Amperometric bio-based sensor for azo compound detection using laccase as biorecognition element

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Abstract

Present paper is about the development of novel electrochemical sensor for the detection of azocompounds using laccase as bioelement and sunset yellow has the model substrate. Detection of azocompounds is important because of their multiple applications in the area of technology and medicine. They are well known for their use as analytical reagents, in the dye industry or as chemotherapeutic drugs. Bioelectrocatalytical method utilizes the laccase immobilized carbon paste electrode for the selective sensing of azocompounds. Amperometric detection system utilizes a three-electrode system i.e. a combination of working electrode, Ag/AgCI (saturated KCI) reference electrode and platinum as auxiliary electrode. The solution conditions and instrumental parameters were optimized to obtain a good sensitivity. The reduction of sunset yellow and oxidation of intermediate product of its reduction in the presence of co-enzyme in 0.IM acetate buffer (pH 5.5) were studied. The quasi-reversible reduction of sunset yellow was evident from results. When coenzyme was present, the intermediate product of azocompound reduction was further oxidized. The reduction of a pre-protonated azo group involving a four-electron process and two peaks or waves, gives amine derivatives in acidic solutions. In alkaline solution the reduction process occurs at more negative potential with the formation of a stable hydrazo compound. The optimum response was realized by both the electrodes constructed using bioelement immobilized in inorganic host matrix and laccase dispersed carbonaceous electrode material at pH 5.5, 0.1M acetate buffer. Operating at these optimum conditions the biosensor had excellent selectivity against sun set yellow. The peak current varied linearly with substrate concentration in the range 5×10^{-5} to 8×10^{-3} M. The proposed bioelectrochemical sensor has found highly sensitive in determination of azocompound selected.

Keywords: Laccase, Carbon paste, sensor, inorganic host matrix, ampherometric detection.

1. Introduction

Azo compounds are widely used as textile dyes, chemotherapeutic agents, coloring agents in foods and pharmaceuticals etc. As a result they can enter the body through the intake of certain foods and drugs that contain these azo compounds. These are also starting materials in the synthesis of many organic compounds. About 50% of the industrial colorants produced in the world are azocompounds. Industrial effluents often contain residual dye, which affects water quality and may become a threat to public health (Abdullah, E., et al. 2000). On the other hand, azocompounds as sulfa drugs have multiple therapeutic uses. They are used in the treatment of inflammatory bowel diseases such as crohn's disease and ulcerative colitis. Used in case of arthritis to reduce inflammation. Thus analysis of these drugs from different matrix is highly desirable. Certain azo dyes or their metabolites may be highly toxic and potentially carcinogenic (Rodriguez et al. 1999). Azo compounds contain a least one nitrogen-nitrogen (N=N) double bond, however many different structures are possible (Zollinger, H., 1987). Synthesis of most azo compounds involves diazotization of a primary aromatic amine, followed by coupling with one or more nucleophiles. Amino and hydroxy groups are commonly used coupling components (Zollinger, 1991). Concern has been voiced about the potential carcinogenicity of compounds

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containing azo group. For the reason quantification of chemicals in the environment is a major issue, among which the azo dyes are the compounds with prime importance (Wesenberg et al., 2003). Therefore, a study on development of simple analytical method for their detection is worthy of investigation.

Compared with chromatographic and spectroscopic methods, developed bioelectrochemical technique of measurement is simple, reliable and practical with low detection limit and suitable for on site measurement. Because the bioelectrochemical reaction occurs on the electrode/solution interface, it is especially suitable for a small amount of sample. Bioelectrochemical method developed is also useful techniques for the study of the interaction of dye with bio-molecules.

Materials and Methods

2. Experimental

2.1. Laccase

Laccase (EC 1.10.3.2) is a polyphenol oxidase, which belongs to the family of blue multicopper oxidases. These enzymes catalyze the one-electron oxidation of four reducing-substrate molecules concomitant with the four-electron reduction of molecular oxygen to water. Laccases oxidize a broad range of substrates, like phenolic compounds; can also oxidize other substrates such as aromatic amines, syringaldazine, and non-phenolic compounds, to form free radicals (Rodriguez et.al.1999, Wong Y, Yu J. 1999, Wesenberg D et al. 2003). In the presence of mediators, fungal laccases exhibit an enlarged substrate range and are then able to oxidize compounds with a redox potential exceeding their own (Yaropolov, A.et al. 1994).

2.2. Apparatus

Cyclic voltammograms were recorded on a model CHI660 electrochemical workstation (CH Instruments, USA) controlled by Chi1101a software that operated under Windows 2000 environment. A three-electrode system was equipped with a laboratory made modified CPE, an Ag/AgCl reference electrode and a platinum-wire auxiliary electrode.

2.3. Chemicals

Laccase from T. versicolor (TV) was purchased from biochemika, Fluka. Bovin serum albumin (BSA), 2, 2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1-hydroxybenzotriazole (HBT), sunset yellow were received from Sigma, Calcium carbonate, K2HPO4, KH2PO4, MgSO4, FeSO4, Na2HPO4, Ammonium acetate, NaH2PO4, graphite powder and mineral oil (white, light) were purchased from Fisher Scientific. All chemicals were of analytical-reagent grade, and were used as received without further purification. Double-distilled water was used throughout the experiments.

2.4. Enzyme characterization

2.4.1. Laccase activity

The specific activity of laccase was assayed spectrophotometrically by monitoring the absorbance increase from oxidation of ABTS at 420 nm (å = 3.6×104 M"1 cm"1) using UV–Vis spectrophotometer at room temperature. The assay mixture consisted of 0.5mM ABTS (Wolfenden and Wilson, 1982) and appropriate amount of laccase in pH 5.2 PBS. The reaction was started by the addition of laccase solution. One unit of activity is defined as the amount of oxidized ABTS (μmol) per minute.

2.4.2. Protein determination assay

Protein determination was done by the method of Bradford using bovine serum albumin as the standard (Bradford, 1976). The range utilized was 12 to 100 μg. Standard or sample (800 μl) solution was pipetted into a test tube. Dye reagent concentrate (200 μl) was added to each tube and mixed well. These were incubated at room temperature for at least 5 min but not longer than 60 min. The absorbance was measured at 595 nm (Bradford, 1976).

2.4.3. Temperature and pH activity profiles

The temperature profile was calculated in 0.1 M Na-acetate and phosphate buffer at pH 5 and 5.5 respectively in the temperature range 30-70 ºC. The

pH profile was studied with in the range of pH 3-8 using different buffers.

2.5. Bioelectrode preparation

2.5.1. Enzyme mixed directly with carbonaceous electrode material

The working carbon paste electrode was prepared by using appropriate quantity of enzyme solution (known protein content) with standardized quantity of riboflavin, 1g of carbon paste containing 75% (w/w) graphite powder and 25% (w/w) mineral oil and hand-mixing of carbon particles with enzyme mixture and mineral oil . The enzyme modified carbon paste was subsequently packed firmly into the electrode cavity to 5mm depth, initial part of which must be filled with plan carbon paste for 5mm depth (3mm diameter ×10mm depth) of a hollow inert borosilicate glass tube. Electrical contact was established via a copper wire. Before each use, the electrode surface of m-CPE electrodes were renewed by a simple polishing procedure, wetted with double distilled water and then thoroughly smoothed with abrasive paper and then with alumina paper .

2.5.2. Immobilized enzyme layer attached to electrode surface

The $CaCO_3$ colloidal suspension (4mg/ml) was prepared by dispersing amorphous $CaCO_3$ in deionized water with stirring overnight. Laccase was also dissolved in deionized water with a concentration of 2mg/ml and riboflavin 2mg/ml. A defined amount of aqueous laccase mixture and $CaCO_3$ (containing (20 + 20) µg laccase, 40µg $CaCO_3$) was spread on the surface of the carbon electrode. The mixture was dried at room temperature, $CaCO_3$ film in which enzymes were entrapped will adhere to the surface of electrode. The resulting electrode was placed in saturated glutaraldehyde vapor at room temperature for 10-15 min in order to induce the chemical cross-linking of the entrapped enzyme molecules. Before use, the enzyme electrode was rinsed with buffer solution to remove the enzyme not firmly immobilized. The amount of enzyme immobilized on the electrode surface was calculated by the difference between the amount of protein initially adsorbed and that detected in washings. Intermolecular interaction between $CaCO_3$ and laccase were studied by infrared spectra.

2.6. Decolorization with laccase system

Dye solutions (0.1 mM; 2.5 ml) buffered with 0.1 M acetate and phosphate buffers, pH 5.5, were incubated with 100 µl of laccase (4.3 mg protein/ml, 6.3 U/ml) and 0.5 ml distilled water in a standard cuvette. Dye absorbance was measured at different times during the experiment and the percentage of decolorization was calculated (Abdullah, E. et al. 2000).

2.7. Biocatalysis with laccase system and Amperometric measurements

The enzyme electrode was fitted into a rotating disk electrode holder (CH instruments), which was placed in a three-electrode cell with an Ag| AgCl (3 M KCl) reference electrode (BAS, Bioanalytical Systems, USA) and a platinum wire auxiliary electrode. The electrodes were connected to Potentiostat controlled by the Chi1101a electrochemical software. All measurements were performed at an applied potential between -1 to +1 V vs. Ag| AgCl with an electrolyte of 0.1M phosphate and acetate buffer.

Results and Discussion

3.1. Temperature and pH activity profiles

The optimal temperature was investigated through assays performed in the range of 30 ºC to 70 ºC. The optimal temperature treatment for laccase at 1 h of incubation is 45 ºC. The optimal pH for laccase is pH 5.5, but a best activity is retained in the pH range of 4 to 6. The experiments were performed with two different types of buffer from pH 2 to pH 9, revealed that phosphate buffer was more sensitive. Results of the study with trametus versicolor species is almost similar as mentioned in the literature stating that laccase activity is maximum at slightly acidic pH and temperature between 30-50 for enzyme those obtained from sources like Trametes hirsuta Sclerotium rolfsii and Pleurotus ostreatus (Durán N et al. 2002, A. Zille et al.2003, A. Zille et al. 2005, A Kandelbauer et al. 2004). Enzyme laccase obtained from few rare species shows optimum activity at very high temperatures. One of literature mentions that crude laccase from Ganoderma lucidum

showed high thermo stability and maximum decolorization activity at 60 °C and pH 4.0 (Yoon-Seok Chang et al. 2006) same in case of mesophilic basidiomycetes (J. Jordaan and W. D. Leukes 2003).

3.2. Decolorization with laccase and laccase/ mediator system

A decolorization percentage of sunset yellow with laccase was almost 89±2. Extend of decolorization was similar in both acetate and phosphate buffer. Oxidative dye decolorization approach using laccase was highly efficient; the redox potential difference between the biocatalyst and the dye is expected to be a relevant indicator of the ability of the enzyme to decolorize the dye. (Fernandes A et al. 2004).

3.3. Laccase activity towards sunset yellow

Fig. 1(a) shows UV–Vis spectra were recorded at different times of the enzymatic reaction. The absorption band at 481 nm was selected for further work. As can be seen in Fig1 (b) the absorbance at that wavelength decreases as a consequence of biocatalysis. The sunset yellow molar extinction coefficient (å) at 481nm was 7.1×103 cm”1 M”1. After the addition of laccase to a given substrate solution, absorbance (A)–time (t) profiles showed the linear decrease in the absorbance and brownish coloration of the solution was observed. This is also evident from the absorption spectra indicating the new peaks at 242 wavelengths with linear increase in the response, followed by a linear decrease in wavelength at 481. This could be interpreted as a gradual consumption of substrate by the enzymatic reaction. From experiments like those described above but performed at different substrate concentrations, the kinetic parameters were evaluated. The Michaelis–Menten constant (KM) and the maximum rate (Vmax) were 1.55×10"3 M and 0.58×10"4 M s”1, respectively. Kinetic values correlated using other kinetic equations also revealed similar results. Kinetic parameters evalued using current kinetics values are given in table 1. These results are clear evidence that sunset yellow is the best substrate of laccase, opening the way to the electro analytical quantification of this azocompound using bioelectrodes.

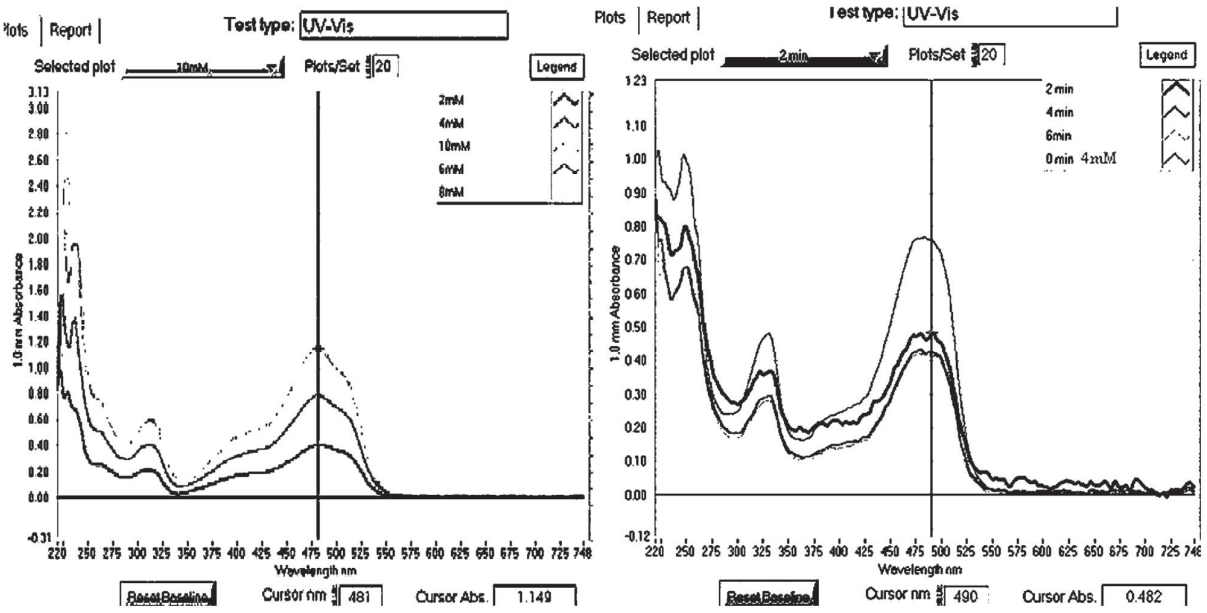

Fig. 1 (a) UV–Vis spectra for sunset yellow at different concentration indicating the absorption maximum at 481nm. Fig. 1 (b) Spectra showing the decrease in absorbance at 481nm at different times (*t*): *t*=0; t=10min after the addition of laccase. C_{SY}=4×10"3 M; enzyme concentration: 40 U ml"1.

3.4. Preparation of bioelectrode using immobilized enzyme layer attached to electrode surface.

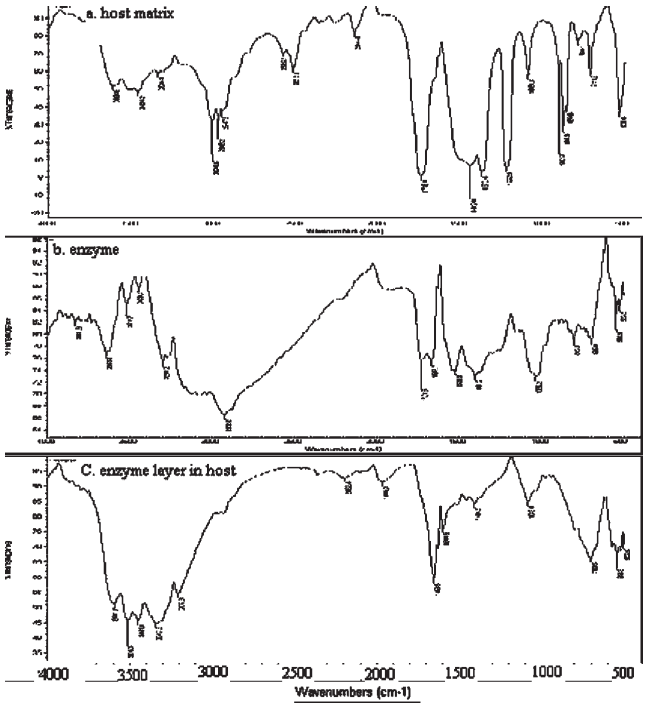

Fig 2. FT-IR spectra of (a)host matrix, (b) enzyme laccase (c) and laccase/ $CaCO_3$ matrix layer.

The interactions between the host matrix and the enzyme were studied by FT-IR spectra. From the Fourier transform infrared spectra of host $CaCO_3$ it can be seen complex absorption band in the region 1490 to 702 cm-1, which is often being associated with calcite. The IR absorption spectrum of enzyme (b) indicating the peaks in the wave numbers 3288 cm^{-1} is assigned to N-H vibration of laccase. The peak at 2922 cm-1 is assigned to *C*–H vibration. And this board peak is may be due to the interactions of different groups. Peaks from 1730 to 1600 cm-1, from 1552 to 1401 cm-1 are due to the presence of different amide groups. The FT-IR spectrum of the laccase/CaCO3 biomembrane presents a combination of both preceding spectra (fig C), spectrum indicating the successful immobilization of laccase molecules with in the $CaCO_3$ host. The multiple absorption bands from 1700 to 1400 cm-1 region because of amide groups which were prominent spectrum b were almost disappeared. Spectrum (c) is indicating the single absorption peak at 1653 cm^{-1} which is because of the C O stretching modes of amide band of the protein. The board absorption peak at 2922 cm^{-1} present in the spectrum was almost neutral at C., while in spectrum c there is only an absorption band at 3432 cm-1 (Simpson,1998). This result shows that there might be intermolecular interaction between enzyme and host matrix-CaCO3.

3.5. Cyclic Voltametric behavior of Laccase modified electrode

Cyclic voltammograms at laccase electrode in acetate buffer (pH 5.5, 0.1 M) without azodye and with increasing concentrations of azodye are shown in Fig. 3. Cyclic voltamograms of thus-prepared composite enzyme mixed electrode in the presence of different concentration of sunset yellow substrate are shown in Fig. 4a. It was observed that the peak current changes steeply on enzyme layer immobilized electrode after dye was added to buffer. Such an increase in peak is due to the oxidation of intermediate species liberated from the enzymatic reaction catalyzed by laccase on enzyme electrode shown in Fig 4 b. Reduction current measured was indicative of enzymatic activity of electrode.

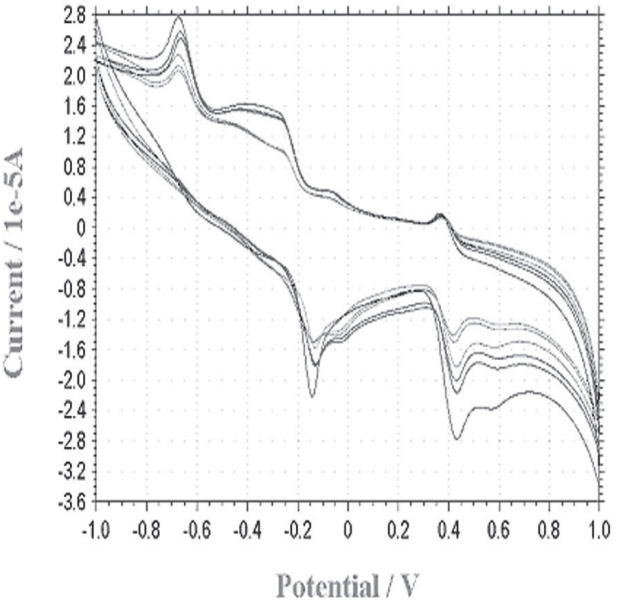

Fig 3. Graph showing the linear change in current with change in substrate concentration

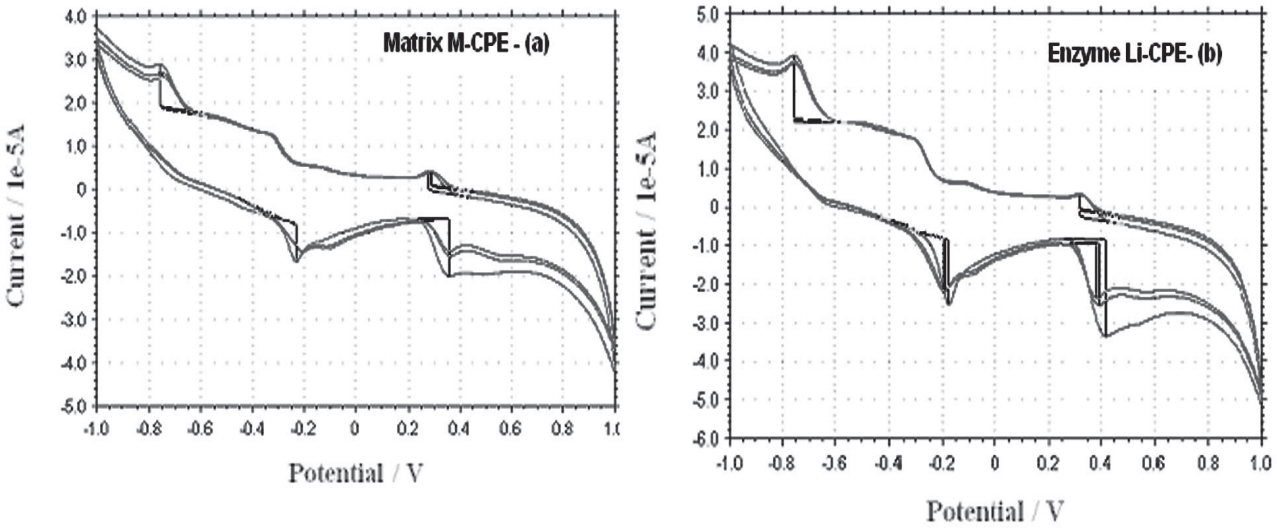

Fig. 4(a). Cyclic voltammograms (repetitive cycles) for the electro-reduction of sunset yellow on matrix modified CPE, Fig. 4(b). Cyclic voltammograms (repetitive cycles) for the electro-reduction of sunset yellow on enzyme layer immobilized CPE under the conditions (a) C_{SY}=4×10"^{3} M; composition acetate buffer, pH=5.2; *v*=0.100 Vs^{-1}; nitrogen saturated solutions.

3.6. Kinetic behavior of laccase enzyme layered modified CPE towards sunset yellow

In order to determine the bioelectrode affinity towards substrate selected, the Michaelis–Menten constant (*K*m) and velocity maximum *V*max were calculated from different kinetic equations. The kinetic constants of matrixed enzyme as well as enzyme layer immobilized electrodes were determined by the oxidation of reference substrate ABTS and sunset yellow (Table 1). The catalytic efficiency of matrixed enzyme is better than layered laccase for the substrate studied. The *V*max of the layered enzyme was found lower than that of the matrixed enzyme with both most reactive substrate ABTS and sunset yellow. The lower catalytic efficiency of immobilized laccase may be due to the partial inactivation of the enzyme by glutaraldehyde during cross-linking and also due to the diffusion limitations in the layered structure. Comparing K_M values for ABTS and sunset yellow, it was concluded that the bioelectrode has high affinity for the substrate.

Table 1: Kinetic constants for the oxidation of sunset yellow by matrixed enzyme (ME) and immobilized layered enzyme (ILE).

Substrate (Sunset yellow)	Km (mM)		Current Max (Imax)(μA)	
	ME	ILE	ME	ILE
Michaelis–Menten	0.98	1.55	4.12	4.83
Lineweaver-Burk plot	1.92	2.5	5.34	6.66
Eadie Hofstee	1.56	2.37	5.21	6.66
Hanes plot	1.21	2.114	4.84	6.406

3.7. Electrode response characteristics

The initial experiments were conducted to establish the optimum pH. Phosphate buffer and acetate buffer of different pH values were used as carrier solution. The maximum response for sunset yellow was obtained at pH 4.5-6. The optimum pH 5.5 value was used throughout the work. A linear response for sunset yellow in acetate buffer (0.1 M,) is shown in Fig 3. Sunset yellow gave a linear plot for the range 5×10^{-5} to 8×10^{-3} M with a linear regression $r2 = 0.9985$. With constant concentrations of sunset yellow, no decrease in response was observed for at least 20-25 cycles in continuous testing. A decrease in response of enzyme electrode was observed for a high concentration (more than 8 mM) of dye, attributed to slow surface fouling by the reaction product (Roessler A 2003). To determine the storage stability, the performance of enzyme electrode was monitored over a period of 1 month. When stored at 4^0C under dry conditions, only a marginal loss of enzyme activity was observed at 1 month.

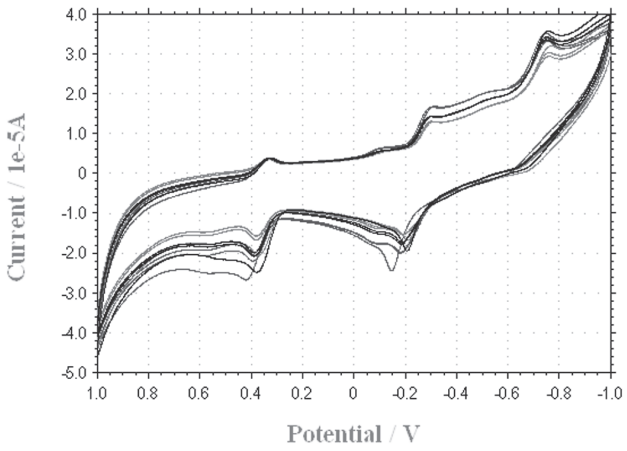

Fig 5. Electrochemical behavior of azocompound sunset yellow at CPE

As showed in figure, a reduction wave appeared with peak potential E p,c –0.718 V on cathodic scan and an oxidation wave with peak potential Ep,a –0.203 V on reverse scan. The difference DEp (Ep,a Ep,c) of the peak potential of oxidation and reduction peaks was 515 mV, and the ratio Ip,a/Ip,c of the peak currents was about 1.4. With the potential scan rate v increasing from 0.1 to 1.0 V/s, the peak current Ip,c of the reduction wave increased linearly. These indicated that the reduction of azocompound was pseudo-reversible process. In addition, the peak current Ip,c increased with pH value increasing from 4.0 to 5.5, and then decreased in the range of 5.5-8.0. On the other hand, the peak potential E p,c shifted linearly to negative direction with pH value increasing in the range of 5.5 – 9.

Conclusion

In the present work, an electrochemical biosensor for sunset yellow was developed using enzyme laccase entrapped and immobilized enzyme layered carbon paste composite matrix. An amperometric biosensor for sunset yellow is described, which was prepared by using enzyme modified CPE. The attractive properties and behaviors of CPE as the composite materials enabled us to obtain sensitive biosensing system. The bioelectrode displayed a rapid and sensitive response to the change in concentration of dye. The amperometric response current displays a linear relationship with respect to the concentrations of dye in the range 5×10^{-5} to 8×10^{-3} M. Detection limit and operational constants are described. The biosensor exhibited good performance in terms of reusability, operational stability, fabrication simplicity and shelf life. This simple, easy to- construct, reagent less electrode is suitable for micromolar quantification of azodye sunset yellow.

Acknowledgement

The authors thank the TIFAC and TEQIP for providing the sophisticated instrumental facilities.

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