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Performance of nanopolyaniline-fungal enzyme based biosensor for water pollution

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ABSTRACT

The Laccases are oxidoreductases belonging to the multinuclear copper-containing oxidases; they catalyse the monoelectronic oxidation of substrates at the expense of molecular oxygen. These essentially ecofriendly enzymes work with air and produce water as the only by-product. Their uses span from the textile to the pulp and paper industries, and from food applications to bioremediation processes. Laccases also have uses in organic synthesis, where their typical substrates are phenols and amines, and the reaction products are dimers and oligomers derived from the coupling of reactive radical intermediates. Laccase from *Pleurotus ostreatus* was extracted from the Shaken flask cultures of *Pleurotus ostreatus* and grown at 25°C with continuous agitation (110 rpm) in baffled Erlenmeyer flasks (1000 mL) containing 200 mL medium. The basal glucose yeast extract peptone agar medium (GYP medium) used for cultures unless otherwise stated contained 20 g glucose L^{-1}, 5 g yeast extract L^{-1}, 5 g peptone L^{-1} and 1 g $MgSO_4.7H_2O$ $L^{-1.}$ The pH was adjusted to 5±0 with H_3PO_4 prior to sterilization. The kinetics of oxidation reactions catalyzed by laccase was studied using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS). The laccase showed lower specific activity. Enzyme modified electrodes were fabricated with polyaniline. Electrochemical polymerization of aniline was performed to get the film of polymer on the surface of glass electrode. First, ITO/PANI electrode was reduced by a 15 min cathodic polarization of the sensor at -500 mV in 0.1 M acetate buffer, pH 5.5. After cathodic polarization, the film was immersed in 0.1 M of acetate buffer, pH 5.5 containing enzyme solution for the deposition of enzyme in polymer layer at +650 mV for 20 min. During this oxidation process laccase become electrostatically attached to polymer film. The ITO/PANI/LAC electrode was rinsed with deionised water to remove any loosely bounded enzyme, and stored in buffer solution at 4°C, when not in use. Conducting polymer/enzyme modified electrodes prepared by immobilization of enzyme were tested for electrocatalytic activities towards amperometric sensing of phenol in industrial effluent. Copyright © 2011 VBRI press.

Keywords: Polyaniline; laccase; nanotechnology; amperometric analysis; phenol biosensor.

Brajesh Singh Kushwah was born on 4 August 1962 at village Sagra in district Bhind of Madhya Pradesh, India. He was awarded Doctorate in Instrumentation Engineering from Dr. B.R. Ambeddkar University, Agra and M. Tech. Degree from Dayalbagh Educational Institute (Deemed University), Agra in System Engineering in 2000. He was awarded B. Tech. Degree from Madhav Institute of Technology and Science, Gwalior affiliated to Jiwaji University (Madhya Pradesh) with Honours in 1988. He worked in various reputed industries specialized for fabrication and installation of Telecommunication cables and wires making machines on different posts from 1982-1996. He has well oriented expertise in manufacturing of Wire drawing, Steel tape armouring, Tubular stranding, Fault detecting ,Glues applicator machines and handling, repairing, installation and maintenance of all tools and machineries related to telecommunication and cables. Er. Kushwah started his Teaching and Research career as Assistant Professor/Lecturer in Mechanical Engineering from Raja Balwant Singh College in January 1996. Later, he joined as Associate Professor/Reader in full fledged Department of Mechanical Engineering in same college from 2005. Since then, he is serving the

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Department as Head and Professor. He is working in the field of "Conduction mechanism of polyaniline polymers for making nanobased biosensors" under Bioinstrumentation. His other fields of interest Nanotechnology, Machine fabrication (Cables and telecommunication wires making machines), Bioenergy and sustainable development. He is Co-investigator of 4 projects sponsored by Department of Biotechnology and Science and Technology. He has 18 research papers published in journals and 30 in Proceedings of National and International Conferences. He has visited Italy, France, Switzerland, Luxor (Egypt), Denmark and Germany under Scientific exchange programmes of DST. He has one National patent on soil analytical kit and two technology transfers to his credit.

Introduction

Laccase (p-diphenol:oxygen oxidoreductase, EC 1.10.3.2) is a multi-copper oxidase, which catalyses the oxidation of various aromatic and some inorganic compounds with the concomitant reduction of dioxygen to water [**1**]. Due to a great variety of reactions catalysed by laccases, this enzyme has gained considerable interest in many fields of research, for example, development of oxygen cathodes in biofuel cells [**2**], labelling in immunoassays [**3**], green biodegradation of xenobiotics, including pulp bleaching [**4**], bioremediation [**5**], biosensors [**6**], and organic synthesis [**7**]. Laccases are either mono or multimeric copper-containing oxidases that catalyse the one-electron oxidation of a vast amount of phenolic substrates. Molecular oxygen serves as the terminal electron acceptor and is thus reduced to two molecules of water. The ability of laccases to oxidise phenolic compounds as well as their ability to reduce molecular oxygen to water has lead to intensive studies of these enzymes. The biotechnological importance of these enzymes can also be attributed to their substantial retention of activity in organic solvents with applications in organic synthesis. Laccases have widespread applications, ranging from effluent decolouration and detoxification to pulp bleaching, removal of phenolics from wines and dye transfer blocking functions in detergents and washing powders, many of which have been patented. The biotechnological application of laccase has been expanded by the introduction of laccase- mediator systems, which are able to oxidize non-phenolic compounds that are otherwise not attacked and are thus able to degrade lignin in kraft pulps.

Laccase is the most widely distributed of all the large blue copper-containing proteins, as it is found in a wide range of higher plants and fungi as well as in bacteria. Laccases in plants have been identified in trees, cabbages, turnips, beets, apples, asparagus, potatoes, pears, and various other vegetables. Laccases have been isolated from Ascomyceteous, Deuteromyceteous and Basidiomyceteous fungi.

Laccases reduce oxygen directly to water in a four-electron transfer step without intermediate formation of soluble hydrogen peroxide in expense of one electron oxidation of a variety of substrates, e.g. phenolic compounds [**8**]. This kind of compounds constitutes a large group of organic pollutants, which are widely distributed throughout the environment. Phenolic compounds are used in many industrial processes, e.g. in the manufacture of plastic, paper, dyes, drugs, pesticides and antioxidants [**9, 10**]. Phenols are also breakdown products from natural organic compounds such as humic substances, lignins and tannins. Certain phenols and related aromatic compounds are highly toxic, carcinogenic and allergenic and due to their toxic effects, their determination and removal in the environment are of great importance. Laccases have wide substrate specificity and a great potential for the determination of phenolic compounds [**11**] and also show broad specificity in the process of oxidizing many compounds (mainly of phenolic type) and can be used for detoxification of a number of aquatic and terrestrial xenobiotics, industrial waste-waters, as well as for biotechnological treatment of industrial products [**12**].

Phenolic compounds identified in industrial effluent include phenols, catechols, guaiacols and cresols. Therefore, their determination is very important due to its toxicity and persistency in the environment [**13, 14**]. In Malaysia, the maximum acceptable limit of phenolic compounds in sewage and industrial effluents is in the range of 1-5 ppm (mg/L) [**15**]. Spectrophotometric, gas chromatography, liquid chromatography and capillary electrophoresis are the commonly used methods for the determination of phenolic compounds. However, these methods are time consuming including complicate sample pre-treatment [**16**]. In addition, the equipment is expensive and is not generally portable. Therefore, there is an interest in developing simple, sensitive, specific, accurate and portable system such as biosensor for determination of phenolic compounds. Many biosensor research papers have been reported previously for the detection of phenolic compounds based on several types of enzyme such as tyrosinase [**17, 18**], laccase [**19, 20**] and horseradish peroxidase (HRP) [**21**]. However, reaction mechanisms of the biosensors based on tyrosinase, laccase and HRP are different for various types of phenolic compounds. For tyrosinase and laccase, the enzyme molecules are reduced by phenolic compounds after being oxidized by oxygen [**22**]. For HRP, it is oxidized by hydrogen peroxide after its reduction by phenolic compounds [**23**]. Tyrosinase based biosensor can be used for the monitoring of phenolic compounds with ortho-position of the phenol ring free of substituent group [**24**]. But for phenolic compounds with para- and meta-position free of substituent group, laccase based biosensor can be used. However its catalytic behaviour is complex [**25**].

The liberated quinone species catalytically oxidized by laccase can be electrochemically re-reduced and electrochemical sensor can be developed (**26-28**). This provides the additional advantages of the enzymatic/electrochemical recycling of the substrate, giving rise to an amplification of the signal.

Rapid growth in biomaterials, especially the availability and application of a vast range of polymers and copolymers associated with new sensing techniques have led to remarkable innovation in the design and construction of biosensors, significant improvements in sensor function and the emergence of new types of biosensor. Nevertheless, in vivo applications remain limited by functional deterioration due to surface fouling by biological components. However, new copolymers based upon biomembrane mimicry have been extensively investigated during the last two decades, raising hopes that the problems related to interactions between foreign surfaces and biological fluids and tissues may soon be solved.

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Conducting polymers have considerable flexibilities in modifying their chemical structures. By chemical modeling and synthesis, it is possible to modulate their electrical and mechanical properties [**29**]. Moreover, the polymer itself can be modified to bind with protein molecules [30]. Conducting polymers are also known for their ability to be compatible with biological molecules in neutral aqueous solutions. Additionally, conducting polymers have the ability to efficiently transfer the electric charges produced by biochemical reactions to electronic circuits [**31-33**].

Polyaniline (PANI) is one of the most important and widely used conducting polymers for biosensor applications [**34**]. Polyaniline is a technologically important conducting polymer due to its unique electrical, electrochemical, and optical properties. Conducting polymer based biosensors have found promising applications in various fields, such as biotechnology, food and agriculture product processing, health care, medicine, and pollution monitoring. The combination of oxidoreductases and amperometric electrodes is most commonly studied biosensor concept. Through various strategies, the enzyme reaction can be easily followed and sensitively measured by electrochemical techniques [**35-37**].

The aim is to generate an electrical signal which relates to the concentration of an analyte. In electrochemical biosensors the analytical capability of electrochemical techniques is combined with the specificity of biological recognition processes. In this configuration, a bio-specific agent is immobilized at the surface of an electrode, which converts the biological recognition process into a quantitative amperometric or potentiometric response. The selectivity of a biosensor is enhanced by immobilizing a sensitive and selective biological element (typically an enzyme) within close proximity of the sensor. Such devices have found a vast range of important applications in the fields of clinical, industrial, environmental, and agricultural analyses. The field of biosensors is interdisciplinary, and advances occur from progress in several disciplines, e.g. the development of microelectronics and microcomputers, research in biotechnology. New transducer technology (such as fiber optics, integrated microelectronic biosensors, piezocrystals, and surface acoustic wave methods), various methods of measurement (such as flow injection analysis), and the use of a large range of biological and biochemical systems (e.g., antibodies, bacteria, cells, organelles, liposomes, and tissues) have greatly expanding the areas of research linked to biosensors, as reviewed in several articles and books.

Experimental

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 *Pleurotus 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 L^{-1} and 1 g $MgSO_4.7H_2O$ L^{-1}. The pH was adjusted to 5±0 with H_3PO_4 prior to 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 laccase synthesis, $CuSO_4.5H_2O$ was added after 64±96 h cultivation so that its final concentration in the medium was 2±0 mM.

Purification of laccase

For purification of extracellular laccase *P. ostreatus* was cultivated in 15 L fermenter on GYP medium using 2 mM copper to stimulate laccase formation. After 200 h cultivation when laccase activity reached its maximum, mycelia were separated by centrifugation (20 min, 10000 g). 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; 10000 g). The clear supernatant was repeatedly dialysed against water and was applied to a Q-Sepharose Fast Flow column (50 × 50 mm; Amersham-Pharmacia), pre-equilibrated with 20 mM sodium acetate buffer, pH 5.0. The column was washed at a flow rate of 5 mL min^{-1} with 2000 mL buffer to remove unbound laccase isoforms and protein. Bound laccase was subsequently eluted from the column with a linear salt gradient (0-0.25 M NaCl in the same buffer) with a flow rate of 5 mL min^{-1}. Elution was simultaneously monitored at 280 and 610 nm for protein and type-1 copper, respectively. Fractions containing laccase activity were pooled, concentrated as above, applied to a Superdex 75 prep grade column (800 × 16 mm; Amersham- Pharmacia) pre-equilibrated with 20 mM sodium acetate buffer, pH 5.0, containing 200 mM NaCl, and eluted at a flow rate of 0.5 mL min^{-1}. 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 laccase from *P.ostreatus* column (Amersham-Pharmacia), equilibrated with 50 mM phosphate buffer, pH 7.0, containing 100 mM KCl, was used.

The column was calibrated with the standard proteins ribonuclease A (*M*r 13700), carbonic anhydrase (*M*r 29000), ovalbumin from chicken egg (*M*r 45000), bovine serum albumin (*M*r 66000) and transferrin (*M*r 81000), each at a concentration of 2 mg ml^{-1}. The flow rate for elution was 0.5 mL min^{-1}.

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. Laccase activity was determined by using 2,29-azinobis-3-ethyl- benthiazoline-6-sulfonate (ABTS). Oxidation of ABTS was measured by determining the increase in absorbance at 420 nm with an extinction coefficient of (λmM = 36 $mM^{-1}cm^{-1}$). One unit of enzyme

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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.

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).

Electropolymerization of aniline

Aniline (Sigma–Aldrich Israel Ltd) was distilled under reduced pressure and stored in darkness before use. As received, HCl (Qualigens, India) and purified double distilled water were used in preparing the electrolyte solution. An electrochemical cell consisting of working ITO glass (anode) and secondary electrodes of stainless steel (cathode) was used. Saturated calomel electrode (SCE) was used as reference electrode. The electrolyte consisted of an aqueous solution of aniline monomer and acid HCl. Polymerization was carried out potentiodynamically using a potentiostat/galvanostat (Analytical Electrochemical Workstation, Biolink Overseas, Co). The deposition was carried up to 10 cycles at a scan rate of 25 mV/s. The potential was varied between –200 mV and 1100 mV for deposition. The pH of the electrolyte was measured by calibrated ELICO LI 120 pH meter.

Immobilization of enzyme

A three step procedures was applied. First, ITO/PANI electrode was reduced by a 15 min cathodic polarization of the sensor at -500 mV in 0.1M acetate buffer, pH 5.5. After cathodic polarization, the film was immersed in 0.1M of acetate buffer, pH 5.5 containing enzyme solution for the deposition of enzyme in polymer layer at +650 mV for 20 min. During this oxidation process Laccase become electrostatically attached to polymer film. The ITO/PANI/LAC electrode was rinsed with deionised water to remove any loosely bounded enzyme, and stored in buffer solution at 4°C when not in use.

Characterization of polyaniline film

Characterization of a material is an important step after its synthesis because it gives useful parameters in determining the properties of polymers. The polymer was characterized by using FT-IR, X-ray diffraction (XRD) and SEM. The optical characterization of polyaniline thin films was carried out using UV-VIS spectrophotometer, Model Shimadzu 160A and FT-IR spectrophotometer of Nicolet, USA, Model 510 P in the region between 350 and 4000 cm^{-1}. Since the deposited polymer films were too thin (around 100 nm) to be studied by specular reflectance, an alternative sample preparation technique was employed. Namely, the direct grinding of potassium bromide powder onto the film surface results in the mixture of the KBr with the crushed film, and this mixture was finally used to prepare the polymer film KBr pellets for FT-IR transmission measurements. The surface morphology of the thin films was studied using Hitachi scanning electron microscope. Electrical conductivity measurement was done by using four-probe method.

Electrochemical measurement

The cyclic voltammetry studies were undertaken using electrochemical interface (Schumbeger Model SI1286) 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 polyphenols and reponses of the laccase biosensor to phenols. All Cyclic voltammograms (CVs) were performed at 5 mVs$^{-1.}$ Synthetically concocted waste water composition; 50 g/L NaCl and 100 g/L phenol in 1 M HCl solution [**38**].

Results and discussion

Isolation of the main laccase isoenzyme was performed from the culture supernatant of *P. ostreatus* laboratory batch fermentation with glucose as the main substrate and stimulating laccase formation by the addition of copper to the actively growing culture [**39**]. Under these conditions approximately 62000 U laccase activity and 350 mg extracellular protein L^{-1}, of which a large fraction is laccase were formed by the fungus.

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, prior to the optimization studies, the effect of enzyme amount for pL based electrode during the deposition process at +0.65 V was tested.

Characterization of polymer

Thickness of PANI film: The thickness of PANI thin films were measured by quartz thickness monitor as given in **Table 1**.

Table 1. Evaporating temperature ranges and the thicknesses of deposited polyaniline films

Fraction	Temperature (°C)	Thickness (pm)
I	230-275	0.18
II	275-325	0.20
III	325-375	0.40
IV	375-450	0.12

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X-Ray diffraction analysis: XRD pattern provides information about crystallinity, crystal size, orientation of the crystallites and phase composition in semi-crystalline polymers. It shows a sharp peak at 2θ = 27.4° (d- value –3.3A°) reappear within the broad band peak of 2θ = 25°, suggesting that polyaniline matrix becomes amorphous. Comparing with the XRD pattern of polyaniline prepared using electrochemical method, the XRD pattern of polyaniline prepared with other methods shows regular diffraction peak, and this indicates that the molecules are in order and rigid (**Fig. 1**). Further, **Fig. 2** shows peaks at 2θ = 8°, 15° and 19° revealing its crystalline nature when PANI was synthesized electrochemically and doped with HCl.

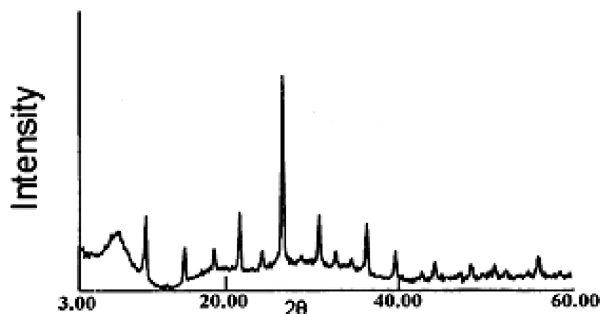

Fig. 1. XRD pattern of PANI prepared using electrochemical method.

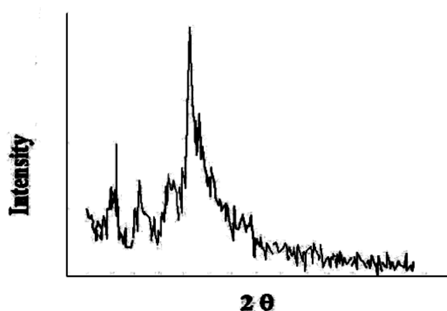

Fig. 2. X-ray diffractogram of electrochemically synthesized PANI/HCl.

FT-IR analysis: The FT-IR spectra of undoped polyaniline show characteristic peaks at 1593, 1495, 1305, 1165, 829 cm^{-1}, 1593 and 1495 cm^{-1}, represent C=C stretched vibration of N-quinoid and N-benzoid rings. Peak at 1305 cm^{-1} is due to C-N vibration bands where as peak found at 465 and 829 are due to C-N double band and C-H vibration bands respectively (**Fig. 3** and **4**). FT-IR spectra of HCl doped PANI shows vibration bands at 3437, 1593, 1495, 1305, 1165 and 829 cm^{-1}. These values characteristic of polyaniline chain are in agreement with theoretical prediction. The peak at 3437cm^{-1} attributed to stretching vibration of secondary amine (**Fig. 4**).

Scanning electron microscopy: It is clear from the SEM of PANI (**Fig. 5**) that the sample is smooth and homogeneous, which is confirmed by XRD patterns. Due to the interaction between electron and sample, considerable amount of heat is generated which causes the development of small cracks during SEM recording. The contrast in the image is a result of differences in the scattering from different surface areas due to geometrical differences [**4**]. The SEM was obtained with films deposited on the glass. It can be seen that the film are similarly compact (**Fig. 5**).

The scanning electron microscopy analysis shows that PANI electrosynthesized in acidic medium in the presence of HCl presents a compact structure. SEM images show that the growth of PANI from acidic media resulted in a sponge-like, branched, porous-structured, high-surface area polymer film on a glass ideal for inclusion of enzyme (**Fig. 6**).

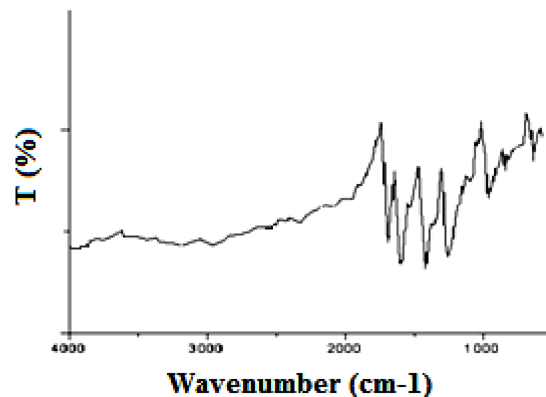

Fig. 3. FT-IR of undoped PANI

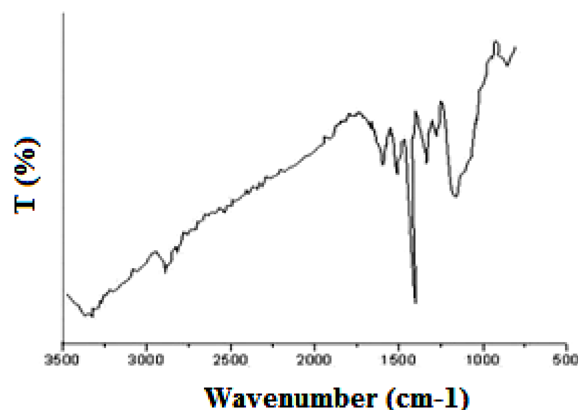

Fig. 4. FT-IR of PANI/HCl

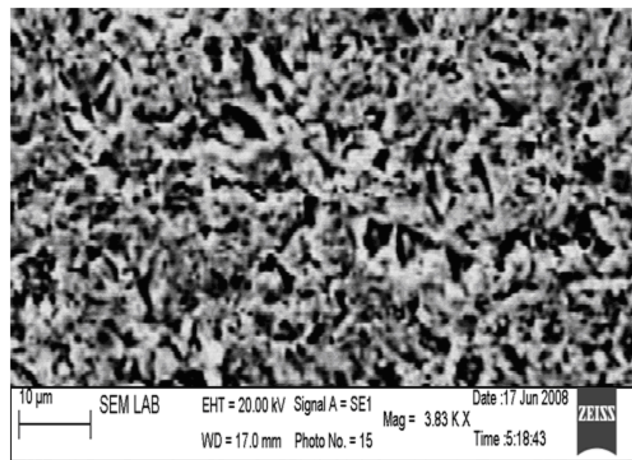

Fig. 5. A micrograph of a PANI film surface deposited by electrodeposition method.

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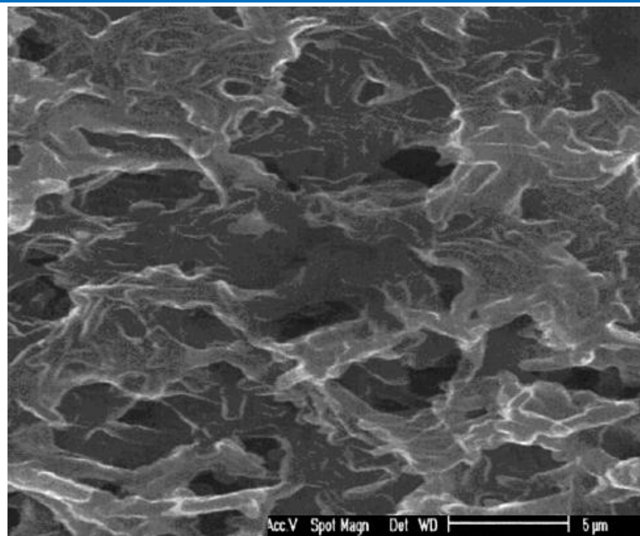

Fig. 6. SEM image of PANI/HCl

Effect of enzyme concentration and deposition time

The effect of enzyme amount for PL based electrodes was studied between 50-450 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 250and 300 U/ml of enzyme amount. A slight decrease was obtained in the presence of higher enzyme amounts. (**Fig. 7**). Moreover, deposition time (2.5-25 min) was searched for *laccase* electrode and 7.5 min was as found to be optimum and up to 15 min sensor response remained constant and then started to decrease slightly.

Similarly 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 up to 25 min. (**Fig. 8**).

Optimization of the biosensor

Effect of pH: According to optimization studies, the optimum pH of pL electrode was obtained as 4-6. In higher pH values, a sharp decrease was obtained. **Fig. 9** shows the results obtained from pH optimization studies of the biosensor. The effect of pH on enzyme based electrodes was studied at different pH ranging from 0-12. 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.

Effect of temperature: For the determination of temperature effect on the response of different biosensor system varying assay temperature (10-50°C) was examined (**Fig. 10**). Optimum temperature was detected as 25- 40°C. According to **Fig. 10**, the biosensor response directly increased with temperature up to 35°C, but on the further increase in the temperature a slight decrease was observed. The experimental range of temperature was selected from 10-50ºC. The acetate buffer solution having pH 5.5 and 4.5µM phenol showed a typical response in relation to temperature according to Arrhenius formula:

$$\ln k = \ln A-(E_a/RT) \quad \text{---------------- } (\mathbf{1})$$

where k is rate constant and Ea is the activation energy. In temperature range 10-35ºC, the response current of biosensor increased with increasing temperature (**Fig. 10**).

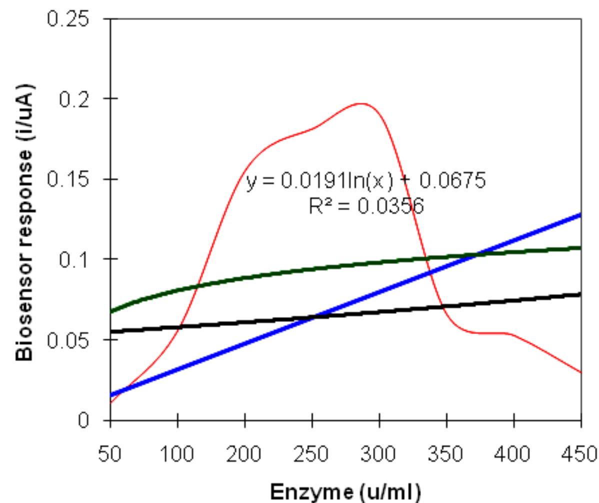

Fig.7. Effect of enzyme concentration.

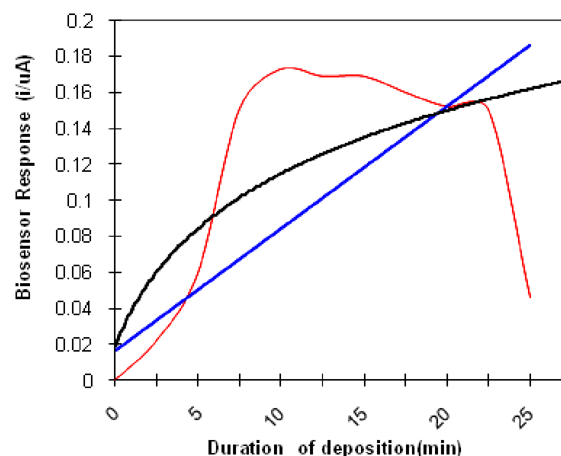

Fig. 8. Effect of deposition time.

Thermal stability: The thermal stability experiments showed that after 13 h period only 7.4% decrease was obtained. During this period approximately 145 measurements have been made and it can be possible to make more measurements in a longer time period.

Effect of phenol concentration: It can be seen from **Fig. 11** that the response current increased with increasing phenol concentration. There was a directly 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 laccase was the first order reaction. But later with continuous increase in phenol

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concentration, the response current increased slowly i.e. enzyme reaction showed a transition from first to zero order. After 8.5 µM phenol concentration, the response showed steep fall.

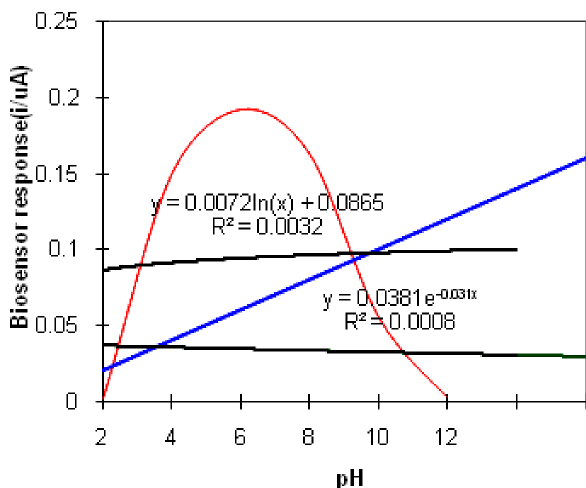

Fig. 9. Effect of pH on biosensor response.

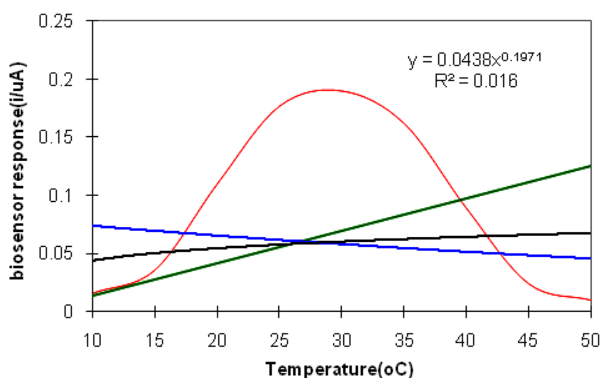

Fig. 10. Effect of temperature on biosensor response

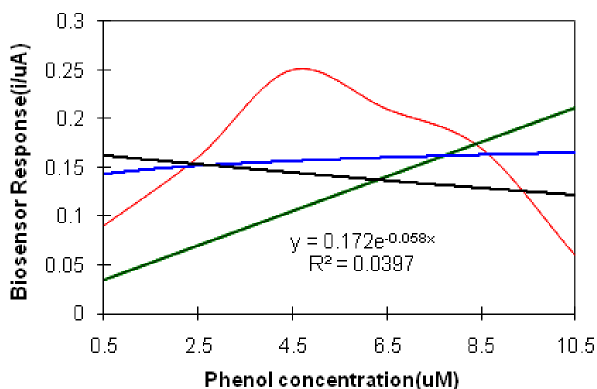

Fig. 11. Effect of phenol concentration on biosensor response.

Effect of potential: The potential was stepped from 0.2-0.6 V in 0.05 V increment in acetate buffer (pH 5.5) solution containing 4.5 µM phenol and 300U/mL laccase enzyme. The biosensor response current increased with the increased potential till 0.4 V and further it depleted with increase in potential.

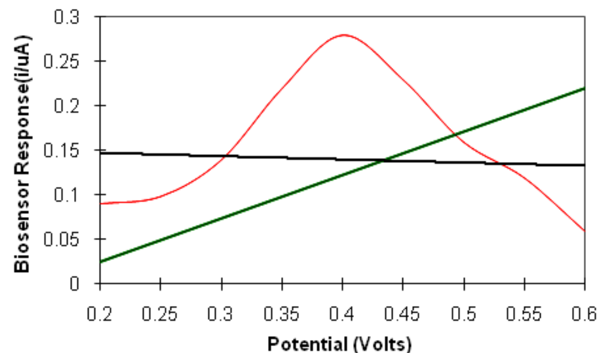

Fig. 12. Effect of potential on biosensor response.

Analytical characteristics

Linear range: Linearity was obtained in concentration range between 0.5-4.5 µM phenol and 0.4-15 µM catechol for pL based enzyme electrode.

Reproducibility and accuracy: A known concentration of phenol was taken (1-5µM) 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 2**.

Table 2. Statistical analysis of biosensor response to varying concentration of phenol.

Phenol conc.	SD	CV
1µM	±.021	1.88
2µM	±.035	2.34
3µM	±.039	2.15
4µM	±.026	1.0
5µM	±.032	1.8

Stability performance: This laccase enzyme PANI based Electrode was kept in acetate buffer pH 5.5, 35ºC, 4.5µM phenol, 300U/mL laccase (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 hours. 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 4ºC and amperometric responses were checked every week at phenol concentration of 5 µM. A decrease in response by 30% was found after 25 days .During this period 175 samples were tested.

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Table 3. Application of the PANI/laccase film electrodes to the waste water samples.

Phenol concentration in waste water sample (µM)	Detected amount (µM)
2.0	2.0±0.03
4.0	3.98±0.038

Sample application

The proposed biosensor was applied in waste water samples. Synthetic waste water samples prepared with known amount of phenol were used as stock substrate solution with different dilution by working buffer and added to the reaction cell after equilibration had occurred and then the change in current was measured following [**40**]. The signals obtained from the waste samples were found to be very similar as shown in **Table 3**. This biosensor was also used to analyse phenol concentration in Yamuna river water. The sample was collected from Etmadaulla, Agra, India. The biosensor showed that the amount of phenol in sample was 3.14µM.

Conclusion

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 laccase according to a direct (mediatorless) mechanism, where oxygen is reduced to water in a four-electron mechanism:

$$O_2 + 4H+ + 4e^- \xrightarrow{\text{Immobilized laccase}} 2H_2O \quad \text{-------} (\mathbf{2})$$

This direct electron transfer mechanism was used as the basis for the creation of efficient biocatalytic oxygen reduction electrodes. The laccase bioelectrocatalytic properties were experimentally investigated in detail using galvanostatic and potentiodynamic techniques by investigating the electrochemical transformation of the copper-containing laccase prosthetic group [**39**]. It was demonstrated that the redox potential of the laccase prosthetic group is about 0.4 V more negative than the zero-current potential of oxygen electroreduction catalyzed by laccase. Thus, the laccase 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 laccase based biosensor showed good sensitivity and stability. In addition, the selectivity of these enzymes allowed the detection and quantification of some phenolic compounds in samples of environmental interest. Continuous measurement of phenolic compounds in the affected natural environment can provide an appropriate feedback during the characterization or remediation of contaminated sites.

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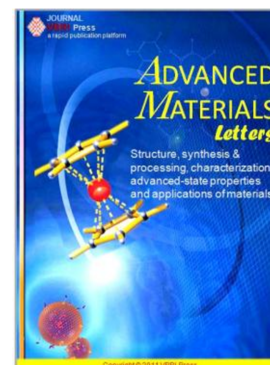

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