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Isolation and Partial Purification of Polyphenol Oxidase from Banana for Biosensor Applications

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Abstract: ***An amperometric biosensor based on the enzyme polyphenol oxidase (PPO), isolated and partially purified from banana, was developed using catechol as substrate. Co-crosslinking method of immobilization was adopted using the protein-based stabilizing agent, bovine serum albumin (BSA) on cellophane and nylon membranes with glutaraldehyde as the crosslinking agent. The parameters measured were the sensitivity, the concentration range of the linear dependence of the sensor response to catechol. The enzyme electrode gave detection limit of 10 x10^{-5} M and 5 x10^{-5} M and linear response range of 20-80 x10^{-5} M and 10-60 x10^{-5} M for catechol employing cellophane as well as nylon membrane respectively. Biosensor response reached steady state within 3 min for both cellophane and nylon membranes and exhibited maximum activity at 25 ^{0}C and pH 6.0.***

Keywords: Polyphenol oxidase (PPO), banana, biosensor, electrode, amperometric.

I. INTRODUCTION

Phenolic compounds are widely used in chemical, petrochemical and pharmaceutical [1] industrial formulations and when discharged in water as a pollutant causes toxicity leading to serious health hazards. Certain catechol derivatives are also essential part of hormones released as part of physiological processes in the human body. It is then crucial to detect them at trace level both in industry effluents and during clinical trials. The traditional techniques used such as HPLC, GC, enzyme immune assays or spectrophotometry are expensive, time consuming and unsuitable for continuous monitoring. The enzyme-based biosensors provide a viable alternative with good selectivity, sensitivity and accuracy [2],[3]. Amperometric biosensors have been developing for phenol and its derivatives, are usually prepared with working electrodes which include polyphenol oxidases (PPO) (tyrosinase and laccase) [4],[5] and enzyme horseradish peroxidase (HRP). Enzyme stabilization and its storage are important criteria in biosensor development. Immobilization is a commonly used method for enzyme stabilization and storage, and it can be reused many times. The varying levels in activity and diffusion limitations occurring with immobilization are mainly dependent on the properties of support material and the immobilization method. Polymeric membranes (such as microfiltration and ultrafiltration membrane) for enzyme immobilization have been widely investigated. It is necessary that they have good adhesion to the transducer surface and should be thin, hydrophilic and porous. Cellulose acetate, cellophane membrane [6], polyvinylalcohol (PVA) and polyurethane are examples of commonly used enzyme membranes. Entrapment within polymer matrices, such as polyacrylamide, gelatin, agarose and poly(N-methyl pyrrole) and have also been reported [7].

This report deals with the studies on the development of amperometric biosensor based on PPO, isolated and partially purified from banana, for the detection of polyphenols using co-crosslinking method of immobilization with BSA as PBSA and glutaraldehyde as the crosslinking agent on cellophane and nylon membranes.

II. MATERIALS AND METHODS

A. Plant Materials and Reagents

Banana was purchased from the local market. Catechol, BSA and cellophane membrane were procured from Himedia and glutaraldehyde from SD Fine Chem., India. All reagents were of analytical grade and used as received. Double-distilled water was used throughout the experiments. Teflon membrane was from M/S Century Instruments, Chandigarh, India.

B. Apparatus

An amperometric principle-based detector system developed in the laboratory was used to amplify and monitor the signals obtained from the enzyme electrode. Clark type of dissolved oxygen electrode was purchased from M/S Century Instruments, Chandigarh, India. A polarizing potential of -650 mV was applied to the gold working electrode.

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C. Enzyme Extraction and Purification

20 g of banana pulp was extracted with 20 ml of 0.1 M phosphate buffer, pH 6 at 4°C. The extract was centrifuged at 13,000 rpm for 15 min. To the supernatant, solid ammonium sulphate was added to obtain 85% saturation at 4ºC. The precipitated protein was separated by centrifugation at 15,000 rpm for 15 min. The precipitate was dissolved in a 0.1 M phosphate buffer, pH 6 and dialyzed against the same buffer at 4ºC in the same buffer.

D. Enzyme Assay

PPO was assayed according to the spectrophotometric procedure of Cosetang and Lee (1987) [8]. The assay mixture consisted of 0.9 ml of 0.05 M Sodium acetate buffer, pH 4.0, 0.1 ml of 0.02 M catechol, and 10-100 *µl* of enzyme. The increase in absorbance at 420 nm was measured as a function of time for 3 min. One unit of enzyme activity is defined as the amount of the enzyme that causes an increase in absorbance of 0.001/min at 25 °C.

E. Protein Estimation

Protein concentration was determined by the dye-binding method of Bradford (1976), [9] and Zor and Selinger (1996), [10]. BSA was used as the standard.

F. Determination of pH Optimum and Stability

PPO activity as a function of pH was determined under standard conditions using 0.05 M catechol as substrate. The buffers used were McIlvaines (citric acid-Na_2HPO_4, pH 2.5-7.5), glycine-HCl (pH 2.5-3.5), sodium acetate (pH 3.5-6.0) and sodium phosphate (pH 6.0-8.0) at 25°C. To determine pH stability, the enzyme was pre-incubated at pH 5.0-7.0 for 10 min at 25°C. At periodic intervals, aliquots of PPO were removed and residual activity was assayed using 0.02 M catechol as substrate under standard conditions.

G. Determination of Temperature Optimum

PPO activity as a function of temperature was determined under standard conditions using 0.05 M catechol as substrate. The optimum temperature for PPO activity was determined by incubating enzyme at different temperatures (32, 35, 40, 45 and 50ºC) in shaking water bath.

H. Preparation of the Biosensor

30 µl of the dialyzed sample was immobilized by cross-linking BSA and glutaraldehyde on cellophane and nylon membranes. After drying for 1 hr the enzyme membrane was washed three times with 0.1 M phosphate buffer (pH 6) to remove excess glutaraldehyde. The immobilized enzyme was sandwitched between an inner teflon and outer cellophane or nylon membranes, and was secured to the electrode using an 'O' ring. This electrode was dipped in 5 ml of 0.1 M phosphate buffer pH 6.0. The contents of the sample cell were kept agitated using a portable air pump. The output voltage from the electrode was amplified through a detector system and measured through a digital voltmeter (Fig. 1).

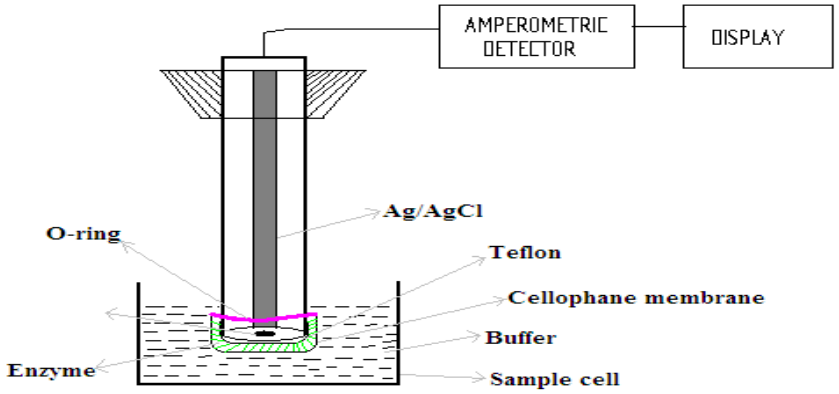


Fig. 1 Schematic representation of biosensor configuration

III. RESULTS AND DISCUSSION

A. Enzyme Extraction and Purification

Ammonium sulphate precipitation of the enzyme was carried out for partial purification of enzyme. The maximum value of activity was found at 85% saturation as shown in Fig. 2.

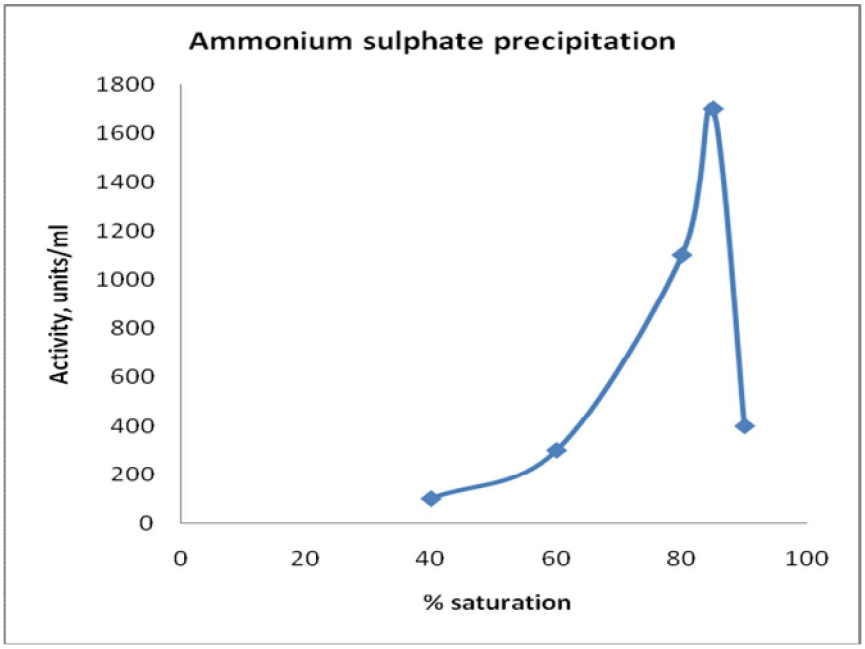


Fig. 2 Ammonium sulphate precipitation

B. Determination of pH Optimum and Stability

The pH optimum for PPO from banana was found to be at (Fig. 3). The rapid deactivation of the enzyme at pH >6 was attributed to the following possibilities: conformational change in the enzyme under alkaline conditions and/or the enzyme may react more rapidly with O-quinone through the Maillard reaction and/or Strecker degradation.

C. Determination of Temperature Optimum

The activity of PPO was measured at different temperatures at pH 6.0 for 10 min. The enzyme showed the highest activity at 25ºC (Fig. 4).

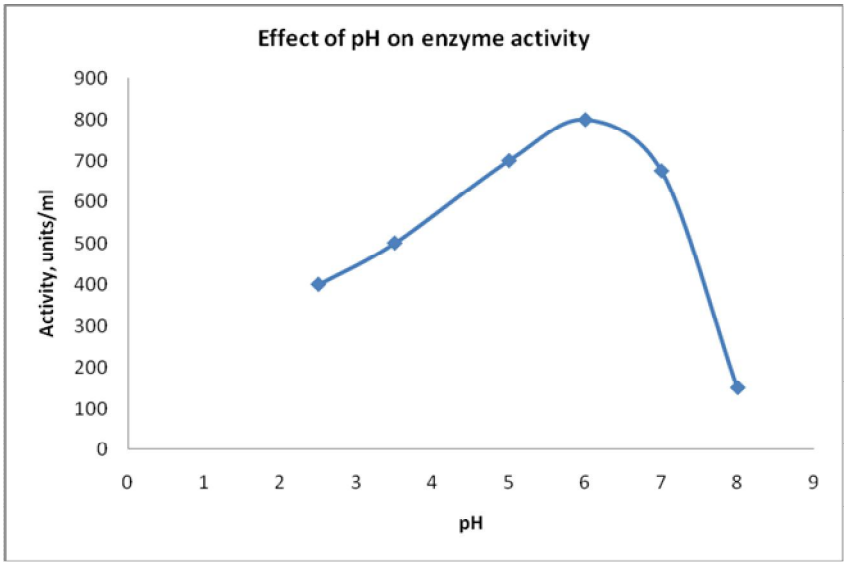


Fig. 3 Effect of pH on enzyme activity

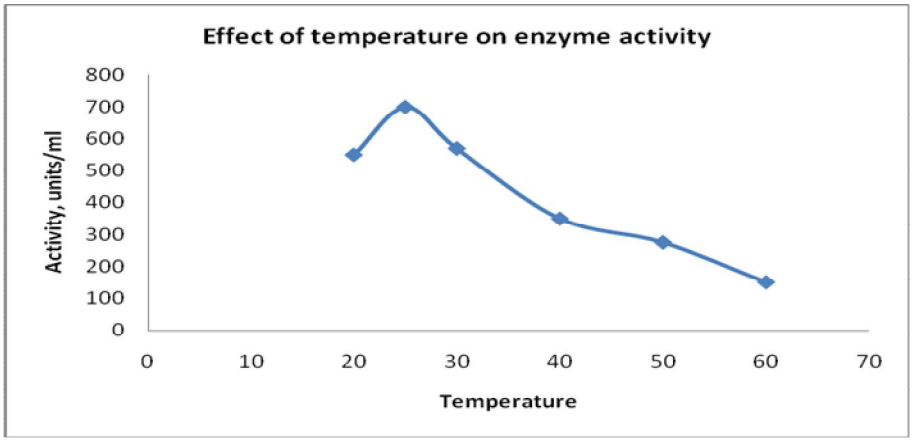


Fig. 4 Effect of temperature on enzyme activity

D. Biosensor Studies

Linearity studies were performed with catechol concentration ranging from 1-200 x10^{-5} M. The steady state voltage response at the end of 3 minutes was recorded and plotted against catechol concentration (Fig. 5). The linearity was best from 20-80 x10^{-5} M and 10-60 x10^{-5} M respectively for cellophane and nylon membranes. The enzyme electrodes gave LOD of 10 x10^{-5} M and 5 x10^{-5} M for catechol employing cellophane as well as nylon membrane respectively. The standard graph showed better linearity (Regression value=0.994) when the enzyme was immobilized on cellophane membrane. Regression value obtained when immobilization was done on nylon membrane was =0.974. Therefore, cellophane membrane was considered superior to nylon membrane for the accurate measurement of phenols. Best sensitivity i.e., low detection limits was achieved by using nylon membrane when compared to cellophane. A broad range of catechol concentrations could be detected when cellophane membrane was used, whereas nylon enabled the detection in a narrow range. Therefore, the range for catechol concentrations that can be detected reduced with an increase in the permeability of the membrane used. Response time was also more for cellophane membrane when compared to immobilization on nylon membrane. This is because the thickness of nylon membrane is less than on cellophane membrane. So, the diffusion barriers are less when nylon membrane is used.

Validation of the test biosensors were successfully performed by comparing the results with conventional HPLC. The proposed biosensor containing banana PPO enzyme immobilized using co-crosslinking method with BSA as PBSA was applied for the analysis. Tap water and textile industry effluent samples prepared with known amount of catechol were used as stock substrate solution with different dilution by working buffer and 50 µl of waste water sample was added to the reaction cell after equilibration had occurred and then the change in voltage was measured. The signals obtained from these 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 1).

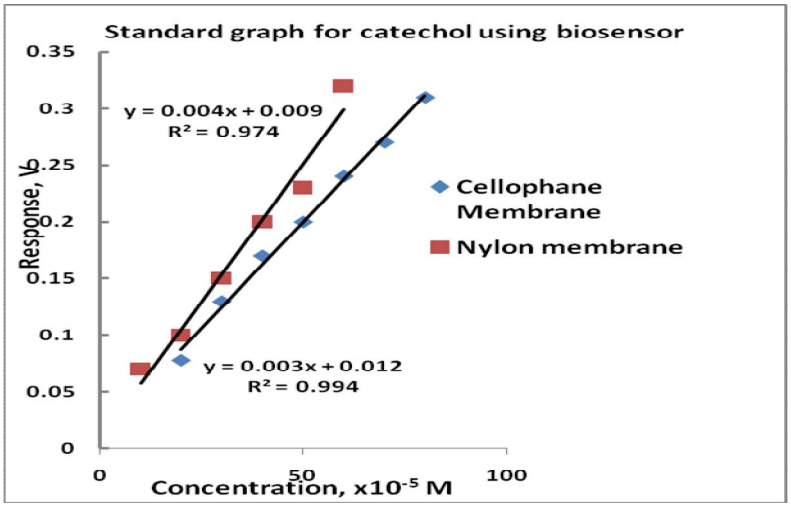


Fig. 5 Standard graph for catechol using biosensor

TABLE 1
REAL WATER SAMPLE ANALYSIS USING TEST BIOSENSORS

Sample	Catechol concentration in waste water sample (x 10^{-5}M)	Detected amount (x 10^{-5} M)		
		Cellophane	Nylon	HPLC
(Tap water)	30	29.92±0.034	29.2±0.016	30.01±0.026
(Industry effluent)	40	39.89±0.04	39.1±0.032	40.03±0.027

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

IV. CONCLUSIONS

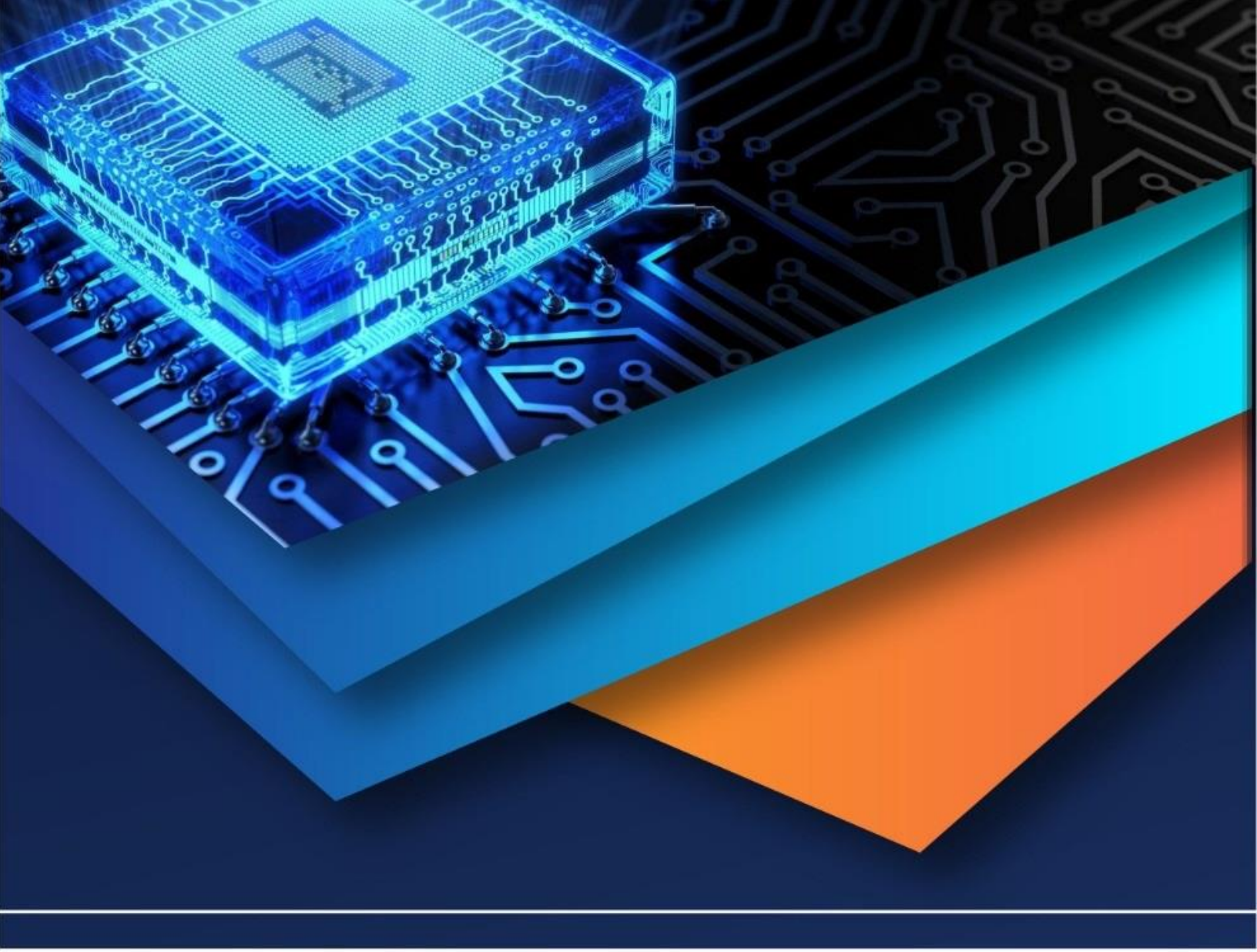
Phenolic compounds have been recognized as toxic substances. Therefore, the determination of phenolic compounds in environmental matrices, including tap and surface water, has become a matter of great concern and scientific interest. Recent research activity has focused on the design and construction of biosensors which are capable of improving the efficiency of site monitoring and can be used for the necessary remediation activities. Polyphenol oxidase-based biosensor reported in this work provides a very good alternative to conventional methods such as HPLC, GC and spectrophotometric techniques with are laborious and time consuming. Banana PPO based biosensor for detection of catechol was developed and biosensor activity was compared on cellophane and nylon membrane support. Results showed that co-crosslinking method of immobilization using BSA on cellophane membrane was considered superior to nylon membrane for the accurate measurement of phenols (R^2 = 0.994). Even though nylon enabled lower detection limits for catechol, the accuracy for detection was less (R^2 = 0.974). Higher permeability of nylon membrane is the reason for better sensitivity when nylon membrane is used. The banana PPO sensor showed very small response time and we could successfully apply the biosensor in detecting catechol in real water samples.

V. ACKNOWLEDGMENT

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