Leland C. Clark Jr. (1918–2005) was an American biochemist born in Rochester, New York. He is most well-known as the inventor of the Clark electrode, a device used for measuring oxygen in blood, water and other liquids. Clark is considered the "Father of Biosensors", and the modern-day glucose sensor used daily by millions of diabetics is based on his research. He is the inventor of Oxycyte, a third-generation perfluorocarbon (PFC) therapeutic oxygen carrier designed to enhance oxygen delivery to damaged tissues.

Chapter VI

IMMOBILIZATION OF AZOREDUCTASE USING SOL-GEL METHOD AND ITS POTENTIAL APPLICATION IN BIOSENSOR

ABSTRACT

A novel potentiometric biosensor was developed by immobilizing *Pseudomonas oleovorans PAMD_1* azoreductase for the determination of azo compounds from the environmental wastes. Azoreductase was immobilized by TMOS (Tetramethoxysilane) sol-gel as revealed by scanning electron microscopy (SEM) investigation and fabricated on an aluminium electrode. Azo compounds were determined by direct reduction of biocatalytically liberated amine species at 0.5 V versus Ag/AgCl as reference electrode. The potentiometric measurements were carried out using a cyclic voltammeter of different dyes such as Orange II, Methyl red and Reactive black with a concentration of 10 mM which shows a reduction peak of 250 μA, 60 μA and 19 μA respectively. The reproducibility of the fabricated biosensor was good with standard deviation value of 2.1 % (n= 10) and a shelf life of more than 15 days when stored at 4 °C. It can be concluded that it is possible to use this azoreductase biosensor for detection of azo dyes in the industrial effluents.

6.1 INTRODUCTION

Textile industry effluents are extremely variable in composition and these industries use water as the principal medium for discharging impurities, applying dyes and finishing agents. Therefore the main concern is about the water quality and quantity discharged and pollutants it carries. The waste from the textile industries may contain dyes of various intense colors, according to the dyes having functional groups of C-N bond, S-O bond, aromatic, alkene, Al-O, Si-O, K-O, N=N (Manikandan *et al.,* 2009). The discharge of effluent containing azo dyes in to natural stream has created significant toxicity due to the soil leaching (Manikandan *et al.,* 2009). In general, textile industry uses more that ten thousand commercial dyes that are incorporated in the Color Index (CI), in which most of the dyes are difficult to decolorize due to their complex aromatic nature (Ozer *et al.,* 2006). Raw dyeing factory effluent at different concentration drastically reduced the percentage germination on crops like rice, maize and nitrogen fixation in green gram (Nigam *et al.,* 2000). Intake of such polluted water results in many pathological effects such as decrease in erythrocyte count and hemoglobin content in human (Subramaniyan *et al.,* 1999).

Therefore, their determination is very important due to its toxicity and persistency in the environmental samples. Currently, several methods are available for the analysis of azo compounds, such as spectrophotometer, gas chromatography, liquid chromatography and capillary electrophoresis. However, these methods are time consuming including complicate sample pre-treatment or high cost

(Rogers *et al.*, 2001; Pascual-Teresa *et al.*, 2000). Therefore, there is an interest in developing a simple, sensitive, specific, accurate and portable system such as biosensor for determination of azo compounds.

Rapid, reliable, low cost, and in some cases, continuous measurement of analytes has been a major challenging goal in analytical sciences. Biosensors are analytical devices for the detection of an analyte by utilizing biological component - a biological or biologically derived material or a biomimic (a synthesized chemical version of a biological substance) coupled to a chemical or physical transducer (electrochemical, mass, optical and thermal). Biosensor offers many advantages over conventional analytical techniques in terms of simplicity, detection limit, specificity and sensitivity. The development of biosensors for analytical purposes has attracted a great deal of interest in recent years.

Enzyme stabilization is an important issue in biotechnological research of enzymes (Ballesteros *et al.*, 1998). As per stability concern, the immobilization is an important approach for biocatalysts (Mosbach, 1987/1988). The method of sol-gel entrapment of biomolecules is a very promising technique for biosensor construction, because of its simplicity, low temperature of the process, large amount and low leakage of entrapped material (Malgorzata *et al.*, 2002).

The aim of our work was to construct a simple biosensor using the azoreductase for the detection of azo compounds. The azoreductase was immobilized by sol-gel method and operation of the biosensor was studied.

6.2 MATERIALS AND METHODS

6.2.1 *Azoreductase production and purification*

Kindly refer chapter II and III

6.2.2 Decolorization assay

The percentage decolorization of the substrate Orange II was determined based on the calculation described by Chen *et al.,* 2003 (detail in third chapter).

6.2.3 *Azoreductase assay*

The activity of Azoreductase was determined based on the procedure described by (Zimmermann *et al.,* 1982) with minor modifications detail in third chapter).

6.2.4 Azoreductase biosensor

6.2.4.1 Immobilization of Azoreductase film

The sol-gel derived silica was achieved based on procedure described by (Lee *et al.,* 2000). TMOS stock solution of sol was prepared by adding 5.0 ml of TMOS, 1.0 ml of deionised water (DI) and 0.05 ml of 0.1M HCl. They were stirred vigorously at room temperature for 15 mins until the transparent homogenous solution was obtained and stored in refrigerator. To induce the hydrolysis, the mixture was sonicated in an ice bath for at least 15 mins. Prior to use, the sol was stored at least 24 hrs at 4 °C. Wet silica gels were made by adding TMOS sol to phosphate buffer solution, which raised the pH and initiated condensation and finally gelation. Azoreductase was introduced within the phosphate buffer solution and become encapsulated upon gelation, then aged in phosphate buffer solution for at least 24 hrs at 4 °C (James Lim *et al.*, 2007).

6.2.4.2 SEM analysis

Microscopic appearance of sol gel immobilized azoreductase enzyme was studied by Scanning Electron Microscopy (Hitachi) at an acceleration voltage of 20 kV. The morphology of the sol-enzyme network was also analyzed.

6.2.4.3 Preparation of electrode

High purity aluminium foil was cut into rectangular shapes of 1 cm in width and 7 cm in length to fit sample holders during the anodization step. Anodizing is an electrolytic passivation process used to increase the thickness of the natural oxide layer on the surface of metal parts, and also treated as anode electrode of an electrical circuit. During anodization process each sample was anodized in 0.3 M oxalic acid at 40 V DC for 10 mins with lead mesh used as a cathode. The anodization was carried out at temperatures below 5 °C to obtain better ordered pore structures. After anodization the foil was rinsed with Millipore water and then immersed into 5 % phosphoric acid for approximately 90 mins to round/widen the pores (Paul Takhistov, 2003).

6.2.4.4 Fabrication of enzyme electrode

Prior to casting of gel, anodized working electrode was polished with alumina powder (Fatma Arslan, 2008; Hyun-Jung Kim *et al.,* 2006), rinsed thoroughly with distilled water. Then the anodized alumina sample was immersed in the sol-enzyme solution for 30 mins at room temperature and allowed to dry in a dust-free hood for 5 mins. The resulting enzyme electrodes were thoroughly washed by double distilled water and stored in 100 mM phosphate buffer (pH 7.0) at 4 °C for further use.

6.2.5 Electrochemical measurement

Electrochemical experiments were carried out in an aerated cell containing 25 ml of 100 mM solution of the phosphate buffer (pH 7.0), at 22 °C. The three-electrode system consisted of the modified enzyme-aluminium electrode as working electrode, an Ag/AgCl (3 M KCl) and a platinum wire as reference and auxiliary electrodes, respectively. The potentiometric measurements were carried out using a cyclic voltameter (CV), a selected potential range of +1.5 to -1.5 V with scan rate of 0.1 ($Vsec^{-1}$) was applied to the working electrode and the background current was recorded until the steady state was reached. The working electrode was calibrated with standard buffer (pH 7.0) before the fabrication in order to see if the buffer has any significant effect on the electrode. The biosensor response was measured as the difference between the total and the background current.

6.2.6 Reproducibility, sensitivity and stability

The operational stability of the biosensor response towards the sample is the essential criterion for selecting the best bioprobe to achieve our research objective. The reproducibility of the enzyme based biosensor was calculated up to ten repeated measurements on the same day, using the same electrode with same working conditions. Similarly the stability of the biosensor was checked by taking repeated measurements everyday up to one week in the optimized conditions, using fresh test samples, and storing the biosensor in its optimum solution at 4 °C when it was not in use.

6.3 RESULTS AND DISCUSSION

Enzymes have been used efficiently in its immobilized form, in order to be economically feasible (Taprab *et al.*, 2005). In order to increase the potential use of enzymes in waste water treatment processes, their immobilization is absolutely necessary for its biochemical stability and reuse (Freire *et al.*, 2000; Cordi *et al.*, 2000). Adequate characteristics including high resistance to thermal denaturation, significant improvement of the enzymatic activity, and its preservation for long periods, have been frequently reported in immobilized form of enzymes.

6.3.1 Azoreductase immobilization

Table 1 shows the stability effect of sol-gel immobilized azoreductase from *Pseudomonas oleovorans PAMD_1.* Highest activity was obtained in sol-gel immobilized azoreductase (82 %) after 5 hrs incubation when compared to free enzyme (39 %). There are several reasons for the highest stability of sol-gel immobilized enzyme: (1) better dispersion of the enzyme in the organic solvent, (2) stabilization of the native enzyme conformation in the sol-gel, (3) a higher content of water in the polar sol-gel matrix (Drik-Jan van Unen, 2000). However, by comparison, control enzyme (without immobilization) showed less activity when compared to sol-gel azoreductase which demonstrated a better stability after sol-gel immobilization, similar results were demonstrated with sol-gel immobilized *P. luteola* (Jyh-Ping Chen *et al.*, 2007).

6.3.2 SEM analysis

Fig 1a and 1b represents the SEM image of free enzyme (control) and sol-gel immobilized azoreductase (test) respectively. Fig 1a and 1b clearly shows that the azoreductase enzyme was immobilized on the sol-gel support. The micrograph of control sol-gel film displayed a clean three-dimensional cluster whereas, the azoreductase immobilized sol-gel showed a very narrow particle size distribution. This structure resulted in a binding of the free enzyme conjugate with immobilizing gel.

6.3.3 Electrode and enzyme fabrication

The three-electrode system used for the construction of biosensor consist an enzyme-aluminium electrode as working electrode, Ag/AgCl as reference and a platinum wire as auxiliary electrodes as shown in Fig 2. After fabrication of the immobilized enzyme with electrode (Fig 3), further electrochemical measurements were carried out.

6.3.4 Electrochemical measurement

The potentiometric measurements were carried out using a cyclic voltameter of different dyes (Orange II, Reactive black and Methyl red) with a concentration of 10 mM. Fig 4a, shows the typical voltammogram of the azo dye Orange II before the fabrication of the electrode with the immobilized enzyme, representing the control. A reduction peak of 16 μA was observed at a potential of -0.5 V. Fig 4b, shows a well defined reduction peak of 250 μA at -0.5 V for the same after the fabrication of the electrode with the sol-gel azoreductase. The elevated reduction peak is attributed to the reduction of azo bond catalyzed by the enzyme.

Similar results were found for the dye reactive black also. In Fig 5a the voltammogram of the reactive black shows a reduction peak at 16 μA at a potential of -0.5 V against the enzyme electrode peak of 60 μA at the potential of -0.5 V (Fig 5b). This indicates the potential range for the reduction of the specific azo bond present in the azo dye. The voltammogram of the methyl red was shown in Fig 6a & 6b. It depicts a reduction peak at 19 μA at -0.5 V against the control (16 μA) attributed to the presence of azo group in that. The decrease in the reduction of peak size may due to the nature of substrate specificity. Hence it proves Orange II is more specific than other dyes for azoreductase (Zimmermann *et al.,* 1982). The comparatively significant reduction in peak observed in the different azo dyes indicates the presence of a wide range of azo groups. The results also suggest the broad range of specificity and cleavage of azo compounds by azoreductase..

Other typical researches in enzymes as biosensors show the impact on immobilization. Cyclic voltammograms of immobilized tyrosinase electrode shows the reduction peak on increasing concentrations of catechol. Such reduction peak is due to the reduction of quinine species liberated from the enzymatic reaction catalyzed by tyrosinase on enzyme electrode (Sanket Tembe *et al.,* 2006). Glucose oxidase was co-immobilized with catalase to broaden the calibration range of the electrode (Malgorzata Przybyt *et al.,* 2002).

6.3.4 Sensitivity, Reproducibility and stability

The azoreductase biosensor exhibits high sensitivity towards Orange II azo dye at a concentration of 2.0 mM (Fig 7a and b) which shows a reduction peak at

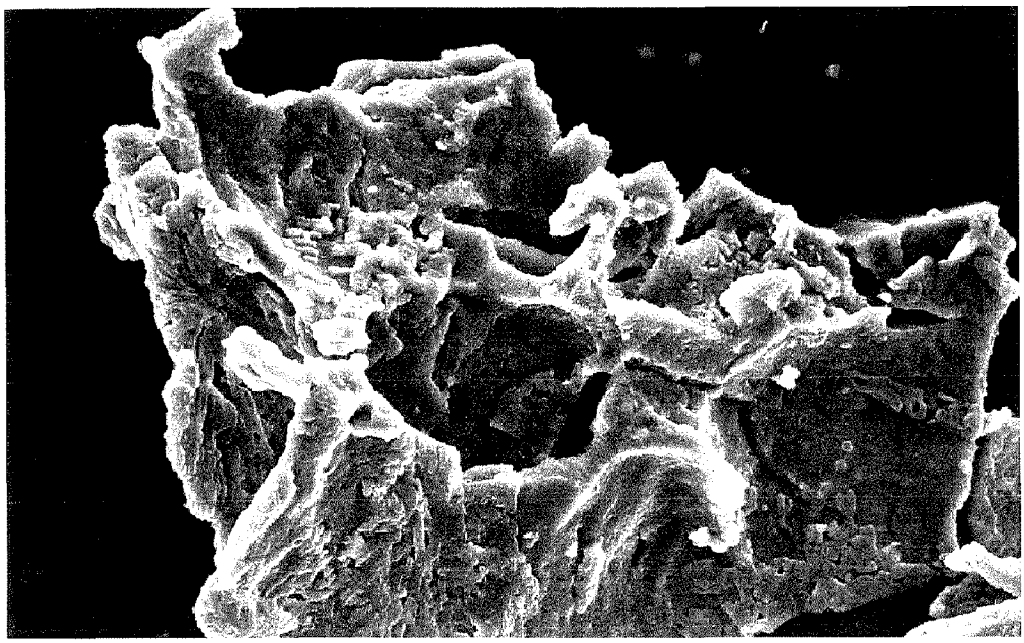
16 and 17 μA at a potential of -0.5 V for control and azoreductase fabricated electrodes respectively. The repeatability of the same sol-gel immobilized azoreductase biosensor was examined by the detection of 10 mM Orange II azo dye. A relative standard deviation (RSD) of 2.1 % (n=10 cycles) was obtained for ten successive determinations, which indicated a good reproducibility of the measurements. The long-term stability of the biosensor was evaluated by measuring its response for one week. It can be seen that over this time period the biosensor maintains more than 60 % of this initial response. The relatively good stability of the biosensor may be due to the immobilized film that could provide a biocompatible microenvironment and the specific ability of azoreductase could be protected effectively (Jin Chen *et al.*, 2005). The large quantities of hydroxyl groups in the sol-gel hybrid material could form strong hydrogen bonds (Yu and Ju, 2002).

6.4 CONCLUSION

The extracted *Pseudomonas oleovorans PAMD_1* bacterial azoreductase shows great potential in environmental biotechnology. It has been investigated for possible applications in decolorization and detoxification of textile effluents and for biosensors.

This work successfully develops a novel amperometric biosensor for assay the azo compounds from the environmental samples by immobilizing azoreductase in a silica based sol-gel thin film. The durability of azoreductase showed as much as in immobilized form than compared with the fee enzyme. A reduction of azo bond by the immobilized azoreductase results in current change, which is proportional to the

concentration of azo compounds in solutions, can be measured. The results described were shown that the immobilized azoreductase biosensor is a very promising tool and it can be used for determination of the impact of azo compounds to the ecosystem. This biosensor shows a good reproducibility and storage stability, can be conveniently prepared in batch.



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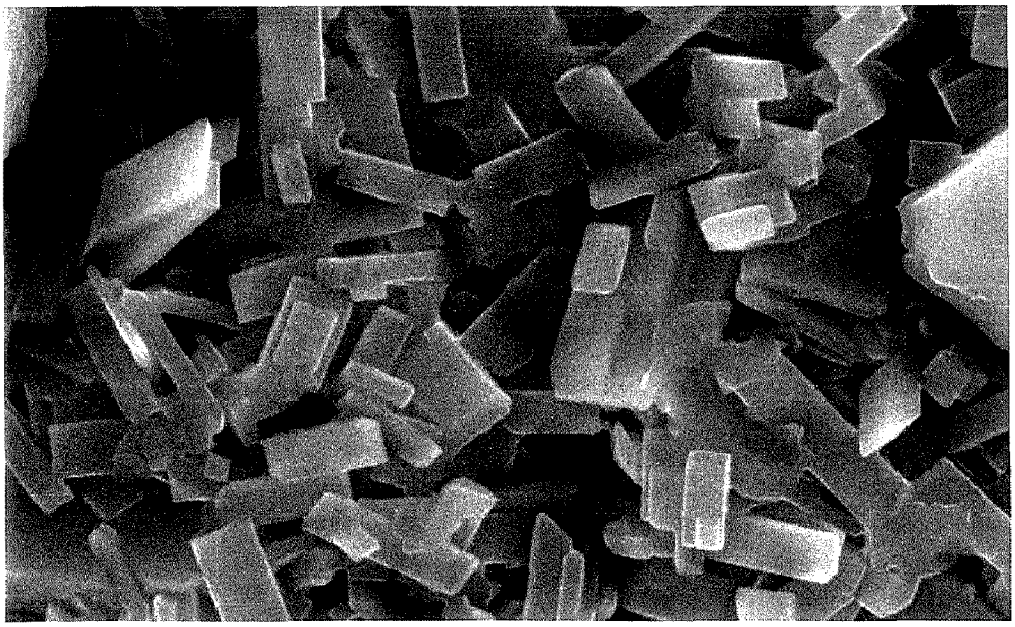


1 (b)

Figure 1: SEM image of sol-gel azoreductase (a) sol-gel without enzyme (control) (b) sol-gel with enzyme

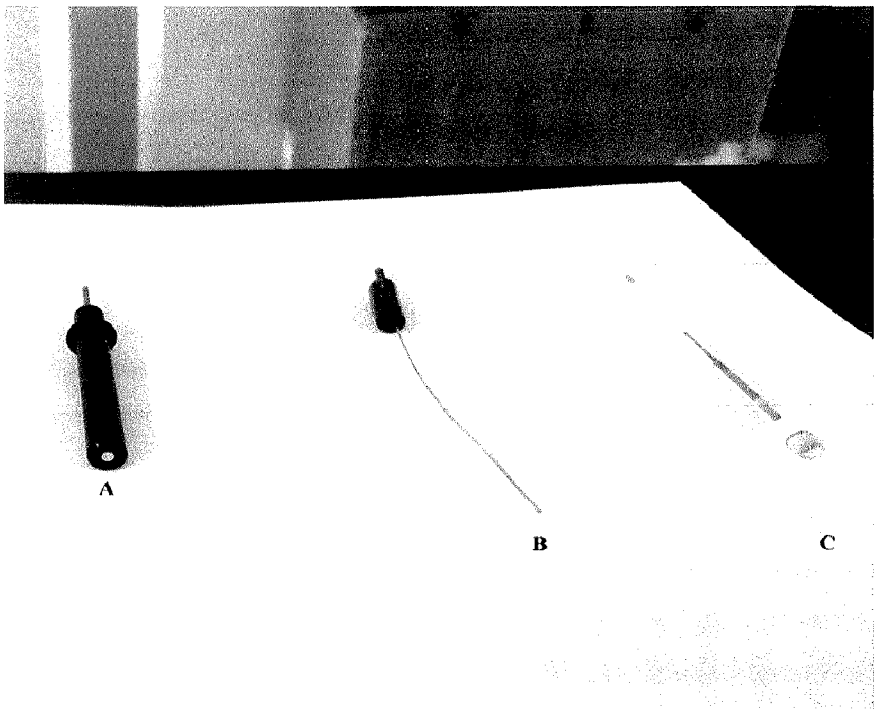


Figure 2: A- Working aluminium electrode, B-Counter electrode, C-Reference electrode

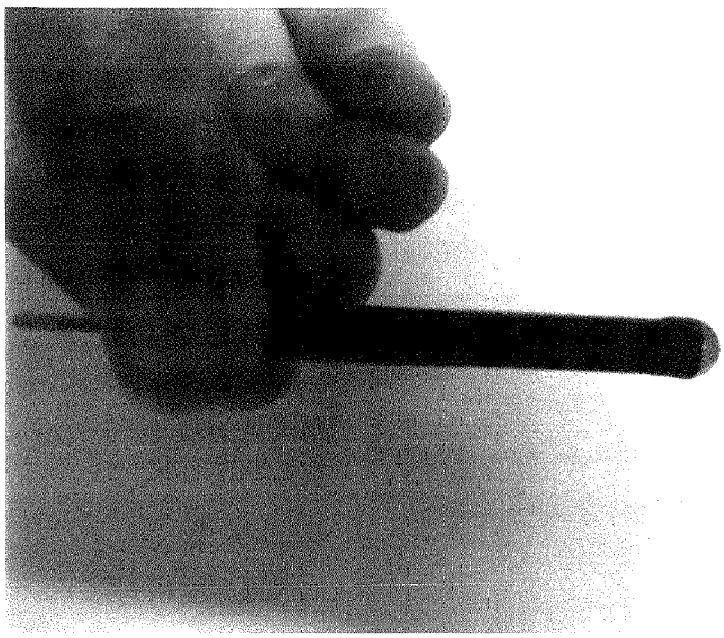
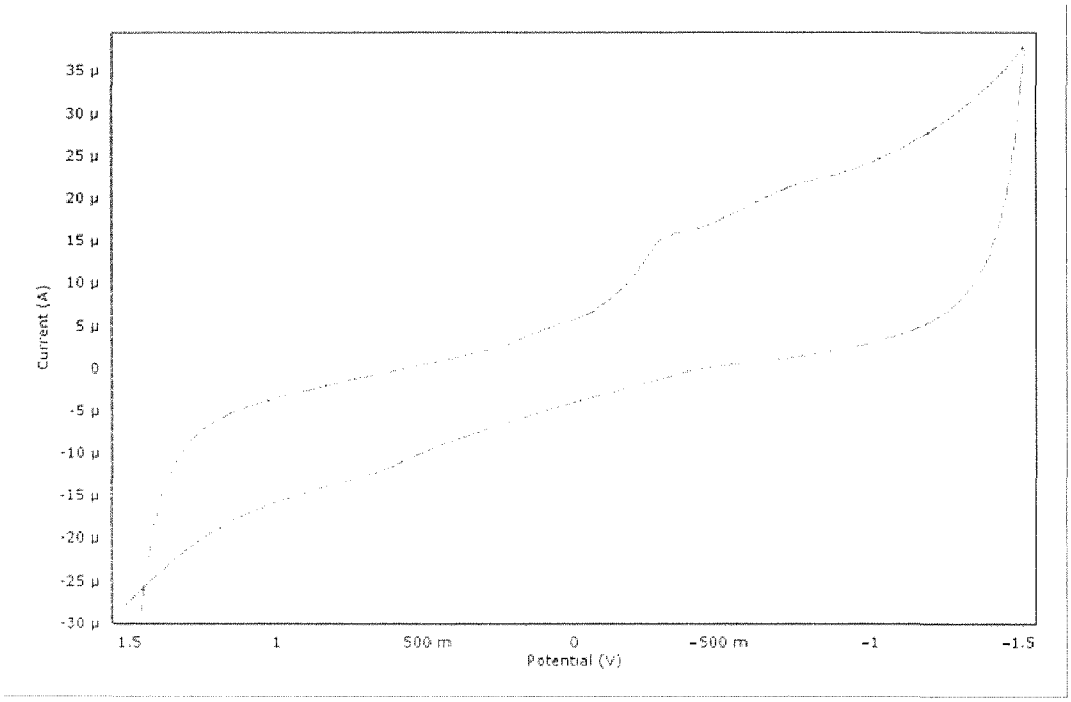
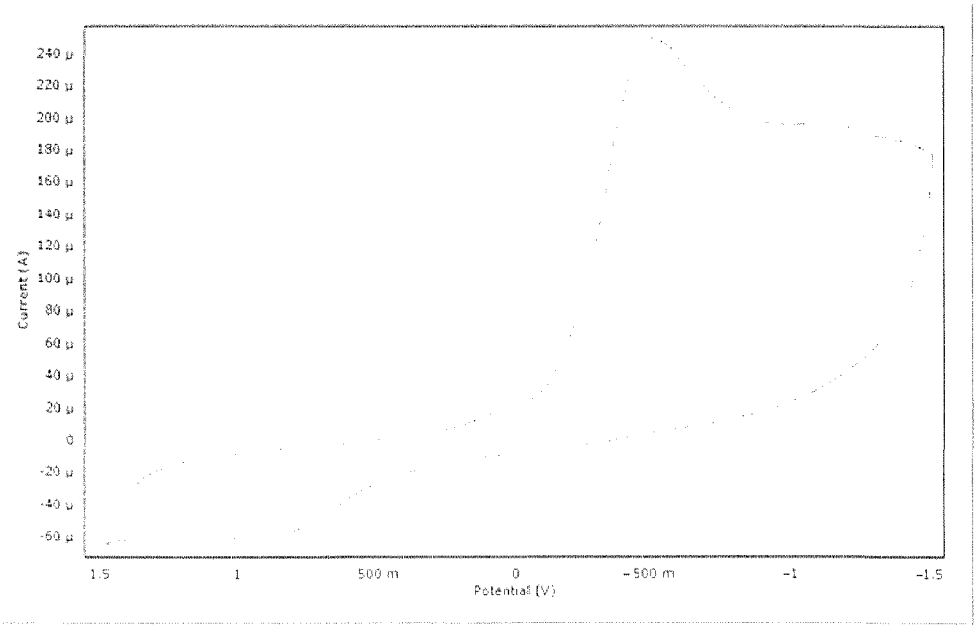


Figure 3: Fabricated working electrode

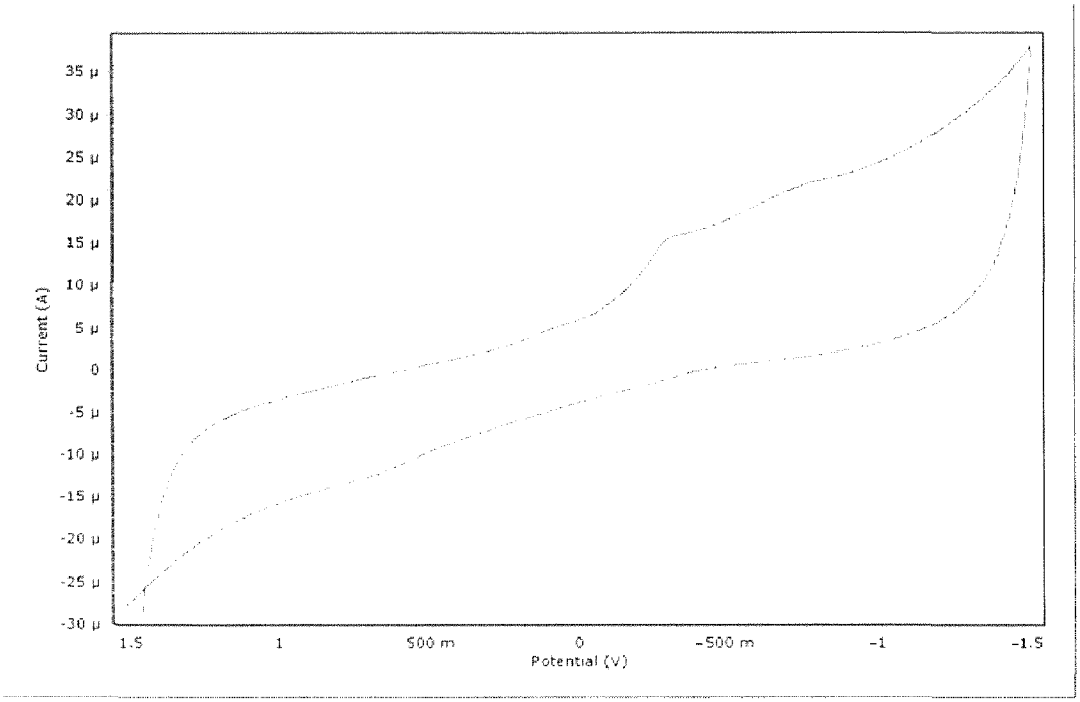


4 (a)

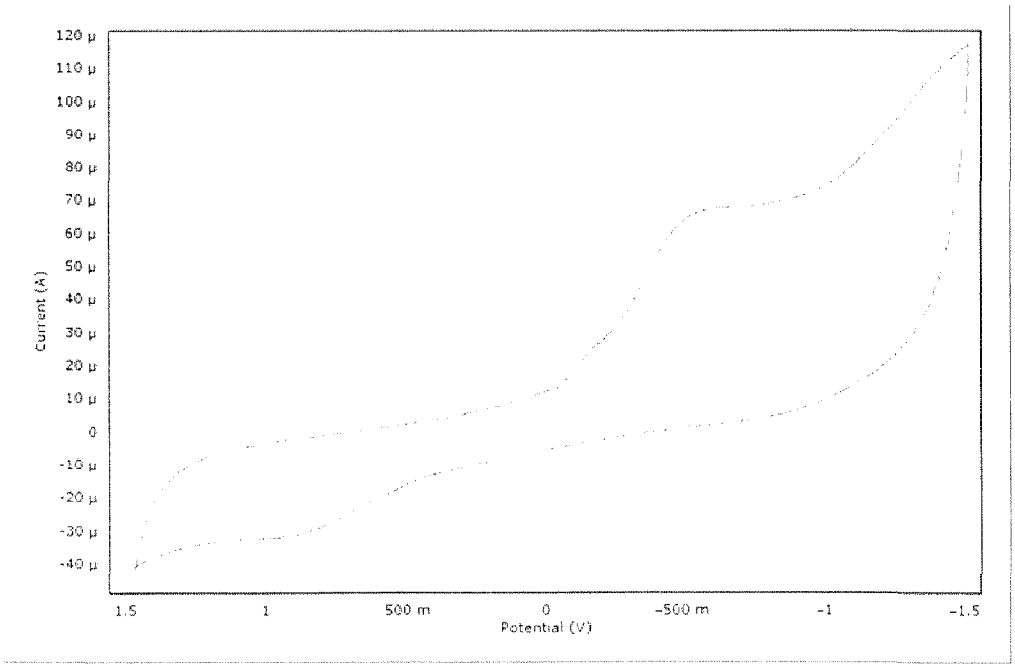


4 (b)

Figure 4: Voltammogram of Orange II a) Control (without enzyme) b) after fabrication of enzyme.

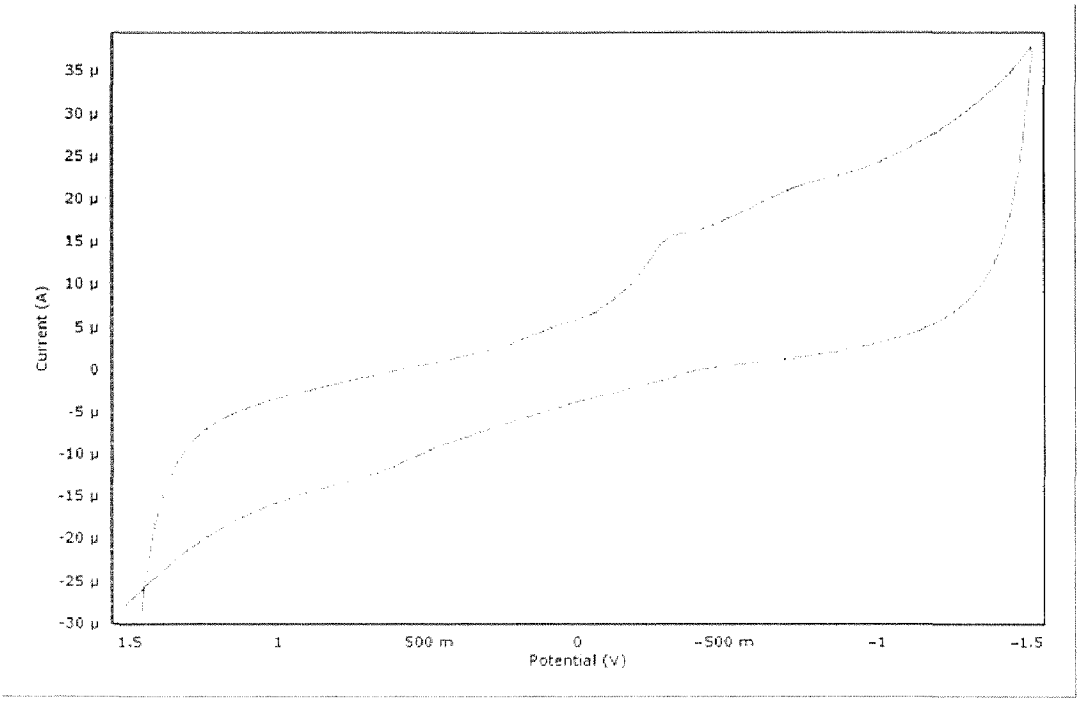


5 (a)

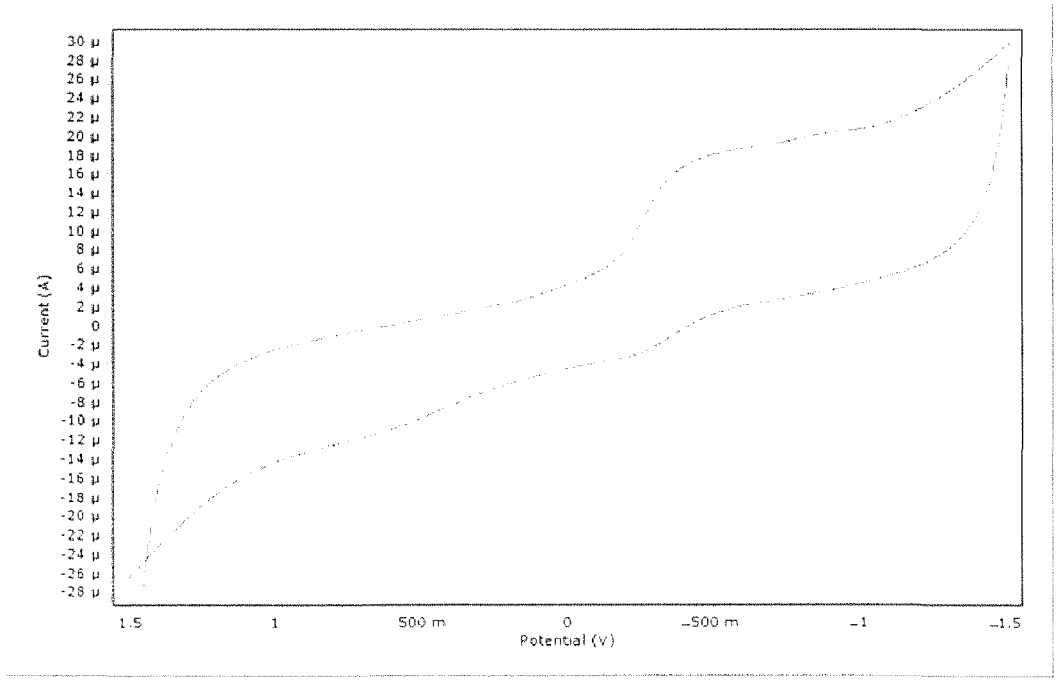


5 (b)

Figure 5: Voltammogram of Reactive black a) Control b) after fabrication of enzyme

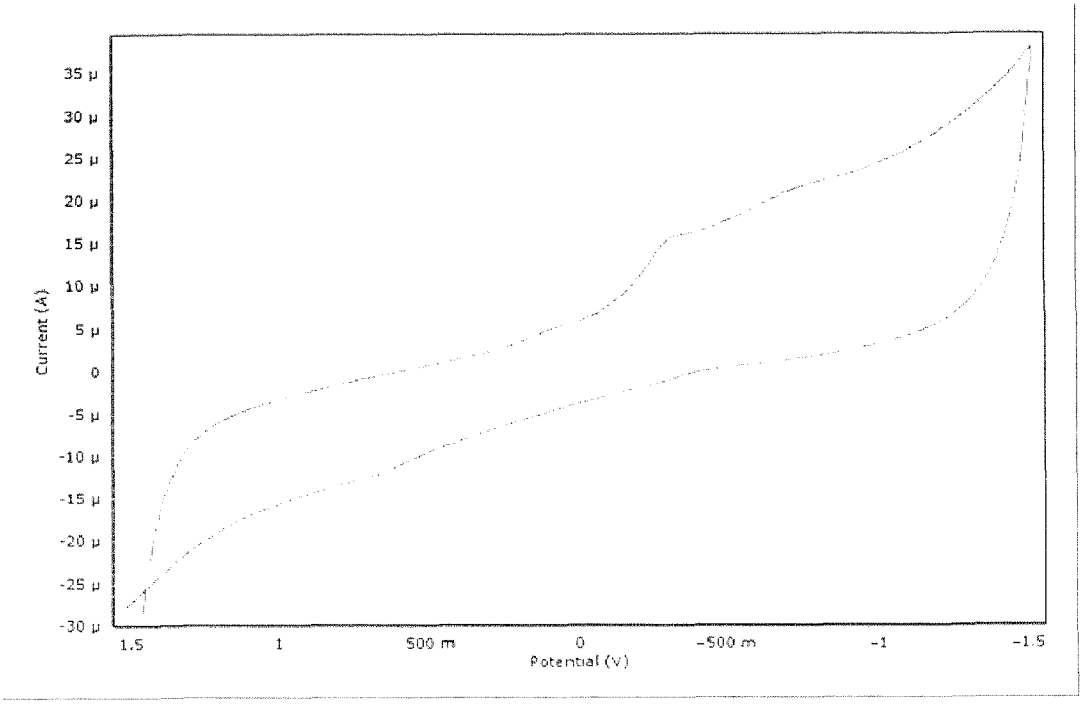


6 (a)

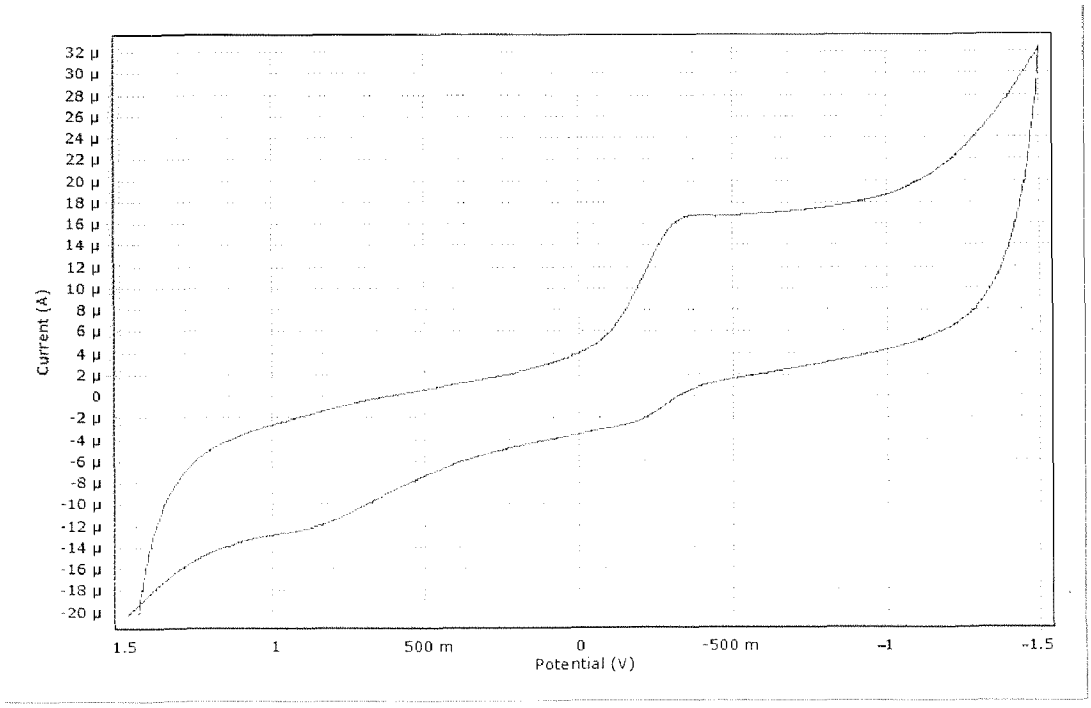


6 (b)

Figure 6: Voltammogram of Methyl red a) Control b) after fabrication of enzyme



7 (a)



7 (b)

Figure 7: Voltammogram of Orange II (2 mM) a) Control b) after fabrication of enzyme

Table 1: Stability of immobilized and free azoreductase at 37 °C after different time incubation

Incubation time (mins)	Residual activity (% decolorization)	
	Sol-gel Immobilized Enzyme	Free Enzyme
60	100	100
120	100	74
180	93	60
240	87	45
300	82	39