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A Simple Laccase Based Amperometric Biosensor for Detection of Phenolic Azo Dyes- A Comparative Study on Different Membranes as Immobilization Supports

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Abstract

Detection of azo compounds and phenolic azo compounds are 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. Most widely used methods for determination of azo compounds are HPLC, GC, voltammetric and capillary electrophoresis. These existing conventional methods are laborious, time consuming and require sophisticated instruments as well as sensitive sample preparation prior to analysis. An effective alternative technique is the use of biosensor. The aim of this work was to detect phenolic azo compound by an amperometric laccase based biosensor with simple oxygen sensing using Eriochrome Black T (EBT) as the model substrate. The optimum operational conditions for the biosensor were investigated and the system was calibrated for detection of phenolic azo dye, EBT. Laccase from *Trametes versicolor* was immobilized on different membranes on Clark type DO sensor by co-crosslinking with bovin serum albumin (BSA) and glutaraldehyde. The behaviour of laccase-based amperometric biosensors on three different membranes like cellophane, nylon and chitosan/TEOS/EG membranes were compared. Among the various membranes tested, nylon membrane was superior to the other methods in terms of sensitivity, limit of detection (LOD), response time while cellophane membrane was found to be the best in terms of operating and thermal stability. Biosensor response reached steady state within 3min and exhibited maximum activity at 45^0C and pH 6.8. Validation of the newly developed biosensor by comparison with HPLC, showed good agreement in results. This method demonstrated excellent selectivity and sensitivity towards the selected phenolic azo compound.

Keywords: amperometric, biosensor, co-crosslinking, eriochrome black-T, laccase.

Introduction

The textile mills daily discharge millions of liters of effluents in the form of wastewater into public drains that eventually empty into rivers. Most of them are recalcitrant in nature, especially azo dyes. The stability and their xenobiotic nature of reactive azo dyes and phenolic azo dyes makes them recalcitrant hence they are not totally degraded by conventional wastewater treatment processes that involve light, chemicals or activated sludge (1). The dyes are therefore released into the environment, in the form of coloured wastewater. This can lead to acute effects on exposed organisms; phytoplanktons form abnormal colouration and reduction in photosynthesis (2, 3). This also alters the pH, increases the biochemical oxygen demand (BOD) and chemical oxygen demand (COD), and gives the rivers intense colourations and public is greatly concerned about water quality. Without adequate treatment these dyes will remain in the environment for an extended period of time and are deleterious not only for the photosynthetic processes of the aquatic plants but also for all the living organisms, since the degradation of these can lead to carcinogenic substances (4).

Azo dyes contain at least one nitrogen-nitrogen (N = N) double bond, however many different structures are possible. The azo groups are generally connected to benzene and naphthalene rings, but can also be attached to aromatic heterocyclic or enolizable aliphatic groups (5). 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 (6). Because of the diversity of dye components available for synthesis, a large number of structurally different azo dyes exist and are used in industry (7).

Reactive azo dyes as well as phenolic azo dyes are widely used in the textile industry to color cellulosic fibers (8). Inefficiencies in the dyeing process result in 10–15% of all dyestuff being lost directly to wastewater. For this reason, it is absolutely necessary to find new detection methods of the dye from the textile wastewater.

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 (9, 10). A number of microorganisms belonging to different taxonomic groups of bacteria, algae, fungi and yeast have been reported for their ability to decolorize azo dyes (11).

The quantification of chemicals in the environment is a major issue, among which the azo dyes and phenolic azo dyes are the compounds with prime importance (12). Several methods have been reported for detection of textile azo dyes, such as HPLC (13), spectrophotometric, voltammetric (14) and capillary electrophoresis (15). These existing conventional methods are laborious, time consuming and require sophisticated instruments as well as sensitive sample preparation prior to analysis. An effective alternative technique is the use of biosensor. Biosensors are accurate, specific, easy to use, less time consuming and do not necessitate sample pretreatment and can overcome the disadvantages of the conventional methods (16, 17).

The aim of this work was to combine the analytical performance of a laccase system for a reactive phenolic azo dye like EBT detection with that of simple oxygen sensing. Use of lower negative potentials for oxygen detection reduces interference from other possible electroactive species that may be present in the sample. As there are reports of oxidative biodegradation of azo dyes by fungal laccase (18, 19), we use laccase based biosensor with oxygen sensing for detection of a phenolic azo dye, EBT.

Laccase is an oxidoreductive ligninolytic enzyme used in various biotechnological and environmental applications. In comparison to other oxidoreductases, such as the peroxidases that need H_2O_2 in its catalytic process, laccase only uses oxygen for the oxidation of its reduced state (20). These enzymes degrade azo dyes as well as phenolic azo dyes through a highly nonspecific free radical mechanism forming phenolic compounds and in certain cases to form quinones, thereby avoiding the formation of toxic aromatic amines (21). Figure1 represents the suggested model for the activity of laccase on phenolic azo dye, EBT (22).

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.

The present report describes the construction of amperometric biosensor containing immobilized laccase from *Trametes versicolor* for the detection of a commercially reactive dye EBT in industry effluents. Co-crosslinking with BSA and glutaraldehyde was done on three different types of membranes like cellophane, nylon and Chitosan/TEOS/EG membranes were compared.

Materials and Methods

Reagents

Laccase (from *Trametes versicolor*) having specific activity 10 IU mg-1 was procured from sigma (USA), Eriochrome Black-T (EBT), Chitosan, BSA, Citric acid, tetra ethyl ortho silicate (TEOS), ethylene glycol (EG) and cellophane membrane were procured from Himedia and glutaraldehyde from SD Fine chem., India. All chemicals were of analytical-reagent grade and were used as received without further purification. Double-distilled water was used throughout the experiments.

Apparatus

A Clark's electrode with an amperometric detection system was used for detection of substituted phenols. An amperometric principle based detector system developed in our laboratory was used to amplify and monitor the signals obtained from enzyme electrode. Clark type of dissolved oxygen electrode was purchased from M/S Century Instruments, Chandigarh, India. The Clark-type electrode consists of a Gold (Au) cathode and a reference Ag/AgCl-electrode covered with saturated KCl electrolyte enclosed within a Teflon membrane. A polarizing potential of -650mV was applied to the gold working electrode.

The current generated from the enzyme electrode depends on analyte concentration, area of electrode and also on the protocol used to develop biosensing membrane. The current was converted to voltage and amplified using signal conditioning circuit. The output of the signal conditioning circuit was measured through a digital voltmeter.

Construction of biosensing membrane and measurement of response

2IU of enzyme was crosslinked with glutaraldehyde (5%-10µl) on three different membranes hereafter referred to using the code A, B and C using 3 mg of BSA. After drying for 1hr the enzyme membrane was washed with 0.1M phosphate buffer (pH 6.8) to remove excess glutaraldehyde. This was attached to the surface of the probe using an inner Teflon membrane by rolling the "O" ring over the end of the probe. This was immersed in a sample cell containing 5ml of 0.1M phosphate buffer of pH 6.8.

A: Cellophane membrane

B: Nylon membrane

C: Chitosan/TEOS/EG membrane: Chitosan solution was prepared by dissolving chitosan (2 g) in citric acid (6% w/v). Chitosan/TEOS/EG blends were prepared by adding TEOS (30% w/w) and EG (10% w/w) in chitosan.

Procedure for azo dye (Eriochrome Black T) detection

The sample cell was continuously saturated with oxygen using a portable air pump. The probe was connected to the amperometric detector system developed in our laboratory. 50µL of standard solutions of EBT of varying concentrations (5-40 ppm) were injected into the buffer solution using micropipette.

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When the substrate is introduced into the sample cell, the enzyme reaction proceeds resulting in depletion of oxygen in the vicinity of enzyme membrane thereby yielding an electrochemical signal of decreasing current. This response is converted into voltage amplified and monitored which is directly proportional to the concentration of analyte. The time taken to reach steady state is 3 minutes.

Optimization of Performance parameters

Optimum pH for Biosensor performance

The effect of pH on sensor response was studied by incubating the immobilized enzyme membrane-A for 30 min in 0.1M buffer of different pHs in the range 4-8 and the response for EBT (20ppm) was measured at that pH. Acetate buffer was used in acidic range and phosphate buffer for neutral and basic range.

Optimum temperature for biosensor performance

Optimum temperature for biosensor performance was determined by incubating the immobilized enzyme membrane-A at temperatures ranging from 27 to 70^{O}C for in 0.1M phosphate buffer pH6.8. Response for EBT (20ppm) when immobilized enzyme membrane was incubated at temperatures ranging from 27 to 70^{O}C was measured.

Operational stability of immobilized enzyme membrane

The operational stability studies of immobilized enzyme membranes A, B and C were carried out at 28 ±2° C. The enzyme membranes were stored up to 30 days by keeping immersed in phosphate buffer of pH 6.8 and the activity was checked daily by injecting 20ppm of EBT and the response recorded as drift in voltage.

Calibration plots, analytical characteristics and immobilized enzyme kinetics

Various analytical features such as linearity range, limit of detection, correlation coefficient (R^2), and sensitivity were studied for different enzyme membranes A, B and C. Kinetic parameters for the immobilized laccase catalysed reaction were also calculated for the three enzyme membranes.

Studies on reproducibility

The reproducibility of the biosensor was studied by using the fabricated biosensor for estimating a known concentration of EBT (20ppm) in 10 replicates each. The observations were statistically analyzed for standard deviation (SD) and coefficient of variation (CV).

Sample application

The laccase based biosensors with three enzyme membranes were applied in synthetic waste water samples.

Result and Discussion

Optimization of parameters for the biosensor performance

Optimum pH for Biosensor performance

The effect of pH on sensor response was studied by incubating the enzyme membrane-A for 30 min in 0.1M phosphate buffer of different pHs in the range 4-8 and the response was measured at that pH. The optimization studies showed that the optimum pH of immobilized enzyme was at pH 6.8 (figure 2). Therefore, for further studies 0.1M phosphate buffer pH 6.8 was used.

Optimum temperature for Biosensor performance

Effect of temperature on the response for EBT

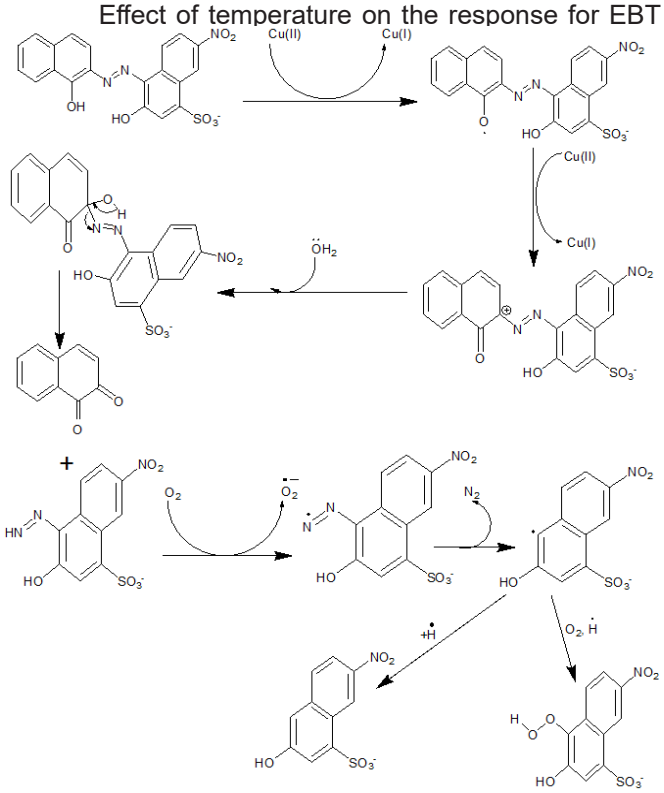

Fig. 1. Proposed mechanism for the degradation of phenolic azo dye, Eriochrome Black T by laccase.

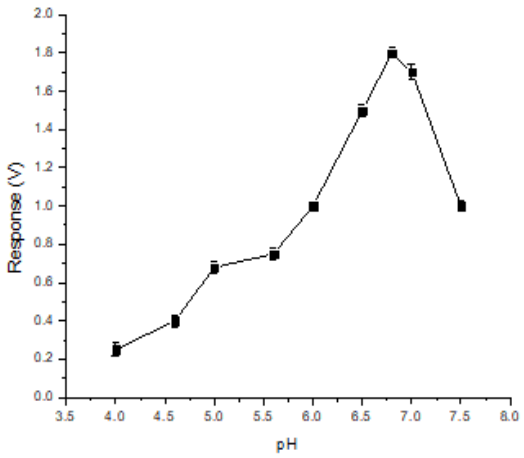

Fig. 2. Optimum pH for biosensor performance

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on immobilized enzyme membrane-A was studied by incubating the enzyme electrode in 0.1M phosphate buffer pH 6.8 at temperatures ranging from 27 to 70^{O}C and the response for EBT (20ppm) was measured. The optimum temperature for biosensor performance was found to be 45^{o}C (figure 3) as the response increased upto 45^{o}C and later it decreased. Results of the study with *Trametes versicolor* species is almost similar as mentioned in the literature stating that laccase activity is maximum at temperature between 30-50 for enzyme those obtained from sources like *Trametes hirsuta*, *Sclerotium rolfsii* and *Pleurotus ostreatus* (23, 24, 25).

Operational stability of immobilized enzyme membrane

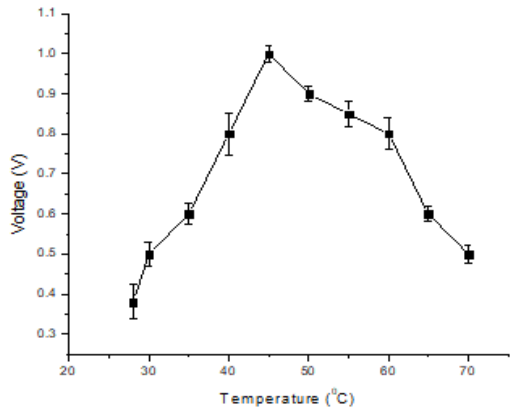

Fig. 3. Optimum temperature for biosensor performance

The operational stability studies of different immobilized enzyme membranes were carried out at room temperature (28 ± 2°C). In order to quantify the operational stability of various enzyme membranes, repeated measurements with 20 ppm EBT were carried out. As shown in the figure 3, as the number of analyses increased, activity of immobilized laccase (in terms of biosensor response) increased initially probably owing to the decreased diffusional barriers for the analyte. Further usage of immobilized enzyme for repeated analysis of EBT leads to sharp decrease in activity. However, enzyme

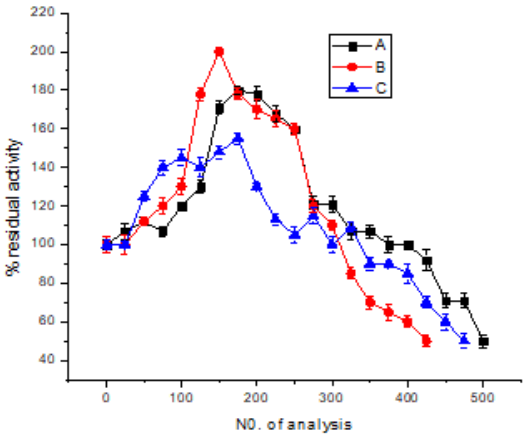

Fig. 4. Operational stability of laccase based biosensor employing various enzyme membranes.

immobilized in cellophane membrane (A) shows a stable high activity upto 175 analyses and thereafter declined to reach 50 % activity around 500 analyses. Thus, co-crosslinking using BSA on cellophane membrane found to be the best when compared to other membranes.

The order of operational stability for the different enzyme membranes are as follows: A>C>B in terms of membrane half life. (Figure 4 & Table 1).

Thermal stability of immobilized laccase

For thermal stability studies on immobilized enzyme membranes, freshly immobilized enzyme

Table 1. Comparison of % activity retained after repeated analysis of EBT for the different enzyme membranes.

Enzyme Membranes	% activity retained after No. of analysis for 20 ppm EBT						No. of analysis possible with 50% activity retention (half life)
	100	200	300	400	450	500	
(A) Cellophane (B) membrane	120	178	121	100	71	50	500
(C) Nylon (D) membrane	130	170	110	60	----	---	250
(E) Chitosan/ (F) TEOS/EG	145	130	100	85	60		200

membranes were kept at 28 ± 2° C overnight in 0.1M buffer solution of pH 6.8. Initial activity 'a' of the enzyme membrane for 20ppm of EBT was measured at 28 ± 2° C. The electrode was then immersed for 15 minutes in 5ml buffer at desired temperature using a constant temperature bath. The enzyme membrane was immediately cooled to room temperature in ice bath for 2 minutes. Then the residual activity 'b' of immobilized enzyme was observed at room temperature by injecting 20ppm EBT. The % residual activity is calculated as [b/a]xl00. Thus the residual activity was calculated at the different temperatures in the range of 40-80° C in steps of 2° C. Transition temperature (Tm), was calculated from a plot of % activity versus temperature at 50 % activity. Figure 5 demonstrates the thermal deactivation behaviour of laccase based biosensor for co-crosslinking method of immobilization using BSA using the enzyme membranes A, B and C at pH 6.8. Enzyme membranes A and C could retain 100% activity whereas membrane B retains 95% at 60^{0}C. This showed that all the three enzyme membranes are exhibiting good thermal stability. Transition temperature (Tm) is found to be 75, 72 and 73 for the enzyme membranes A, B and C respectively.

Immobilized enzyme kinetics

Immobilized enzyme kinetics was studied using EBT as model substrate. Apparent Michaelis-Menten constants (K_m app.), and the maximum rate of reaction (Vmax) were calculated from the corresponding Lineweaver-Burk plots as shown in (Table 2).

Determined from experimental results, Km. app. covers all parameters responsible for the immobilized enzyme behavior (enzyme denaturation or inhibition, enzyme active sites availability and other parameters

Table 2. Kinetic parameters for the laccase catalysed reaction using enzyme electrode employing various enzyme membranes with EBT as substrate.

Types of enzyme membranes	Vmax(V)	Km.app. (ppm)	Vmax/Km.app.
A	0.28	40	0.0070
B	0.32	26	0.0123
C	0.22	30	0.00733

affecting enzyme capability to recognize its substrate) (26). Kam.app. is an indicator of the affinity that an enzyme has for a given substrate and, hence, the stability of the enzyme-substrate complex. The kinetics of laccase catalysed reactions is first affected by the affinity between enzyme and the substrate. An estimation of this influence can be done by amperometric measurements in terms of the Vmax./Km.app. ratio. These parameters are often calculated in the design of enzymatic sensors to evaluate the sensitivity of the system proposed, which is related to the low or high affinity of the enzyme towards a specific substrate. Here enzyme membrane B showed more affinity towards EBT when compared to other membranes. Observed Km with immobilization on enzyme membrane B was 26 ppm and immobilization on membrane A and C were 40 and 30 ppm respectively. The substrate concentration required for the reaction to proceed at half maximal velocity was more for membranes A and C compared with membrane B. Vmax for enzyme membranes A and C were 0.28 V/ min and 0.22 V/min as against 0.32 V/ min with immobilization on nylon membrane. The reasons for high Vmax and low Km for immobilization on membrane (B) is because nylon is more permiable to substrate. Therefore, the concentration of substrate in the microenvironment of the enzyme will be more when nylon is used instead of cellophane and chitosan/TEOS/EG.

Calibration plots and analytical characteristics

Table 3 summarises the characteristics of the calibration plots obtained for EBT tested with laccase electrodes using different membranes A, B and C under the optimized working conditions of temperature and pH. Therefore, co-crosslinking method with BSA on

Table 3. Analytical characteristics of laccase based biosensors for the different enzyme membranes with EBT as substrate.

Types of enzyme membranes	Linearity range (ppm)	LOD (ppm)	Response time (min.)	Recovery time (sec.)	Equation	R^2
A	20-70	8	5	60	y=0.0025x + 0.036	0.994
B	8-25	3	3	40	y=0.0054x + 0.006	0.991
C	15-40	5	5	60	y=0.003x + 0.013	0.998

nylon membrane was found to be the best in terms of sensitivity, LOD, response time to detect EBT.

The calibration graph for EBT showed better linearity (R^2-value =0.994, 0.991, 0.998) when the enzyme was immobilized on membranes A, B and C (Figure 6, 7 & 8). Best sensitivity i.e., lower limits of detection could be achieved by using enzyme membrane

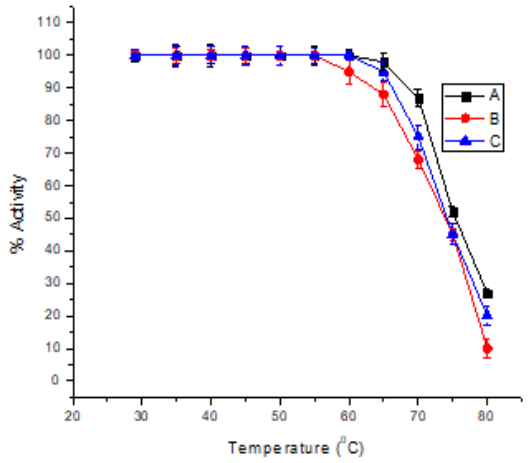

Fig. 5. Thermal inactivation of laccase based biosensor using various enzyme membranes.

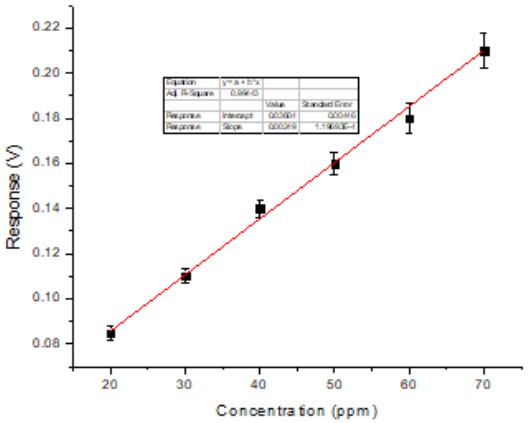

Fig. 6. Calibration graph of laccase based biosensor (A)

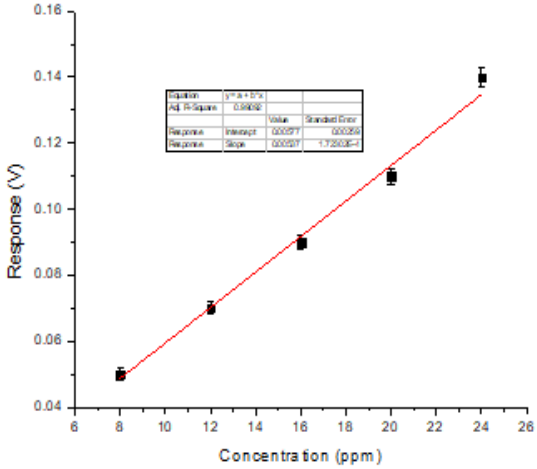

Fig. 7. Calibration graph of laccase based biosensor (B)

B when compared to A and C and response time was less (3 min.) for immobilization on membrane B because

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the diffusion barriers are less when nylon membrane is used.

Reproducibility

To check reproducibility and accuracy of the biosensor, a known concentration of EBT was taken (20

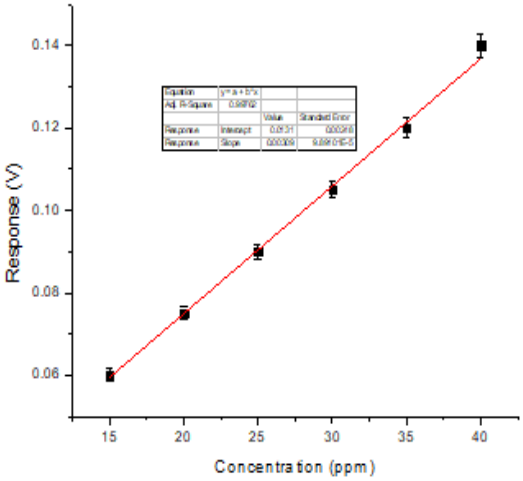

Fig. 8. Calibration graph of laccase based biosensor (C)

ppm) in 10 replicates each and fabricated biosensor was used for its estimation.

The observations were statistically analyzed for standard deviation (SD) and coefficient of variation (CV) given in Table 4 .The results showed that the determinations were consistent in terms of reproducibility, accuracy and reusability for all types of enzyme membranes.

Sample application

Validation of the test biosensor was successfully performed by comparing the results with conventional HPLC. The proposed biosensor containing laccase enzyme immobilized using co-crosslinking method with BSA on membrane B was applied for the analysis of EBT in simulated effluents. 50µl of simulated effluents were added to the reaction cell after equilibration had occurred and then the change in current was measured. The signals obtained from the effluent samples were found to be very similar with that of the reference compound solutions having the same concentration. Good correlation was observed between results obtained with the test biosensor and those with HPLC (Table 5).

Table 4. Statistical analysis of biosensor response for the various type of enzyme membranes using 20 ppm of EBT.

Type of enzyme membranes	Standard Deviation (S.D.)	Coefficient of variation(CV)
(A) Cellophane membrane	0.0055	0.0375
(B) Nylo membrane	0.0038	0.0246
(C) Chitosan/TEOS/EG	0.0045	0.00524

Therefore, the system could be easily applied for the screening of phenolic azo dye in industrial effluents.

Table 5. Simulated effluent sample analysis using test biosensor

Sample	Concentration of EBT (ppm)	Detected amount (ppm)	
		Biosensor	HPLC
1	10	11±0.02	10±0.03
2	14	13±0.03	14±0.02

Note: Results are expressed as ±S.D.., n=5

Conclusion

An amperometric biosensor containing immobilized laccase from *Trametes versicolor* was constructed for the detection of a reactive phenolic azo dye like EBT in industry effluents. Co-crosslinking with BSA and glutaraldehyde was done on three different types of membranes like cellophane, nylon and Chitosan/TEOS/EG membranes and compared. The kinetic analytical properties and thermal behaviour of the resulting biosensors were also tested. Based on the studies it can be concluded that laccase modified electrodes can be used for detection of phenolic azo dyes from textile effluents. Moreover, these biosensors can be used for the continuous monitoring of the dye content in effluent and can be coupled with the decolorization process for a better control of the total dye content in the effluent. Reason for the selection of BSA as stabilizing agent is that BSA stabilizes the enzyme by hydrophobic interactions. Hydrophobic interactions are considered as the single most important factor in stabilization of enzyme structure. Therefore strengthening of these interactions should impart structural rigidity to the enzyme molecules and thus make them more resistant to unfolding. Moreover, the detachable membrane unit used by us for these studies has enabled a convenient method to follow the activity of the immobilized enzyme over a long duration using a number of enzyme membranes, but using a single dissolved oxygen probe. This has made relatively large number of analysis possible in a convenient and economical way. The biosensor technique has been useful to track the operational stability of the immobilized enzymes used this study. Validation was successfully performed by comparing the test results of real samples with HPLC. Good correlation was observed between results obtained with the test biosensor and those with HPLC. The results obtained from these studies are the first ones obtained using a simple DO sensing laccase based amperometric biosensor for the detection of azo dyes using EBT as the representative molecule.

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