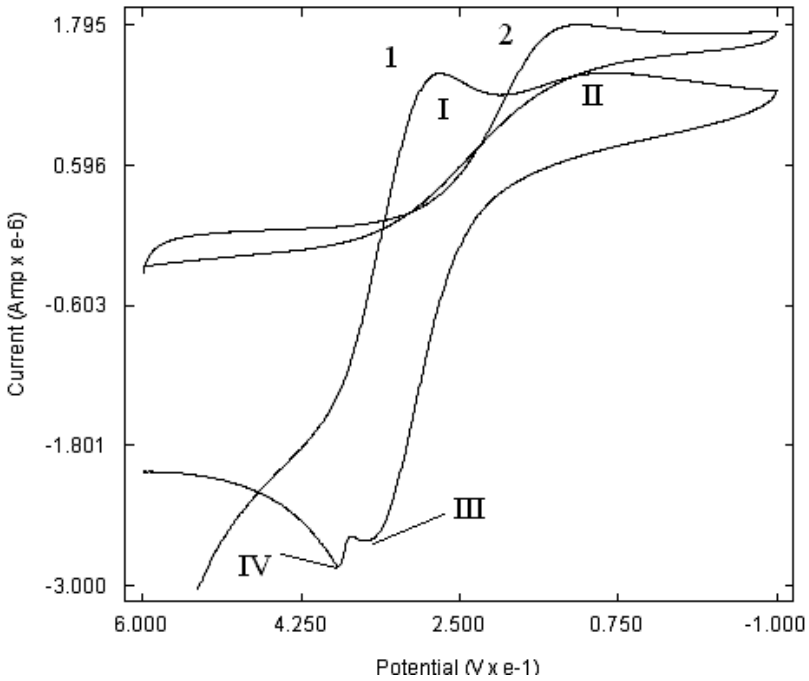


Fig. 1. Cyclic voltammograms for Fc-COOH in 0.1 M phosphate buffer, (1) buffer alone and (2) 5mM Fc-COOH in buffer; **I** to **IV** are peaks

Fig. 2 shows cyclic voltammograms of ferrocene monocarboxylic acid (Fc-COOH) at different scan rates, ν, in a cathodic scan (polarographic notation)[8]. From the voltammograms, it implies that Fc-COOH has significant electrochemical reversibility as indicated in Table-1 showing peak potential (E_p) and currents (I_p) obtained from the voltammograms. The forward scan was anodic and the peaks (E_p^A) were all more positive than the corresponding cathodic peaks (E_p^C). The potential difference between the anodic and cathodic peaks ($\Delta E_p = E_p^A - E_p^C$) were greater than 59/n mV by a factor of 1.2 to 2.0 (with an average of about 1.6) times the expected value of 59 mV for a one electron redox reaction (*i.e.* 59/n when n = 1). One may be tempted to conclude that the redox process of Fc-COOH involves 1.5 electrons. On the other hand this may be an artefact due to possible inconsistency of the experimental conditions which result in the anodic (E_p^A) sweep to record artificially higher values than the cathodic scan (reverse direction) which may be caused by electrode fouling. The current intensities showed an increase with scan rates but the trends for both anodic and cathodic values were both irregular. As a result, the current ratios $|I_p^A/I_p^C|$, are shown to be *ca.* 1 for all scan rates. A value of $|I_p^A/I_p^C| = 1$ imply that the system is electrochemically reversible.

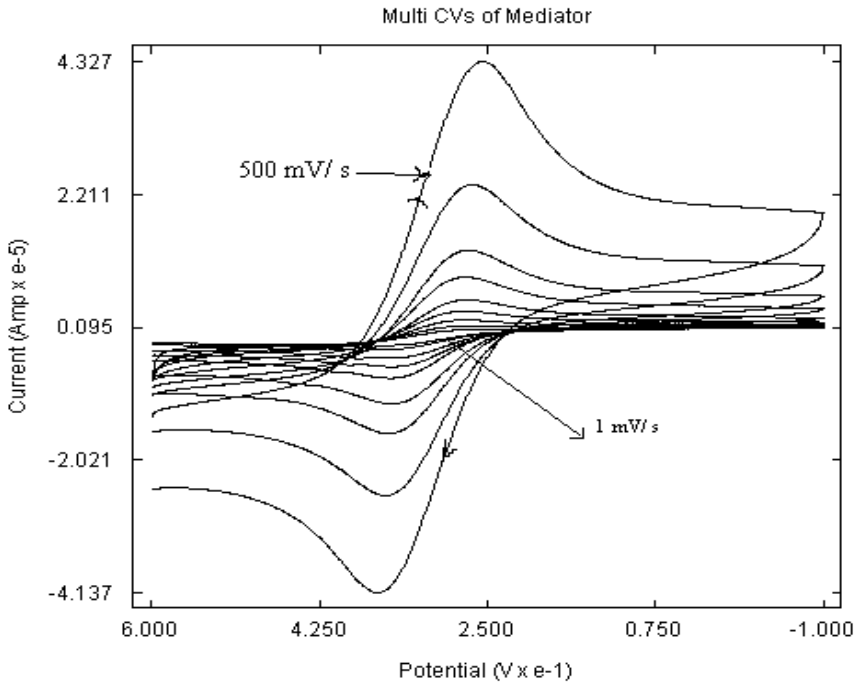


Fig. 2. Cyclic voltammograms of Fc-COOH in phosphate buffer at different scan rates from 1 to 500 mV s^{-1}.

TABLE-1
PEAK POTENTIAL (E_p) AND CURRENT INTENSITY (I_p) OBTAINED FROM THE CYCLIC VOLTAMMOGRAMS OF FERROCENE MONO-CARBOXYLIC ACID IN FIGURE 2 AT DIFFERENT SCAN RATES, ν (1 to 500 mV s^{-1})

ν(mV/s)	1	2	5	10	20	50	100	250	500
E_p^C(mV)	249	263.00	277.00	273.00	271.00	275.00	270.00	265.00	256.00
E_p^A(mV)	370	379.00	349.00	349.00	351.00	350.00	353.00	356.00	365.00
ΔE_p(mV)	121	116.00	72.00	76.00	80.00	75.00	83.00	91.00	109.00
E°'(mV)	310	321.00	313.00	311.00	311.00	313.00	312.00	311.00	311.00
I_p^C(μA)	2	1.80	2.80	4.60	7.00	11.10	16.10	26.40	43.70
I_p^A(μA)	2	2.40	3.50	5.30	6.80	11.20	15.10	25.10	38.90
I_p^A/Ip^C	1	1.33	1.25	1.15	0.97	1.01	0.94	0.95	0.89
$I_p^A/\nu^{1/2}$	2	1.70	1.57	1.68	1.52	1.58	1.51	1.59	1.73

Fig. 3 is a plot of I_p *vs.* $\nu^{1/2}$ for Fc-COOH mediator using the Randle-Seucik equation with a slope of $2.69 \times 10^5\ n^{3/4}C^oD^{1/2}A$. If n ≈ 1, electrode surface area A of glassy carbon electrode = 0.1104 cm^2; the diffusion co-efficient D is obtained from the expression, $D^{1/2}$ = Slope/{2.69×10^5

$n^{¾}$CoA}, *i.e.*, $D = 1.5790 \times 10^{-8}$ cm^2 s^{-1}. The mechanism for redox behaviour of the Fc-COOH mediator is that during the cathodic (reduction) sweep, the ferricenium ion (Fc^+) in Fc-FOOH is reduced to ferrocenium (Fc^0). The latter is then oxidized during the anodic sweep (oxidation)[6].

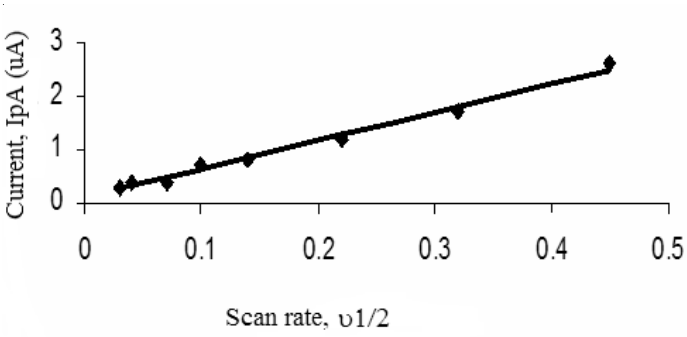
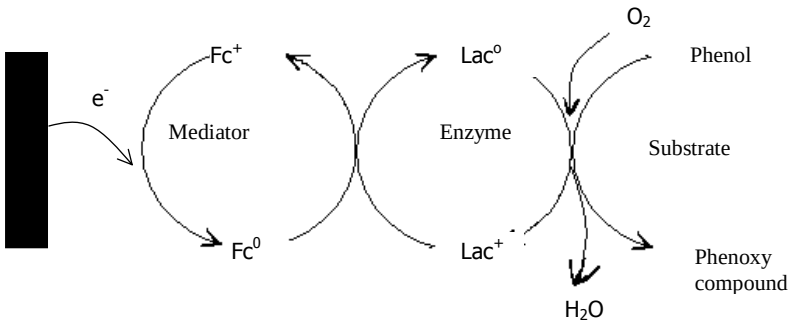


Fig. 3. Plot of anodic peak current *vs.* $v^{½}$ for ferrocene monocarboxylic acid

The general mechanism of the enzyme catalysed oxidation of phenol is that, during the cathodic scan, the electrode supplies electrons to the mediator system which in turn are picked by laccase enzyme as shown in the suggested **Scheme-I**. In the next step, the enzyme passes the electron to the dissolved oxygen (in aerated solution) which in turn removes hydrogen (oxidation) from the phenol substrate and gets reduced to water. In the anodic scan, the electron density of the enzyme/mediator system is reduced as the electrons are removed by the electrode. Addition of phenol which reacts with oxygen also results in consumption of electrons. Thus the anodic current intensity should decrease as the phenol concentration is increased.



Scheme-I. Diagram showing ferricenium/ferrocenium (Fc^+/Fc^0) mediated catalytic oxidation of phenol by laccase enzyme (Lac^o/Lac^+)

Fig. 4 shows two CVs of (1) a mixture of laccase and mediator and (2) a mixture of laccase, mediator and phenol substrate. The forward sweep (scan rate 5 mV s^{-1}) was a cathodic process while the reverse one was

anodic. The mixture of laccase and mediator in the absence of phenol enzyme gave one peak (1) at 272 mV with a current of 2.2 µA in the forward scan while in the reverse scan two unresolved peaks were recorded at 337.5 mV (current 2.7 µA) and 352 mV (current 3.0 µA). In the presence of phenol, laccase and mediator mixture (2) gave a similar pattern of peaks for both the forward and reverse directions but with the difference that slightly higher currents were recorded and also that only a single peak in each direction (forward and reverse) was observed. Thus in the forward direction (cathodic), a peak at 275 mV (**I**) with current of 2.6 µA (compared to 2.2 µA in the absence of enzyme) and in the reverse scan (anodic) a peak at 337.5 mV (**II**) (with current 2.9 µA compared to 2.7 µA in the absence of enzyme), was observed. Thus in general, it was observed that the presence of substrate phenol in the enzyme/mediator system, resulted in increased currents for both cathodic and anodic processes. This is expected to be the case due to the catalytic oxidation of phenol by the enzyme.

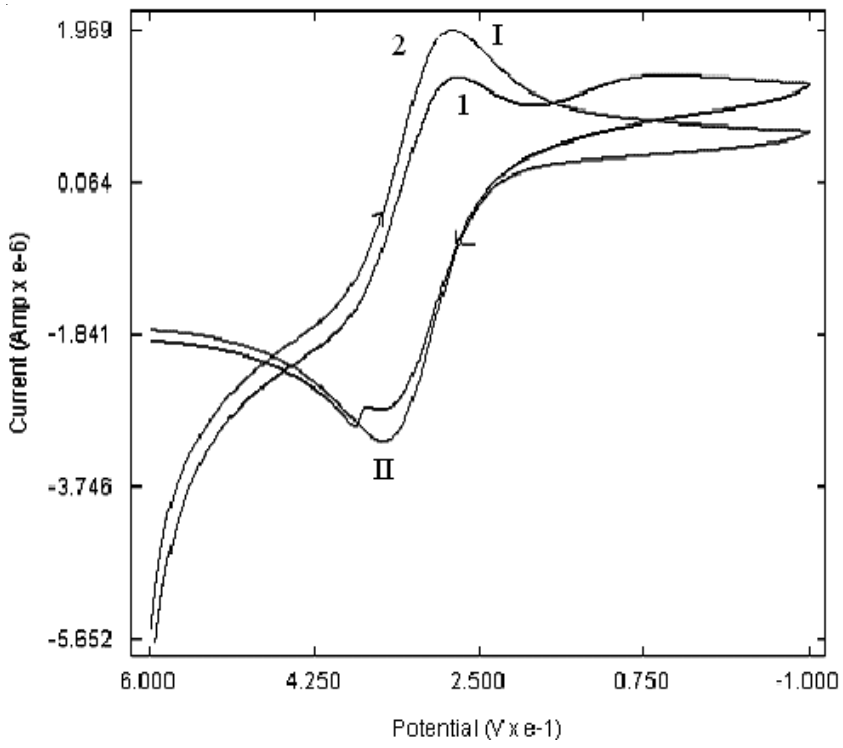


Fig. 4. Cyclic voltammograms scanned at 5 mV s^{-1}; (1) mixture of 4 mg laccase and 5 mM Fc-COOH in buffer; (2) mixture of 4 mg laccase, 5 mM Fc-COOH and 1 µM phenol in buffer: **I** and **II** are peaks

Response characteristics of laccase biosensor

Optimization of catalytic oxidation of phenol by laccase enzyme in the presence of ferrocene monocarboxylic acid under different pH and mediator concentration is discussed below. Fig. 5 illustrates the effect of increasing mediator concentration on the peak current intensity of the mediator for the anodic and cathodic scans, respectively. In both scans, the current increases with increase in the concentration of Fc-COOH up to 5 mM. Beyond this concentration, the steady state condition is attained where the current levels off. The reason for attaining the steady state current in Fig. 5 could be due to the saturation effect of the diffusion layer at the electrode surface.

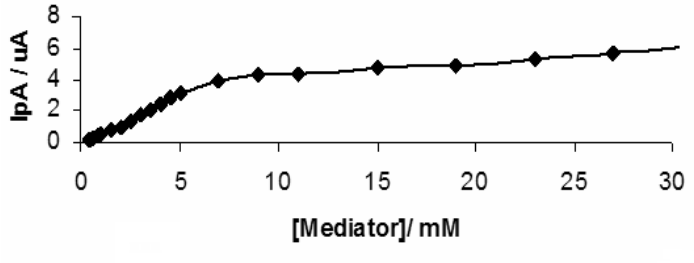


Fig. 5. Plot of anodic current intensity *vs.* concentration of ferrocene monocarboxylic acid (mediator)

Fig. 6 shows the effect of pH of blank buffer in the range 5.84 to 8.22, on the performance of the laccase biosensor in catalytic oxidation of phenol. It was observed that, as the pH increased from pH 5.84 to 6.21, the current intensity remained fairly constant (at about 2.5 μA) but showing a slight decrease. However, increasing the pH from 6.21 to 6.51 showed an increase in current to a maximum value of about 2.7 μA and then a drop from this value to *ca.* 0.1 μA between pH 6.51 to 7. 33. Between pH 7.33 and 8.22, the current level remained fairly constant. Thus the optimum pH was taken as 6.5 (2 significant figures).

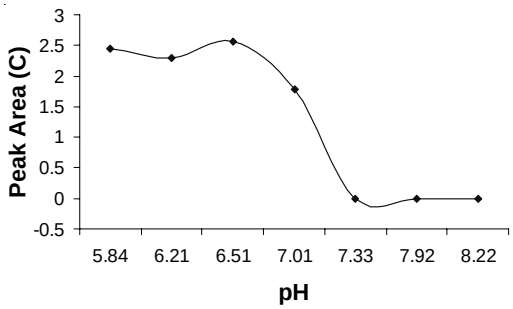


Fig. 6. Effect of buffer pH; plot of peak area *vs.* pH blank buffer containing a mixture of enzyme, mediator and phenol

Determination of phenol in real sample using differential pulse voltammetry

Fig. 7 shows typical DPV scans obtained for the real sample and the spiked phenol concentrations (nine standards) ranging 1.0 to 100 pM. It was observed that as the spiked phenol concentration increases, the peak height decreases. Thus the sample gave the highest peak followed by the lowest phenol concentration spiked (1.0 pM) while the highest concentration of phenol spiked (100 pM) had the lowest peak current intensity. The decrease in peak current with increasing phenol concentration (an example of indirect determination[17]) may possibly be due to a reverse mechanism of the redox processes illustrated in **Scheme-I**. Thus the DP voltammograms in Fig. 7 involve oxidation reactions. In the latter process, electrons are removed from the enzyme system by the electrode. This means that the amount of current recorded should decrease as phenol molecules consume the electrons supplied by the ferrocene mediator thus depleting the electron density of the enzyme/mediator system. A plot of peak area (μC) *vs.* the concentration of added phenol gave the calibration curve in Fig. 8. The concentration of phenol in the unknown sample is calculated either through extrapolation or by statistical method (*i.e.* [y-intercept]/[slope of the graph] = concentration of unknown sample)[18].

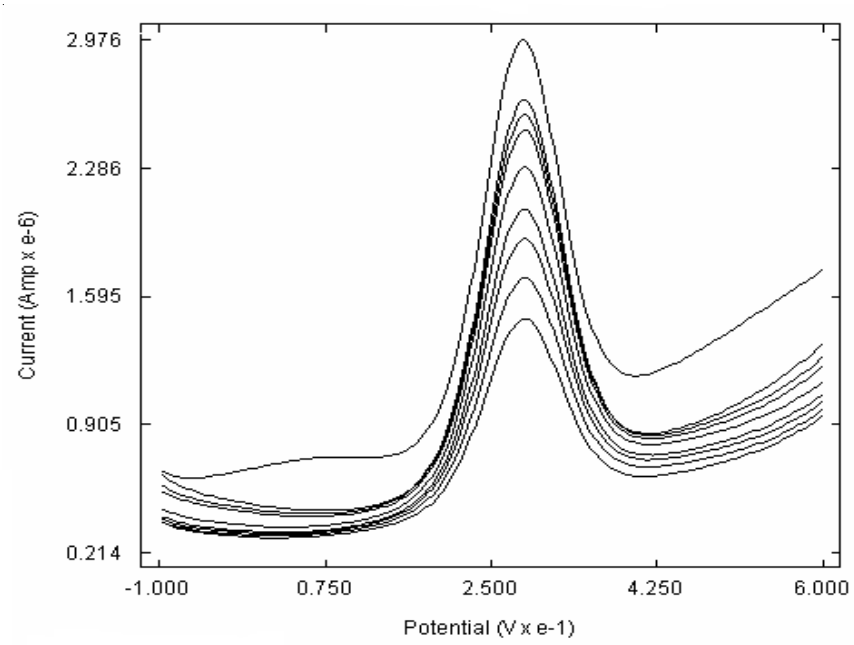


Fig. 7. Plot of DP voltammograms of varying phenol standard concentrations (nine) added to the real sample: (1) 1.0 pM and (9) 100 pM phenol. NB. Scan direction is anodic giving positive current as per IUPAC notation[8]

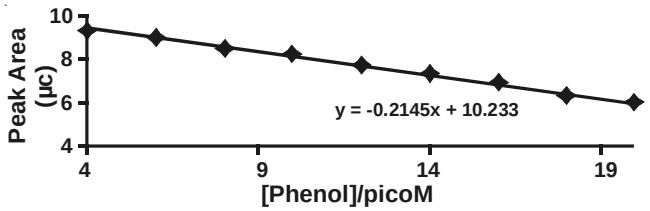


Fig. 8. Plot of peak area (current intensity, µC) *vs.* concentration of phenol (picomolar, pM)

The laccase biosensor was found to respond to different concentrations (1×10^{-12} - 100×10^{-12} M or 1 to 100 pM) of phenol. The average concentration (n = 3 samples) of the real samples was calculated as 47.7 pM phenol (using the linear equation in Fig. 11 *i.e.* 10.233/0.214 = 47.7 pM). This value is lower than the guideline value by USEPA quoted as 3.5 µg L^{-1} or 37 nM phenol[19], implying the method developed in this study is suitable for trace analysis of phenol in environmental samples.

Conclusion

The cyclic voltammograms obtained for ferrocene monocarboxylic acid mediator showed the latter to be electrochemically reversible with a diffusion coefficient (D_o) of 1.5790×10^{-8} cm^2 s^{-1}. The laccase electrode responded to different concentrations of phenol (1×10^{-12} to 100×10^{-12} M) using differential pulse voltammetry. The coal waste water sample was found to contain 47.7 pM phenol, a value lower than the toxic level for humans of 1.0 µg L^{-1} (or 10 nM phenol)[2].

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