Development of Biosensor for Phenol Detection Using Agarose–Guar Gum Based Laccases Extracted from *Pleurotus ostreatus*

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ABSTRACT: Laccases from *Pleurotus ostreatus* was extracted from the Shaken flask cultures of *Pleurotus ostreatus* grown at 25°C with continuous agitation (110 rpm.) in baffled 1000 mL Erlenmeyer flasks containing 200 mL medium. The basal GYP medium used for cultures contained 20 g glucose l^{-1}, 5 g yeast extract l^{-1}, 5 g peptone from casein l^{-1}, and 1 g $MgSO_4.7H_2O$ l^{-1}. The pH was adjusted to 5.0 with H_3PO_4 before sterilization. The kinetics of oxidation reactions catalyzed by laccases was studied using 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid). The laccases showed lower specific activity and higher activity in nonpolar organic solvents. A biosensor using laccases was constructed for the determination of phenol. The enzyme was extracted from *Pleurotus ostreatus* and entrapped in agarose–guar gum composite biopolymer matrix. Phenol was determined by direct reduction of biocatalytically liberated quinone species at −0.1 V versus Ag/AgCl (3*M* KCl). The response was found to be linear and concentration dependent.It has a shelf life of more than 2 months when stored at 4°C. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 115: 1358–1365, 2010

Key words: biomaterials; biopolymers; conducting polymers; nanotechnology

INTRODUCTION

A biosensor may be considered as a combination of a biorecepter, the biological component, and a transducer, the detection method. The total effect of a biosensor is to transform a biological event into an electrical signal. Biosensors found extensive applications in medical diagnostics, environmental pollution control for measuring toxic gases in the atmosphere, and toxic soluble compounds in river water. These pollutants include heavy metals, nitrates, nitrites, herbicides, pesticides, polychlorinated biphenyls, polyaromatic hydrocarbons, trichloroethylene etc. Pollutant sensitive biocomponents have been used with a variety of detection modes for their quantitative estimation.[1–3] An enzyme sensor may be considered as the combination of a transducer and a thin enzymatic layer, which normally measures the concentration of a substrate. The enzymatic reaction transforms the substrate into a reaction product detectable by a transducer. The sensitive surface of the transducer remains in contact with an enzymatic layer, and it is assumed that there is no mass transfer across this interface. The external surface of the enzymatic layer is kept immersed in a solution containing the substrate under study. The substrate migrates toward the interior of the layer and is converted into reaction products when it reacts with the immobilized enzyme. Different strategies are followed for the immobilization of molecular recognition agent in sensor devices particularly in biosensors. Polymers are the most suitable materials to immobilize the enzyme, the sensing component, and hence to increase the sensor stability. The use of enzyme sensors can help in the direct measurement of such compounds, including organic pollutants for environmental control. As hydrogen peroxide used in food, textile, and dye industries for bleaching and sterilization purposes can be directly measured by enzyme sensors as per the following equation, with the liberated oxygen detected by oxygen electrode:

$$H_2O_2 \xrightarrow{\text{Catalase}} H_2O + \frac{1}{2}O_2$$

This technique is faster and more convenient than the classical colorimetric and volumetric methods. By far the largest group of direct electron-transfer biosensors is based on coimmobilization of the

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enzyme in a conducting polymer, namely polypyrrole and polyaniline. Various epoxy cements are somewhat similar. Sensor devices have been made from classical semiconductors, solid electrolytes, insulators, metals, and catalytic materials. As the chemical and physical properties of polymers may be tailored by the chemist for particular needs, they gained importance in the construction of sensor devices. Although a majority of polymers are unable to conduct electricity, their insulating properties are utilized in the electronic industry. A survey of the literature reveals that polymers also acquired a major position as materials in various sensor devices among other materials. Either an intrinsically conducting polymer is being used as a coating or encapsulating material on an electrode surface or a nonconducting polymer is being used for immobilization of specific receptor agents on the sensor device.[4]

Enzymatic oxidation techniques have great potential within a variety of industrial fields including the pulp and paper, textile, and food industries. Enzymes recycling on molecular oxygen as an electron acceptor are the most interesting ones. Thus, laccases (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) is a particularly promising enzyme for the aforementioned purposes. The laccases molecule is a dimeric or tetrameric glycoprotein, which usually contains four copper atoms per monomer distributed in three redox sites.[5] This enzyme catalyzes the oxidation of ortho and paradiphenols, aminophenols, polyphenols, polyamines, lignins, aryl diamines, and some inorganic ions coupled to the reduction of molecular dioxygen to water.[6] Laccases-mediated systems have been applied to numerous processes such as pulp delignification,[7] oxidation of organic pollutants,[8] and the development of biosensors.[9,10] During the past two decades, bioelectrochemistry has received increased attention. Progress on bioelectrochemistry has been integrated into analytical applications, e.g., biosensors working as detectors in clinical and in environmental analysis.[11] Among many analytical methods for measuring phenolic compounds, electrochemical biosensors based on immobilized laccases have received the major share of attention. As laccases are able to catalyze electron transfer reactions without additional cofactors, their use in biosensors has also been studied to detect various phenolic compounds, oxygen, or azides. With regard to laccases, the immobilization has an important influence on the biosensor sensitivity.[12] Martele et al.[13] have shown that micropatterning is an efficient method for the immobilization of laccases on a solid surface to develop a multifunctional biosensor. Also, Roy et al.[14] found that crosslinked enzyme crystals of laccases from *Trametes versicolor* could be used in biosensor applications with great advantage over the soluble enzyme. More recently, Cabrita et al.[15] have immobilized laccases from *Coriolus versicolor* on *N*-hydroxy succinimide-terminated self-assembled monolayers on gold. This procedure could be useful for the further development of biosensors. In addition, an enzyme electrode based on the coimmobilization of an osmium redox polymer and a laccases from *T. versicolor* on glassy carbon electrodes has been applied to ultrasensitive amperometric detection of the phenolamine neurotransmitters dopamine, epinephrine, and norepinephrine, attaining nanomolar detection limits.[16] In view of major concern with regard to toxicity, considerable attention has been given to the reliable quantification of phenols in complex environmental matrices.

Tembe et al.[10] reported the development of electrochemical biosensor for l-dopa and dopamine using a composite biopolymer matrix, agarose and guar, as they are naturally occurring biopolymers having high permeability toward water. The composite material provides natural microenvironment to the enzyme and also gives sufficient accessibility to electrons to shuttle between the enzyme and the electrode. Its good film forming and adhesion ability, together with its nontoxicity and biocompatibility, has developed growing interest in using it for laccases entrapment and subsequent sensor fabrication.

In this article, the potential application of this laccases-polysaccharide bioelectrode in environmental pollution monitoring is described using phenol as a representative of phenols. Here, we developed electrochemical biosensor for phenol using enzyme laccases entrapped in agarose–guar gum composite biopolymer matrix, and analytical performance of the biosensor was studied.

MATERIALS AND METHODS

Culture of *Pleurotus* and enzyme purification

Pleurotus ostreatus was isolated from a Rose Wood tree (*Dalbergia sissoo*) growing in Bichpuri Campus of Raja Balwant Singh College, Agra (India) and was maintained through periodic transfer at 25°C on potato dextrose agar plates. Shaken flask cultures of *P. ostreatus* were grown at 25°C with continuous agitation (110 rpm.) in baffled 1000 mL Erlenmeyer flasks containing 200 mL medium. The basal GYP medium used for cultures unless otherwise stated contained 20 g glucose l^{-1}, 5 g yeast extract l^{-1}, 5 g peptone from casein l^{-1}, and 1 g $MgSO_4.7H_2O$ l^{-1}. The pH was adjusted to 5.0 with H_3PO_4 before sterilization. Several agar plugs cut from the actively growing, outer circumference of a fungal colony growing on potato dextrose plates were used as inocula. For stimulating laccases synthesis,

$CuSO_4 \cdot 5H_2O$ was added after 64–96 h of cultivation so that its final concentration in the medium was 2.0 m*M*.

Purification of laccases

Mycelia were separated by centrifugation (20 min; 10,000 × *g*) after 200 h cultivation, when laccases activity reached its maximum, and the culture supernatant was frozen, thawed, and filtered to remove precipitated polysaccharides. The enzyme solution was then concentrated using a 30 kDa ultrafiltration membrane. Precipitate was removed by centrifugation (20 min; 10,000 × *g*). The clear supernatant was repeatedly dialyzed against water and was applied to a Q-Sepharose Fast Flow column (50 × 50 mm; Amersham-Pharmacia) pre-equilibrated with 20 m*M* sodium acetate buffer, pH 5.0. The column was washed at a flow rate of 5 mL/min with 2000 mL buffer to remove unbound laccases isoforms and protein. Bound laccases was subsequently eluted from the column with a linear salt gradient (0–0.25 *M* NaCl in the same buffer) at a flow rate of 5 mL/min. Elution was simultaneously monitored at 280 and 610 nm for protein and Type-1 copper, respectively. Fractions containing laccases activity were pooled, concentrated as earlier, applied to a Superdex 75 prep grade column (800 × 16 mm; Amersham-Pharmacia) pre-equilibrated with 20 m*M* sodium acetate buffer, pH 5.0, containing 200 m*M* NaCl, and eluted at a flow rate of 0.5 mL/min. Active fractions were pooled, desalted, filter-sterilized, and stored at 4°C. For molecular mass determination of the native protein, a Superdex 75 HR 10/30 (major laccases from *P. ostreatus)* column (Amersham-Pharmacia) equilibrated with 50 m*M* phosphate buffer, pH 7.0, containing 100 m*M* KCl was used.

The column was calibrated with the standard proteins ribonuclease A (*M*r 13,700), carbonic anhydrase (*M*r 29,000), ovalbumin from chicken egg (*M*r 45,000), bovine serum albumin (*M*r 66,000), and transferrin (*M*r 81,000) each at a concentration of 2 mg/mL.The flow rate for elution was 0.5 mL/min.

Enzyme activity assay

To determine extracellular enzyme activity in agar medium, plugs containing mycelia from the center of the fungal colony were added to the reaction buffer (at a ratio of 50 mg of plug per mL of reaction buffer). Boiled agar plugs (10 min) served as controls. To detect activity in submerged cultures, culture supernatant was used in the reaction mixtures. Blocks of fresh fruiting bodies from various developmental stages were used for the measurement of enzyme activity. Laccases activity was determined by using 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) Oxidation of ABTS was measured by determining the increase in absorbance at 420 nm with an extinction coefficient of 36 m*M*/cm. One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 mmol of ABTS per min. All the reactions were performed at 30°C. Enzyme activity was expressed as units per gram of medium or fresh fruiting body.

Other analyses

Protein concentrations were measured using the Bradford dye-binding assay (Coomassie blue, Bio-Rad) and bovine serum albumin (fraction V) as the standard. Glucose and fructose concentrations were determined using commercially available test kits (Boehringer Mannheim).

Phenol and agarose were from Sigma ChemicalS, St. Louis. Guar gum was procured from Sisco Research Laboratory (SRL, India). All other chemicals were of analytical grade, and all solutions were prepared with water from Millipore Milli-Q system.

Preparation of immobilized laccases film

Laccases was entrapped in agarose–guar gum composite as described by Tembe et al.[10] Briefly, aqueous solutions of agarose (3%) and guar gum (1%), of 1 mL each, were mixed and the appropriate amount of laccases (2000 U) was then added. The mixture was spread uniformly on glass plate over the surface area of 9 cm^2 and kept overnight in a dust free hood for drying. The thin membrane thus obtained was stored at 4°C under dry conditions.

Fabrication of enzyme electrode

The working electrode was a laccases modified glassy carbon electrode prepared by immobilizing the laccases in agarose–guar gum composite. Before casting of gel, glassy carbon electrode was polished with alumina powder and rinsed thoroughly with distilled water. Composite gel (30 μL) containing 250 U of enzyme extract [25.2 μg as determined by Lowry method[17]] was directly cast on the active surface of glassy carbon electrode. The electrode was then allowed to dry in a dust free hood. When not in use, electrode was stored at 4°C.

Electrochemical measurement

Cyclic voltammetric and differential pulse voltammetric experiments were performed with Autolab 100 potentiostat by standard three electrode configuration comprising of an ITO glass plate as a working electrode, a platinum plate as a counter electrode

and Ag/AgCl as a reference electrode. This cell was used to measure the catalytic reduction of phenol and responses of the laccases biosensor to phenol. All Cyclic voltammograms (CVs) were performed at 5 mV/s. Determination of phenol was carried out electrochemically by measuring the intensity of current that corresponds to the electrochemical reduction of the enzymatically generated quinone. This was done by immersing the working electrode in a 0.1*M* phosphate buffer at pH 6.0 and applying a polarization voltage of +100 mV to the platinum electrode against an Ag/AgCl (sat. KCl). When the background current had stabilized, an appropriate amount of phenol was introduced in an electrochemical cell. All the measurements were carried out at room temperature under continuous stirring. A sensitive cathodic reduction peak was used for quantitative determination. A good linear relationship was observed between cathodic peak currents and phenol concentration.

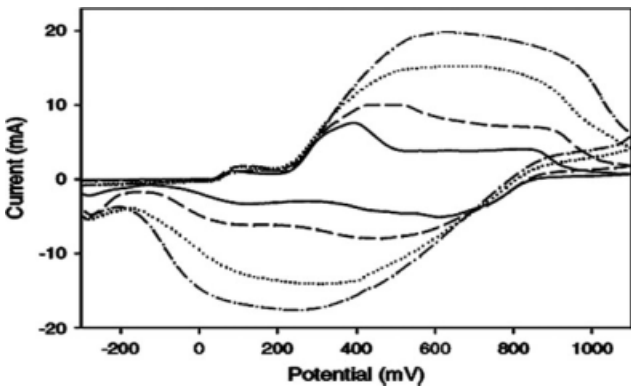

Figure 1 Cyclic voltammogramms obtained at agarose–guar gum entrapped laccases electrode for solutions of increasing phenol concentration from 0 μ*M* (undotted line), 100 μ*M* (dashed line_ _ _), 200 μ*M* (dashed dotted line_._._.), and dotted line (……..) agarose–guar gum membrane without laccase for 400 μ*M* phenol. Scan rate: 100 mV/s. Electrolyte: phosphate buffer (0.1*M*, pH 6.0).

RESULTS AND DISCUSSION

Enzyme details

Isolation of the main laccases isoenzyme was performed from the culture supernatant of *P. ostreatus* laboratory batch fermentation with glucose as the main substrate and stimulating laccases formation by the addition of copper to the actively growing culture. Under these conditions, approximately, a large fraction of laccases were formed by the fungus with 62,000 U laccases activity and 350 mg of extracellular protein. *P. ostreatus* secreted at least eight multiple isomeric forms of laccases ranging from 2.6 to 6.0.

Cyclic voltammetric behavior of laccases modified electrode

CVs of laccases electrode in sodium phosphate buffer (pH 6.0, 0.1*M*) without phenol and with increasing concentrations of phenol are shown in Figure 1. It was observed that the reduction peak increased after phenol was added to phosphate buffer on enzyme immobilized electrode. Such an increase in reduction peak is because of the reduction of quinine species liberated from the enzymatic reaction catalyzed by laccases on enzyme electrode.

Electrode response characteristics

To determine the concentration of phenolic compounds, oxygen consumption that occurred in the enzymatic reaction was detected. By using a thermostatic reaction cells, all the measurements were done at 35°C under continuous and constant magnetic stirring and varying substrate concentration in steady state condition. The working electrode was polarized at −0.7 V versus the reference electrode. On the other hand, 300 s was necessary for each analysis, a base line was obtained in the presence of working buffer in 200 s after that substrate solution was added, and enzymatic action was completed in 100 s. The current changes were registered by a potentiostat. After completion of the measurement, the electrode was rinsed with distilled water. In our study, before the optimization studies, the effect of enzyme amount for PL based electrode during the deposition process at +0.65 V was tested.

Effect of enzyme concentration and deposition time

The effect of enzyme amount for PL based electrodes was studied between 50 and 450 U/mL. The deposition was performed for 15 min in the presence of various enzyme amounts. Maximum sensor response was found in the presence of 250 and 300 U/mL of enzyme amount. A slight decrease was obtained in the presence of higher enzyme amounts (Fig. 2). Continuous dotted line shows behavior of enzyme for biosensing that reached to maximum 0.198 i/μA at 300 U/mL enzyme concentration. This response depleted to 0.03 i/μA at 450 U/mL. Enzyme saturation and biosensing was correlated with a linearity $R^2 = 0.9958$. Current response increased with laccases concentration between 100 and 300 U/mL; however, electrodes grown with 350 U/mL had a lower response. This is in agreement with previous reports.[18,19] This decrease in response is attributed to a transition between kinetically controlled and diffusion controlled regimes. As the concentration of enzyme increases, substrate is consumed in an outer

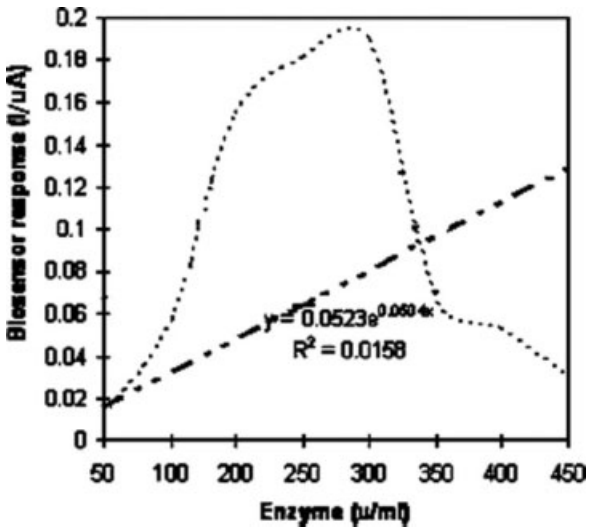

Figure 2 Effect of enzyme loading upon the sensitivity of the biosensors. Dotted line curve (. . ...) shows the response, and dashed dotted line (_._._.) represents the linear trend to enzymatic load.

layer of the immobilization matrix and larger amounts of phenol, the product of the enzymatic reaction, diffuse back to the bulk of the solution instead of reaching the surface of the electrode.

Moreover, effect of deposition time of laccases enzyme electrode on biosensor response was studied within range of 2.5–25 min. The optimum deposition time for PL was recorded to be 7.5 min. This response remained constant upto 17.5 min and then started decreasing upto 25 min (Fig. 3). Laccases catalyze the oxidation of phenol group to *o*-quinone, thus allowing a variety of phenolic compounds to be used as substrates of this enzyme. It is well known that this enzyme presents broad substrate specificity in respect to duration of deposition. Therefore, it is reasonable that this sensor may be used to selectively detect phenol without interference.

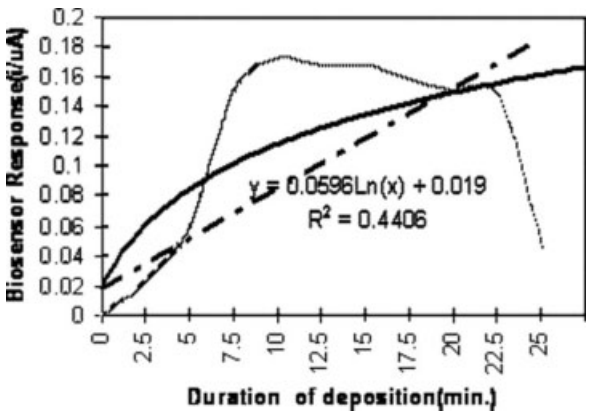

Figure 3 Effect of deposition time of enzyme on biosensor response. Undotted Line denotes the order of reaction.

Optimization of the biosensor

Effect of pH

The initial experiments were conducted to establish the optimum pH. The effect of pH on enzyme based electrodes was studied at different pH ranging from 0 to 12. Phosphate buffers at different pH values were used as carrier solution. According to optimization studies, the optimum pH of PL electrode was obtained as 4–6. In higher pH values, a sharp decrease was obtained. Figure 4 shows the results obtained from pH optimization studies of the biosensor. The regression equation revealed linearity ($R^2 = 0.9892$) and correlation with enzymatic activity and biosensor response. Biosensor response was found feeble below 3 where as at higher pH range 8–12 steep decrease in the response was noticed. The buffers used for pH range 3.5–5.5 were acetate buffer and for 6.0–8.0 pH range it was potassium phosphate buffer.

The maximum response for phenol was obtained at pH 6.0. The optimum response of the biosensor was obtained at pH 6, which is also the optimum pH for the enzyme activity. Thus the immobilization of the enzyme laccases did not alter its optimum pH for catalytic activity.[12,20] This finding is in agreement with the work reported for immobilized laccases on carbon fiber electrode,[12] graphite electrode,[11,21] redox hydrogel polymer,[22] and platinum treated silane.[23] In these studies, the optimum response was in the pH range 4.0–6.0. Therefore, pH 6.0 was chosen in subsequent experiments. A linear response for phenol obtained in phosphate buffer (0.1*M*, pH 6.0) is shown in Figure 4.

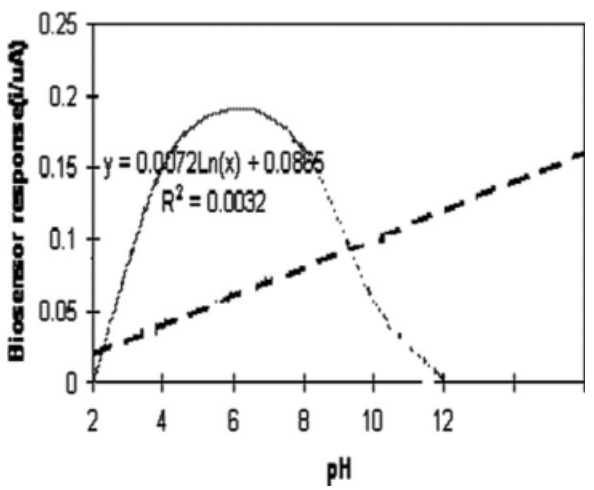

Figure 4 Effect of pH on biosensor response. Dotted line curve (. . ...) shows the response, and dashed dotted line (_._._.) represents the regression expression of enzyme to pH.

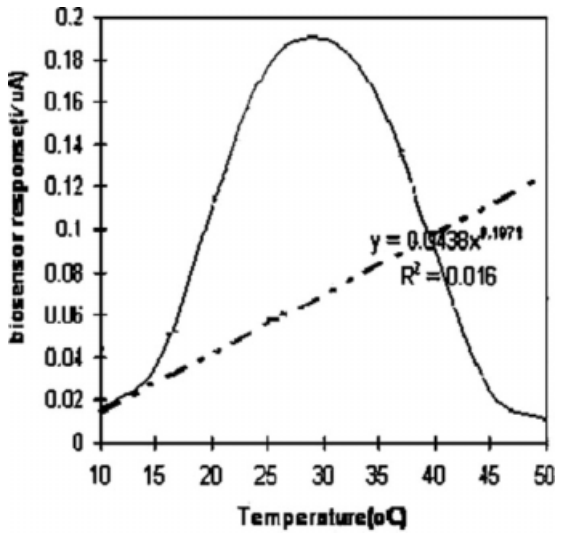

Figure 5 Effect of temperature on biosensor response. Undotted line curve shows the response, and dashed and dotted line represents the regression expression of enzyme to temperature.

Effect of temperature

Temperature affects the rate of reaction and thereby alters the response time; higher temperature gives faster response at the same time it also shifts equilibrium potential and gives nonlinear response.[24] The response of biosensor, at different temperatures ranging between 10 and 50°C has been observed. From the Figure 5, it is clear that the biosensor output increases with an increase in temperature. The maximum linear response was observed between the temperature ranges of 20–35°C, and later it decreases. Performance of the biosensor was better at temperature 25–32°C, and it has been considered for experimentation. According to Figure 5, the biosensor response directly increased with temperature upto 35°C, but on the further increase in the temperature a slight decrease was observed. The experimental range of temperature was selected from 10 to 50°C. The acetate buffer solution having pH 5.5 and 4.5 μ*M* phenol showed a typical response in relation to temperature according to Arrhenius formula-

$$\ln\ k = \ln\ A - (E_a/RT)$$

Where, k is rate constant and E_a is the activation energy. In temperature range 10–35°C, the response current of biosensor increased with increasing temperature (Fig. 5).

Enzyme maintains its native conformation at elevated temperature and having lower tendency to aggregate. Energy must be put into the system to disrupt these contacts, so that additional energy is required to break the covalent crosslinks between agarose-gaur-polyanilne-laccases and to dissolve and then denature. The stabilization is also because of these contacts rather than chemical crosslinks. At 25°C, the activity increased and then it started decreasing. The halflife of laccases was found to be 45 min at 35°C. The thermal stability started decreasing above 40°C. The activation energy (E_a) of laccases was found to be −55.04 kJ $kmol^{-1}$ K^{-1}. Inactivation rate constant k_r was determined from the slope of the plot log % residual activity versus time of incubation.

Arrhenius plot of thermal inactivation showed that enzyme had a steep inactivation curve from 50 to 40°C. The increased thermal stability in comparison with others may be because of the preordered arrangement of the molecules by inter- and intramolecular crosslinks between the agarose-gaur-polyanilne-laccases and hence the rigidity of the three-dimensional arrangement of molecules.[21,25] Agarose-gaur-polyanilne-laccases offers major advantages to the biosensor designers to perform the laccases catalyzed reactions at even higher temperature thereby increasing the reaction rate.

Thermal stability

The thermal stability experiments showed that after 13 h period only 7.4% decrease was obtained. During this period, ~145 measurements have been made, and it can be possible to make more measurements in a longer time period. The maximum response was observed at pH 7.0 and at temperature 25°C. With reference to the cathodic reaction, there may be a tendency that the net change in H^+ ions may lead to change in pH level. To overcome this, anode was cleaned with buffer solution after every 36 h of usage or after every 20 number of analyses.

Effect of phenol concentration

It can be seen from Figure 6 that the response current increased with increasing phenol concentration. There was a direct proportional relationship in which response current appeared to be in a linear relation with the change in phenol concentration. Thus, this enzyme catalytic reaction of laccases was of the first order reaction. But later, with continuous increase in phenol concentration, the response current increased slowly, i.e., enzyme reaction showed a transition from first to zero order. After 8.5 μ*M* phenol concentration, the response showed steep fall. Phenol sensor gave a linear plot for the range 4×10^{-5} to $8 \times 10^{-4} M$ with a linear regression equation $y = 0.001x + 0.5402$, $r^2 = 0.0397$ where, y represents the current (μA) and x represents the substrate concentration. A detection limit of $8 \times 10^{-6} M$ is achieved. A calibration curve for phenol is shown in Figure 6. An apparent K_m value of 5.5 μ*M* was

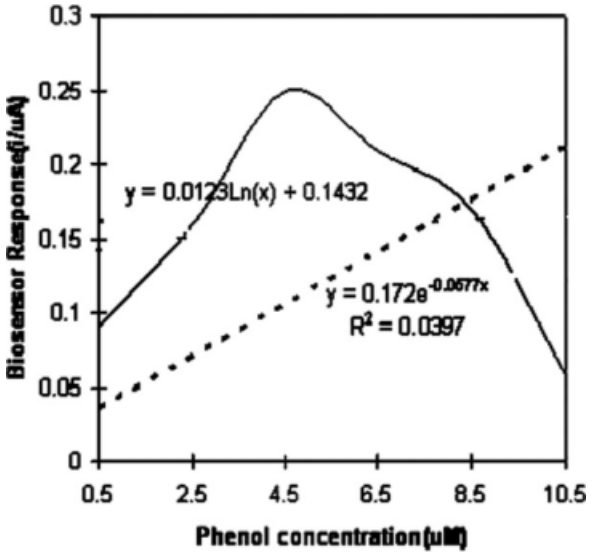

Figure 6 Effect of phenol concentration on biosensor response. Undotted line curve shows the response, and dotted line represents the regression expression of enzyme to phenol concentration.

obtained for phenol. With low concentrations of phenol, no decrease in response was observed for at least 20 cycles in continuous testing. A decrease in response of enzyme electrode was observed for a high-concentration (8.5 μ*M*) of phenol, attributed to slow surface fouling by the reaction product.

Effect of potential

The potential was stepped from 0.2 to 0.6 V in 0.05 V increment in acetate buffer (pH 5.5) solution containing 4.5 μ*M* phenol and 300 U/mL laccases enzyme. The biosensor response current increased with the increased potential till 0.4 V and further it depleted with increase in potential (Fig. 7).

Analytical characteristics

Linear range

Linearity was obtained in concentration range between 0.5 and 4.5 μ*M* phenol laccases based enzyme electrode.

Reproducibility and accuracy

A known concentration of phenol was taken (1 μ*M*, 2 μ*M*, 3 μ*M*, 4 μ*M*, and 5 μ*M*) 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 I.

The reproducibility of the biosensor was investigated at phenol concentration of 4.5 μ*M*. A relative standard deviation of 5.6% ($n = 8$) was achieved.

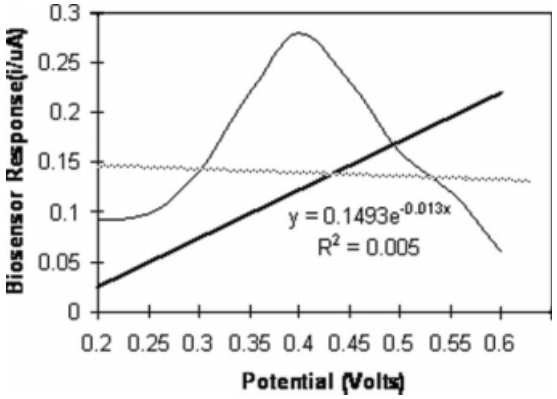

Figure 7 Effect of different operating potentials on biosensor response. Thick line represents regression expression of enzymatic sensor to various potentials, whereas thin line represents the biosensor response to potentials. Thin wavy line shows the linearity in response.

The biosensor exhibited good storage stability for at least two months when stored dry at 4°C and almost 90–98% of their initial activity was retained after 2 months of storage at 4°C. A laccases biosensor based on immobilization of enzyme on glassy carbon electrode modified with redox carboxylic group also demonstrated similar shelf-life.[26] A good correlation was obtained between standard APHA methods ($r = 0.9957$, slope = 1.037).

Stability performance

This laccases enzyme electrode was kept in acetate buffer pH 5.5, 35°C, 4.5 μ*M* phenol, 300 U/mL laccases (deposition time = 7.5 min) and potential 0.4 V. The response current was measured after 10 h. It was found that the response current remained constant till first 5 h. Later, it depleted 2.75% in next 2 h. and further to 3.30% in last 10 h. Further, this enzyme electrode was stored at 4°C, and amperometric responses were checked every week at phenol concentration of 5 μ*M*. A decrease in response by 6.0% was found after 25 days. To determine the storage stability, the performance of enzyme electrode was monitored over a period of 4 months. When stored at 4°C under dry conditions, only a marginal loss of enzyme activity i.e. 3.2% was observed after 2 months. The decrease in sensitivity is possibly

TABLE I
Statistical Analysis of Biosensor Response to Varying Concentration of Phenol (cF)

cF [μ*M*]	SD	CV
1 μ*M*	± 0.021	1.88
2 μ*M*	± 0.035	2.34
3 μ*M*	± 0.039	2.15
4 μ*M*	± 0.026	1.0
5 μ*M*	± 0.032	1.8

because of the leakage of enzyme during electrochemical measurement. The enzyme may also be degrading with time.

CONCLUSIONS

Selective biosensors are an alternative to promote an efficient screening of a broad range of phenolic compounds, which will give more useful chemical information than the total phenol content in the effluent. All data showed that this system could be used for the detection of phenolic compounds in waste water samples. It was demonstrated that the oxygen is reduced at a carbon electrode with immobilized laccases according to a direct (mediatorless) mechanism, where oxygen is reduced to water in a four-electron mechanism:

Immobilized laccases

$$O_2 + 4H + 4e^- \xrightarrow{\text{Immobilized laccases}} 2H_2O$$

This direct electron transfer mechanism was used as the basis for the creation of efficient biocatalyst oxygen reduction electrodes. The laccases bioelectrocatalytic properties were experimentally investigated in detail using galvanostatic and potentiodynamic techniques by investigating the electrochemical transformation of the copper-containing laccases prosthetic group. In this work, an electrochemical biosensor for phenol was developed using enzyme laccases entrapped in agarose–guar gum composite matrix. The analytical characteristics of this sensor are described. The biosensor exhibited good performance in terms of reusability, operational stability, fabrication simplicity, and shelf life. This simple, easy to-construct, and reagentless electrode is suitable for micro molar quantification of phenol. It was demonstrated that the redox potential of the laccases prosthetic group is about 0.4 V more negative than the zero-current potential of oxygen electroreduction catalyzed by laccases. Thus, the laccases prosthetic group cannot be simply considered as a redox mediator entrapped in the protein structure of the enzyme, with the electron transfer from the electrode to the substrate occurring through it. This indicates that the role of the protein globule of the enzyme is essential for its electrocatalytic activity. The response of the biosensor was linear with substrate concentrations and sensor exposure time. The use of the novel materials to immobilize laccases has altered the selectivity of the enzyme to phenolic compounds. Immobilization in this material has enabled the biosensor to be more selective to phenol compared with the nonimmobilized enzyme. This shows that by a careful selection of different immobilization matrices, the selectivity of an enzyme can be modified to yield a biosensor with good selectivity toward certain targeted analytes. The laccases based biosensor showed good sensitivity and stability. Continuous measurement of phenolic compounds in the affected natural environment can provide an appropriate feedback by the characterization or remediation of contaminated sites.

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