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Detection of Orange G Using Novel Bioelectrocatalytical Method

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Abstract: This research discuss the detection of azocompounds by novel bioelectrocatalytical method using orange G 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 biosensor for the detection of azocompound utilizes a three-electrode system, i.e., a combination of working electrode and an Ag/AgCI (saturated KCl) reference electrode and platinum auxiliary electrode were used. The optimum operational conditions for the biosensor were investigated and the system was calibrated for detection of azodye. Linear sweep voltammetry was used to study the influence of pH on the peak current and peak potential. The solution conditions and instrumental parameters were optimized to obtain a good sensitivity. The optimum response was realized by electrode constructed using 10 μL of laccase for one gram of carbon paste and operating at -0.293 V (versus Ag/AgCl (3M KCl) as reference electrode) in pH 5.5, 0.1 phosphate buffer. Operating at these optimum conditions the biosensor had excellent selectivity against Orange G. The peak current varied linearly with dye concentration in the range between 5×10^{-2} to 2×10^{-3} mM. The bioelectrocatalytical method demonstrated excellent selectivity and sensitivity towards the selected azocompound.

Key words: Laccase, carbon paste, sensor, electrochemical, amperometric, detection

INTRODUCTION

Azo dyes constitute the largest chemical class of dyes used regularly for textile dyeing, color photography, paper printing and other industrial applications. About 50% of the industrial colorants produced in the world are azo dyes. Industrial effluents often contain residual dye, which affects water quality and may become a threat to public health (Abdullah *et al*., 2000). Certain azo dyes or their metabolites may be highly toxic and potentially carcinogenic (Rodriguez *et al*., 1999). Azo dyes contain a least one nitrogen-nitrogen (N = N) double bond, however many different structures are possible (Zollinger, 1987). The azo groups are generally connected to benzene and naphthalene rings, but can also be attached to aromatic heterocyclic or enolizable aliphatic groups (Zollinger, 1987). These side groups are necessary for imparting the color of the dye, with many different shades and intensities being possible (McCann and Ames, 1975). When describing a dye molecule, nucleophiles are referred to as auxochromes, while the aromatic groups are called chromophores. Together, the dye molecule is often described as a chromogen. Synthesis of most azo dyes 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). Because of the diversity of dye components

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available for synthesis, a large number of structurally different azo dyes exist and are used in industry (Banat *et al*., 1996).

The azo bond in azo colorant molecules is however vulnerable to reductive cleavage. Due to its significance in toxicological and eco-toxicological terms and its potential for eliminating color impact in azo dye containing effluents, the biologically-mediated decolourisation of azo dyes through azo bond reduction has been extensively investigated in the past 20 years.

In the present context of increasing concern for the survey of chemicals releases to the environment, azo dyes have an unquestionable place. However, market pressure towards the continual introduction of new products has led to the present, extensive range of commercialized dyes.

On the other hand, the 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). Compared with spectroscopic method, Developed bioelectrochemical technique of measurement is simple, reliable and practical with low detection limit and a wide dynamic range. Because the bioelectrochemical reaction occurs on the electrode/solution interface, it is especially suitable for a small amount of sample. Bioelectrochemical method developed is useful techniques for the study of the interaction of dye with bio-molecules.

MATERIALS AND METHODS

Present study was conducted during December 2004-Jan 2008, in Department of Chemical Engineering, Industrial Biotechnology Division of National Institute of Technology Surathkal, Karnataka, India.

Experimental

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 mines, syringaldazine and non-phenolic compounds, to form free radicals (Rodriguez *et al*., 1999; Wong and Yu, 1999; Wesenberg *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 *et al*., 1994).

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.

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), Orange G were received from Sigma, K_2HPO_4, KH_2PO_4, $MgSO_4$, $FeSO_4$, Na_2HPO_4, Ammonium acetate, NaH_2PO_4, 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.

Enzyme Characterization

Laccase Assay

Laccase activity was determined by the oxidation of ABTS (Wolfenden and Wilson, 1982). In a reaction mixture containing 1 mM ABTS in 0.1 M sodium acetate buffer, pH 5, with a 5 to 50 µL enzyme. The oxidation was followed at 30°C and at 436 nm, the enzyme activity was calculated using the molecular extinction coefficient €436 is 29300 M^{-1} cm^{-1} and expressed in µmol/min for ABTS. Activity was expressed in units defined as 1 µmol of substrate oxidized per minute at 30°C. Triplicate values were used during characterization.

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).

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

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 10 µL of laccase (4.3 mg protein mL, 6.3 U mL^{-1}) and 0.5 mL distilled water in a standard stirred cuvette. Dye absorbance was measured at different times during the experiment and the percentage of decolorization was calculated (Abdullah *et al*., 2000).

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 and measured at scan rate of 50 mV vs. Ag| AgCl with an electrolyte of 0.1M phosphate and acetate buffer.

RESULTS AND DISCUSSION

Temperature and pH Activity Profiles

The optimal temperature was investigated through assays performed in the range of 30 to 70°C. The optimal temperature treatment for laccase at 1 h of incubation is 45°C. 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 *Trametes 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 *et al*., 2002; Zille *et al*., 2003, 2005; 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 (Jordaan and Leukes, 2003).

Decolorization with Laccase and Laccase/Mediator System

A decolorization percentage of orange G with laccase was almost 91±3. 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 *et al*., 2004).

Laccase Activity Towards Orange G

In order to determine whether the orange G is the substrates of laccase, spectrophotometric experiments were made. Figure 1a shows UV-Vis spectra were recorded at different times of the

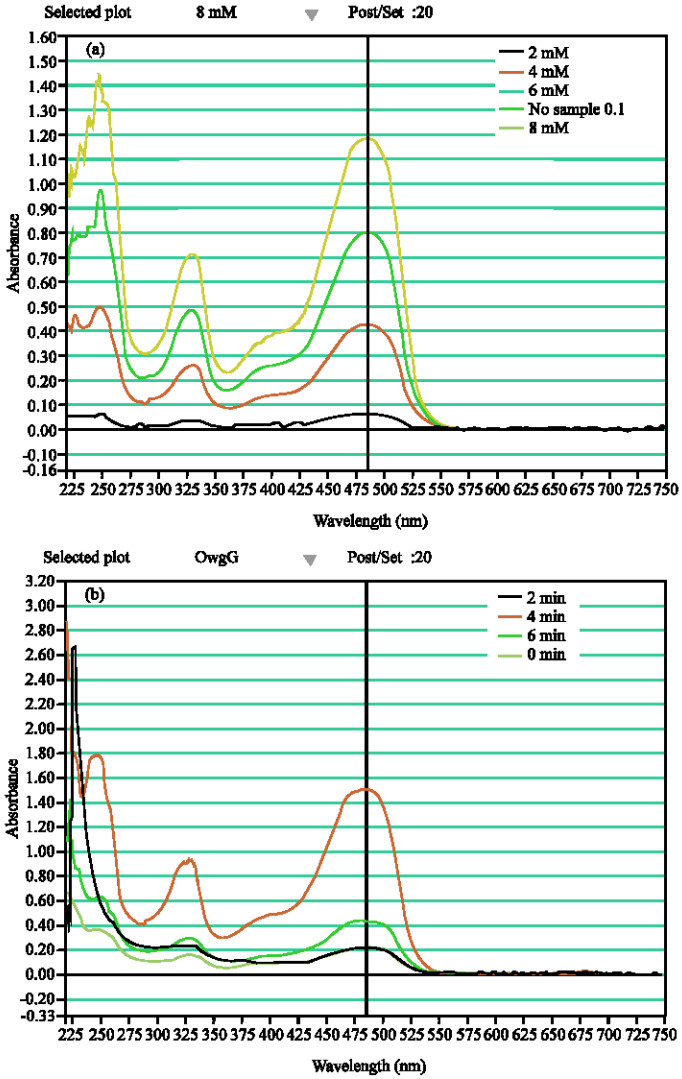

Fig. 1: (a) UV-Vis spectra for orange G at different concentration indicating the absorption maximum at 480 nm. (b) Spectra showing the decrease in absorbance at 480 nm at different times (t): t = 0; t = 10 min after the addition of laccase. $C_{OG} = 10\times10^{-3}$ M; enzyme concentration: 40 U mL^{-1}

enzymatic reaction. The absorption band at 480 nm was selected for further research. As can be seen in Fig. 1b the absorbance at that wavelength decreases as a consequence of biocatalysis. 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 linear decrease in the response, at the maximum absorption wavelength. 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 (K_M) and the Current maximum (I_{max}) were given in Table 1. Kinetic values correlated using available literature revealed similar results (Zille *et al*., 2003). These results are clear evidence that orange G is the best substrate of laccase, opening the way to the electro analytical quantification of this azocompound using bioelectrodes.

Cyclic Voltametric Behavior of Laccase Modified Electrode

It was observed that the peak current changes steeply on enzyme immobilized electrode after dye was added to buffer (Fig. 2). Such an increase in peak is due to the reduction of intermediate species liberated from the enzymatic reaction catalyzed by laccase on enzyme electrode. Cyclic voltamograms of thus-prepared composite enzyme mixed electrode in the presence of different concentration of orange G substrate are shown in Fig. 3. Reduction current measured was indicative of enzymatic activity of electrode.

Table 1: K_M and I_{max} value of Orange G from different Kinetic plots

Substrate (Orange G)	K_M (mM)	Current max (μA)
Michaelis-Menten	3.270	6.569
Lineweaver-Burk plot	3.289	8.928
Eadie Hofstee	3.048	9.002
Hanes plot	2.760	8.703

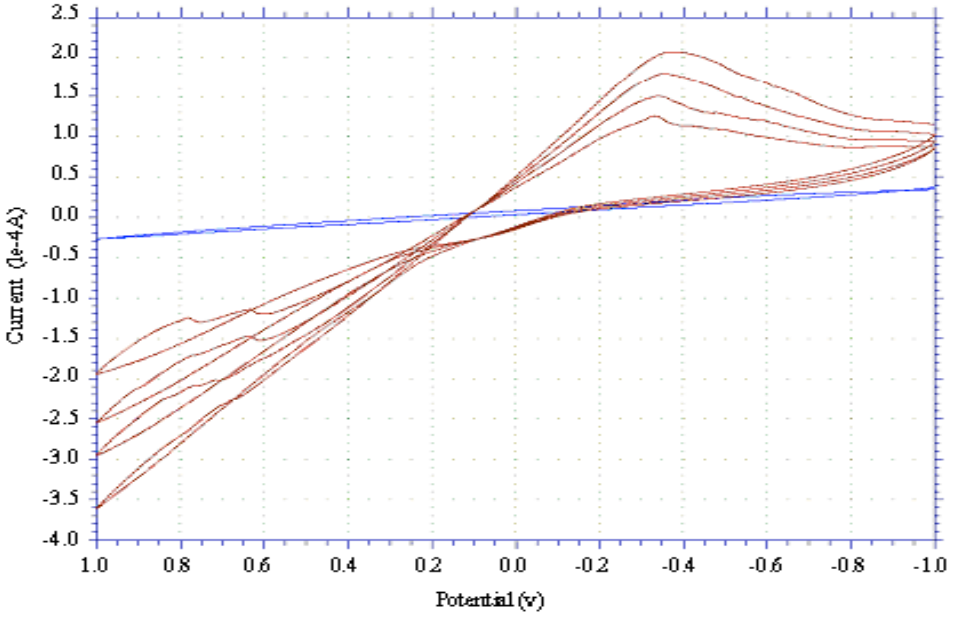

Fig. 2: Cyclic voltammograms at laccase electrode in phosphate buffer (pH 5.5, 0.1 M) with increasing concentrations of dye orange G

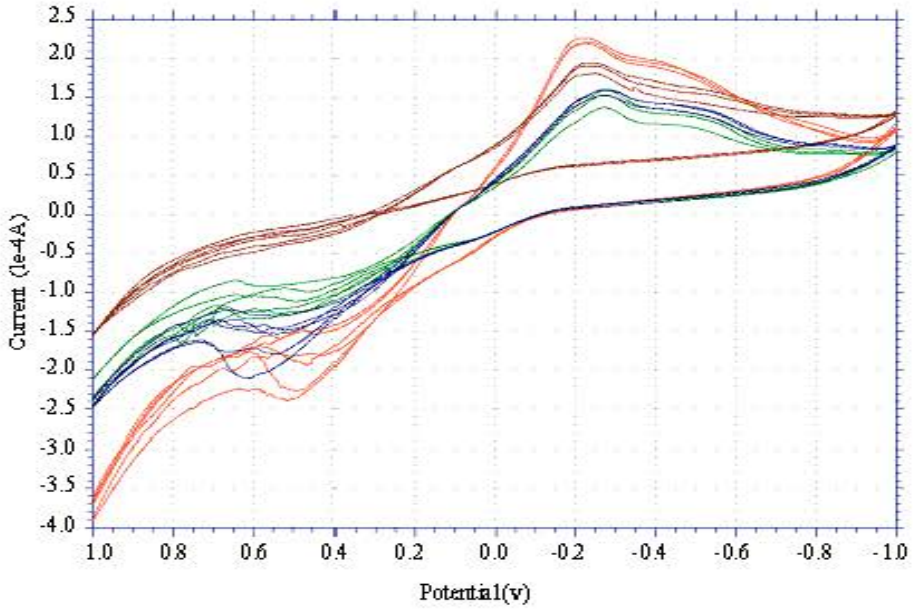

Fig. 3: Cyclic voltammograms showing current response to change in concentration of Orange G. Measurement was carried out using a reaction volume of 8 mL at supporting solution pH was 5.5 and measurement was done at 30°C

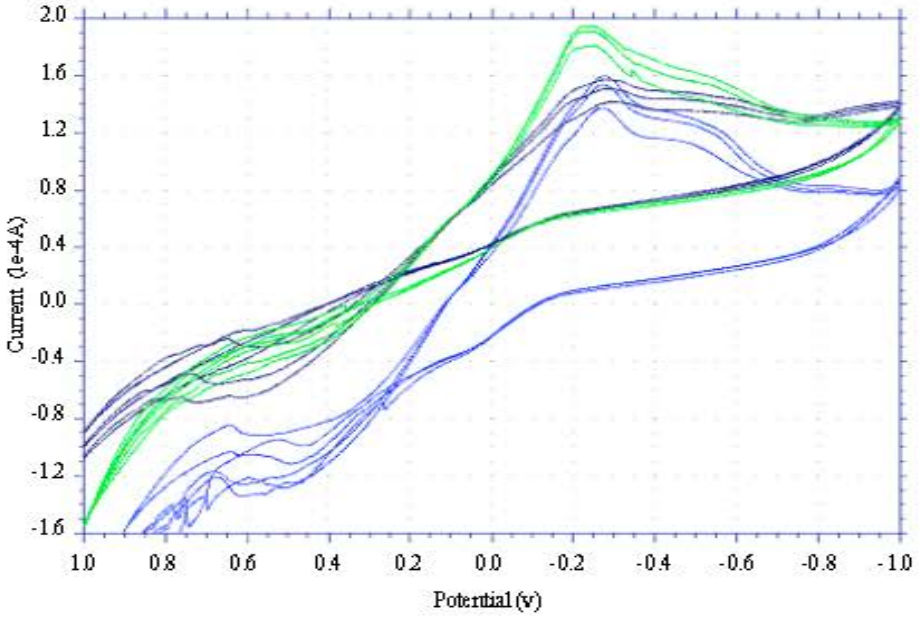

Fig. 4: Effect of pH on response current , maximum response for orange G was obtained at pH 4.5-6. The optimum pH 5.5 showing highest response for 800 (ppm) of Orange G. A linear response for orange G was obtained both in acetate and phosphate buffer (0.1 M)

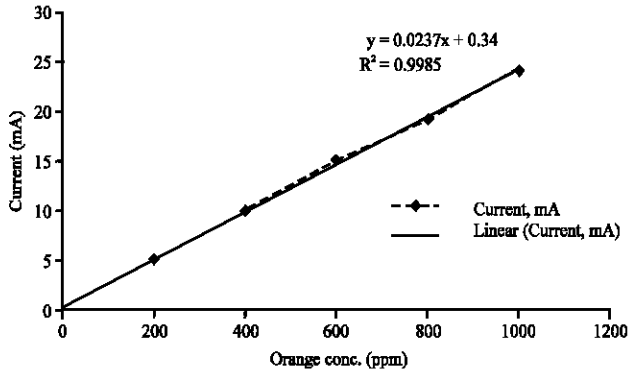

Fig. 5: Calibration graph for Orange G obtained with laccase modified graphite electrode in 0.1 M phosphate buffer pH 5.5, at 50 mV scan rate vs. Ag/AgCl electrode

Electrode Response Characteristics

The initial experiments were conducted to establish the optimum pH. Phosphate buffers and acetate buffers of different pH values were used as carrier solution. The maximum response for orange G was obtained at pH 4.5-6. The optimum pH 5.5 value was used throughout the work. A linear response for orange G was obtained both in acetate and phosphate buffer (0.1 M,) is shown in Fig. 4 inset. Orange G sensor gave a linear plot for the range 5×10^{-5} to 2×10^{-3} M with a linear regression equation y = 0.0237x+0.34, r^2 = 0.9985 where *y* represents the current (mA) and x, the substrate concentration. A calibration curve for Orange G is shown in Fig. 5. With constant concentrations of orange G, no decrease in response was observed for at least 15 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 and Crettenand, 2003). To determine the storage stability, the performance of enzyme electrode was monitored over a period of 3 months. When stored at 4°C under dry conditions, only a marginal loss of enzyme activity was observed after 1 month.

CONCLUSION

In the present study, an electrochemical biosensor for orange G was developed using enzyme laccase entrapped in carbon paste composite matrix. An amperometric biosensor for orange G 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^{-2} to 2×10^{-3} mM. 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, reagentless electrode is suitable for micromolar quantification of azodye orange G.

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