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COVALENT IMMOBILIZATION OF SAPINDUS PEROXIDASE ON POLYANILINE

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ABSTRACT

Covalent immobilization of peroxidase is convenient in continuous processes to improve them economically. In the present study, peroxidases from *Sapindus mukorossi* were immobilized and used for removal of 4-chlorophenol (4-CP) by the polymerization pathway. Five milligram of vacuum dried polyaniline-glutaraldehyde composite was incubated in 1ml sodium acetate buffer (50mM;pH 5.0) containing 0.5 mg/ml of peroxidase, at 4°C, and the mixture was maintained under orbital agitation. When free and immobilized peroxidases were tested for their ability to remove 4-CP from aqueous solution, temperature and pH optima for both was recorded at 40°C and 5.0 respectively. K_m and V_{max} with respect to H_2O_2 were calculated to be 0.44mM and 134.7 E.U and with respect to guaiacol were determined to be 3.5mM and 70.54 E.U. respectively.The removal of 4-CP was maximum when the immobilized enzymes were incubated in a batch reaction for 90 minutes. The percentage removal of 4-CP (0.2mM solution) was reported to be 100, 50, 26, 0 in the 1^{st}, 2^{nd}, 3^{rd}, 4^{th} cycle respectively in the batch reaction.

KEYWORDS: ***Sapindus mukorossi,*** **Covalent immobilization, Polyaniline-glutaraldehyde (PAniG), Sapindus peroxidase, 4-Chlorophenol (4-CP)**

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INTRODUCTION

Enzyme catalysis is an effective way of degrading harmful pollutants present in the industrial effluents and treatment of phenolic wastes by peroxidases is a very well-known example of such a process. Peroxidases (EC 1.11.1.7; donor: hydrogen peroxide oxidoreductase) catalyze the oxidation of various electron donor substrates such as phenols and aromatic amines in the presence of H_2O_2[1]. However, the use of soluble enzymes for waste removal is limited due to their reusability, stability, sensitivity to various denaturants and applications in continuous reactors. Also oxidation of phenols by peroxidases in the presence of H_2O_2 resulted in the formation of free radicals, which might inactivate the enzyme[2]. These limitations can be overcome by using immobilized enzymes on several supports like celite, sephadex and sepharose and using various protective additives, such as borate, gelatin and polyethyleneglycol (PEG), to decrease enzyme inactivation[3], enhanced stability, easier product recovery and purification, proteolysis and reduced susceptibility to contamination[4]. Enzyme immobilization is physical attachment to a solid support over which a substrate is passed and converted to product. Principle methods for immobilizing enzymes are adsorption, cross-linking and entrapment. Adsorption is also known as carrier-binding method as carrier matrices are used but leaching of enzyme occurs from the surface[5].Cross-linking ofenzyme provides easy control over the leaching in which intermolecular interaction between protein molecules or to functional groups on an insoluble support matrix is done by means of bifunctional or multifunctional reagents such as glutaraldehyde[6]. Entrapping method is based on enclosing enzymes in semi permeable membranes such as cellulose acetate fibres which differs from the adsorption and cross-linking method in that the enzyme itself does not bind to the gel matrix or membrane (www.scribd.com). In recent years, the utilization of biodegradative abilities of some fungi (*Caldariomyces fumago, Lentinula edodes, Phanerochaete chrysosporium, Pleurotus pulmonarius, Aspergillus oryzae, Trametes versicolor, Chrysonilia sitophila* [TFB-27441]) and plants (horseradish, turnip, tomato, soybean, bitter gourd, white radish chayote, and *Saccharum uvarum*) seems to be promising[7]. They have extracellular nonspecific free radical-based enzymatic system which can completely eliminate a variety of xenobiotics, including synthetic dyes, giving rise to non-toxic compounds[8].

In the present work, the method for covalent immobilization of Sapindus peroxidase on polyaniline (a polymer of aniline) was established and various experiments were performed to check the efficiency of immobilized enzymes for the removal of 4-CP.

MATERIALS AND METHODS

Chemicals and plant material

Sodium acetate, sodium borate, glutaraldehyde, ammonium persulphate and 4-CP were purchased from HiMedia, Mumbai, India. Glacial acetic acid, 4-aminoantipyrine (AAP) and potassium ferricyanide were bought from Merck, Mumbai, India.

Fresh leaves of *Sapindus mukorossi* were obtained from the botanical garden at the Panjab University, Chandigarh, India.

Preparation of the crude enzyme

Twenty five grams of fresh *Sapindus mukorossi* leaves were homogenized in sodium acetate buffer (50mM; pH 5.0), with addition of Polyclar AT (1.0g/10 g of tissue) as phenolic scavenger. The suspension was filtered through four layers of cheesecloth and centrifuged at 12,000 rpm for 20 minutes. The supernatant was collected and designated as crude Sapindus peroxidase. All procedures were carried out at 4°C.

Crude extract was precipitated four times with chilled methanol and centrifugation at 10,000 rpm for 30 minutes. The supernatant was discarded and the pellet was air dried. The dried pellet was dissolved in the acetate buffer to form a concentrated as well as relatively purified enzyme solution. This methanolic extract was further used up in all analysis.

Protein assay

The amount of protein in the extract was determined by the Foln-Lowry method[9]. A standard curve using known concentrations of BSA was prepared beforehand.

Enzyme assay

The enzyme was assayed for activity by the method described by Bergmeyer[10]. The rate of decomposition of hydrogen peroxide by peroxidase, with guaiacol as hydrogen donor, was determined by measuring the rate of colour development at 470 nm.

Immobilization of enzyme

A). Preparation of immobilization support

0.44M aniline and 0.68M ammonium persulphate were allowed to react in equal amounts at 4°C for 2 hours. The green coloured precipitate of polyaniline was washed with 0.1 M HCl solution and activated by 2.5% glutaraldehyde for 4 hours at 4ºC. The resulting polyaniline-glutaraldehyde (PAniG) composite was thoroughly washed with acetate buffer and dried under vacuum.

B). Immobilization of the enzyme

Five milligram of vacuum dried PAniG composite was incubated in 1 ml of enzyme extract (0.5mg/ml) at 4°C. The mixture was maintained under orbital agitation at 100 rpm for 60 minutes. The pH of the medium was set at 5.0 using 50 mM acetate buffer.

C). Assay for immobilized enzyme

The immobilized enzyme was also assayed according to the Bergmeyer's method[10]. One millilitre reaction mixture containing PAniG-enzyme composite, buffer and guaiacol was prepared. The reaction was initiated by addition of H_2O_2 and absorption was read after a minute at 470 nm.

Characterization of free and immobilized Sapindus peroxidase

A). Effect of temperature

The free and immobilized enzymes were incubated at different temperatures (30-70°C) for 60 minutes. The enzyme activity was assayed and plots were drawn for both.

B). Effect of pH

Free and immobilized enzymes were incubated at 40°C in 50mM buffers to see the effect from pH3.5-5.0 (sodium acetate buffers) and to observe the effect from pH 6-8 (sodium phosphate buffers) and plots were drawn for the same.

C). Enzyme kinetics

Free/immobilized enzymes were taken in excess and the amount of H_2O_2 and guaiacol was varied for each reaction. The enzyme activity was recorded and Lineweaver-Burk plots were prepared to determine the values of K_m and V_{max}.

Optimization of process parameters for the removal of 4-CP from aqueous solutions

Removal of 4-CP

The percentage removal of 4-CP was determined by the 4-AAP method. Three hundred microliter of the batch reaction mix was centrifuged at 3000 rpm for 30 minutes to pellet out any phenol polymer formed. One hundred microlitre of supernatant was safely removed and then 800µl of sodium borate and 50µl of 4-AAP were added. Fifty microliter of potassium ferricyanide was added to initiate the reaction. The mixture was turned upside down at least thrice to achieve a uniform colour. The absorbance at 510 nm reflected the amount of phenol. A control without containing the enzyme was used to set blank.

Percent removal of 4-CP was defined as:

$$[A_c – A_r/A_c] \times 100$$

Whereas:

A_c is the absorbance of the control and

A_r is the absorbance of the reaction.

Efficiency of immobilized enzymes at different concentrations of hydrogen peroxide and 4-CP, varying contact time and multiple cycles

A batch reaction consisting of 4-CP (0.4mM), 5 mg of immobilized enzyme and 0.4-4.0 mM H_2O_2 was prepared and allowed to react at 40°C for one hour. The samples were withdrawn to determine the percentage removal of 4-CP.Similarly, the removal of 4-CP was studied at different concentrations (0.2-1.4 mM) by incubating the immobilized enzyme (5mg) for one hour in the presence of H_2O_2 (3mM) at 40°C.The samples were withdrawn to calculate the percentage removal of 4-CP.To study the effective reaction time (30-150 minutes) for the optimum removal of 4-CP, a batch reaction was set up by mixing the immobilized enzyme (5mg), 4-CP (0.2mM) and H_2O_2 (3 mM) at 40°C. The samples were withdrawn after 30 minutes of intervals and the percentage removal of 4-CP was measured.The re-usability of immobilized enzyme was tested by washing the used enzyme with acetate buffer and using it again for removal of 4-CP from aqueous solution. The re-use efficiency of the immobilized enzyme was determined by plotting the percentage removal of 4-CP in the subsequent cycles.

RESULTS AND DISCUSSION

Characterization of free and immobilized Sapindus peroxidase

A). Effect of temperature

Effect of temperature on the activity of free and immobilized Sapindus peroxidase were shown in Figure1. The free enzyme lost its activity substantially at 60°C (32 E.U.) and almost all its activity at 70°C (6 E.U.). Comparatively, enzyme activity was too low at 80°C in Turkish black radish peroxidise[1] and deactivated at 60°C and retained only 10% of its initial activity in HRP[11]. However, the immobilized enzyme retained moderate activity at 60°C (43 E.U.) and 70°C (20 E.U.). In other study, immobilized HRP shows maximum activity at 40°C and deactivated at 60°C retaining only 29% of its initial activity[11]. Also at 55 °C, the free and immobilized HRP retained their activity to a level of 28 and 63%[12]. Similarly, temperature optima for immobilized lignin peroxidase was 40°C[13] and on 50°C, HRP immobilized on graphene oxide have about 72% activity after 20 minutes of incubation[14].

B). Effect of pH

The variation in the activity of the immobilized enzyme with changing pH of the immobilization medium was plotted on a graph (Figure 2). The enzyme was found immobilized best at pH 5. It was found that the free enzyme activity came down to 68 E.U. as the pH reached 8.0. However, the immobilized enzyme retained its activity upto 84 E.U. throughout the pH range of 5.0-8.0. Similarly, immobilised HRP shows maximum activity at pH 5.0 and retained its activity upto pH 8.0[11]. Comparatively, higher pH optima (at pH 7.0) was observed with immobilized HRP[12] and lower pH optima (at pH 4.0) was seen with immobilized lignin peroxidise[13]. In another study, optimum pH for the immobilized HRP is around 7.0 and retained about 36% activity at pH 10[14].

C). Enzyme kinetics

For the free enzyme, Lineweaver-Burk plots (Figure 3A & 3B) were drawn with respect to H_2O_2 and guaiacol. The K_m and V_{max} of the free enzyme with respect to guaiacol was determined to be 1.023 mM and 81.55 E.U. respectively. Comparatively, Km and V_{max} for purified peroxidase from sunflower (*Helianthus tuberosus* L.) tubers were 0.263mM and $33.3x10^5$ E.U.[15], from Turkish black radish were 0.036mM and 38728.17 E.U.[1] and from the leaves of spinach were 17.35mM and 1234 E.U.[16]. Similarly, K_m and V_{max} with respect to H_2O_2 was calculated to be 0.44mM and 134.7 E.U. respectively for Sapindus peroxidase. Comparatively, K_m and V_{max} activity of purified peroxidase from Jerusalem artichoke (*Helianthus tuberosus* L.) tubers were 1.143 mM and 0.213×10^5 E.U.[15] and Turkish black radish were 0.0084mM and

35122.23 E.U.[1]. The K_m and V_{max} of the immobilized enzyme with respect to both guaiacol and H_2O_2 was determined by Lineweaver-Burk plots (Figure 3C & 3D). The K_m and V_{max} with respect to guaiacol (Figure 3C) were determined to be 3.5mM and 70.54 E.U. respectively. In another study, K_m and V_{max} of immobilized chitosan-HRP were determined to be 25 mM and 133 $mM^{-1}min^{-1}mg$ of enzyme using toluene as solvent[17]. With respect to H_2O_2, K_m and V_{max} were calculated to be 0.39mM and 67.39 respectively for Sapindus peroxidase (Figure 3D). Similarly, K_m and V_{max} for immobilized HRP were 368 µM and 128 U/mg[12], for immobilized Chloroperoxidase (CPO), the value were 47.2µM and 245.2 U/mg protein[18] and for lignin peroxidise (LiP), the value were 10.56 mg/ml and 120.49 U/mg of protein[13].

Efficiency of immobilized enzyme at different concentrations of hydrogen peroxide and 4-CP, varying contact time and multiple cycles

A). H_2O_2 concentrations

H_2O_2 concentration at 1mM and 3mM was optimized the best for the remove of 4-CP in one hour for free and immobilized enzymes respectively (Figure 4A). In another comparative study, HRP in the presence of 20µL H_2O_2 took 100 minutes for the removal of 2mM phenol[19].

B). 4-CP concentrations

Efficiency of immobilized enzyme for the removal of different concentrations of 4-CP was checked. The percentage removal of 4-CP was observed to be 100, 20, 18, 12 at 0.2, 0.6, 1.0 and 1.4mM concentrations respectively (Figure 4B). The removal of 4-CP was maximum when lower concentration (0.2 mM) of 4-CP was incubated along with the enzyme, but when the concentration was increased beyond 0.2 mM in the reaction mixture the rate of removal of 4-CP was tremendously decreased.

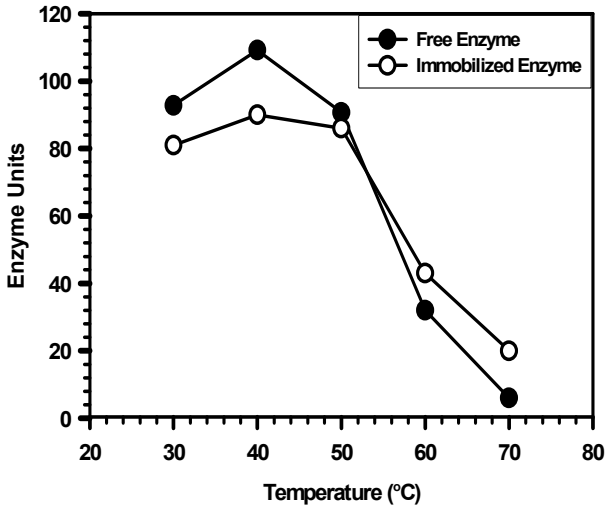
C). Contact time

The variation in the enzyme activity of the immobilized enzyme with respect to variation in the reaction time was plotted on a graph and the optimum time was found out to be 90 minutes (Figure 4C).In another study, optimum time of 48 h was required for the maximum immobilization of HRP on chitosan[17].

D). Re-usability of immobilized enzyme

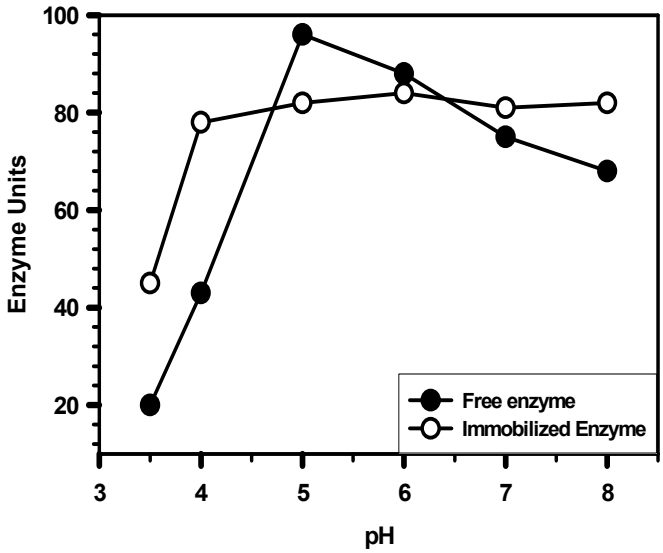
The re-usability of immobilized enzyme was a bottleneck. The immobilized enzyme lost its complete activity in the 4^{th} re-use cycle (Figure 4D). In case of immobilized HRP, it was recycled for 5 times and phenol removal efficiency was reduced to higher than 75% of its initial value[19] and in other case, enzyme activity dropped to 25% of its initial activity after 7^{th} cycle[14]. The irreversible inhibition of peroxidase by high concentration of hydrogen peroxide is a well-known characteristic of this enzyme[20]. The activity loss of the immobilized enzyme was due to leaking of loosely bound enzymes during these runs from the enzyme-adsorbed membrane[21].So, use of a spacer molecule which is flexible part of a molecule, providing a connection between two other parts of enzyme like polyethylene glycol (PEG) (www.scribd.com). Also, Polyacrylonitrile (PAN) membrane surfaces were modified with chemical polymerization of conductive polyaniline (PANI) called composite PAN/PANI membrane maintain upto 63% of initial activity at the end of 20 runs[21].

Figure 1
Effect of temperature on the activity of free and immobilized enzyme.



The free and immobilized enzymes were incubated (30-70°C) at different temperatures for 60 minutes. The enzyme activity was assayed and plots were drawn for both. Each value represents the mean of three independent experiments performed in duplicate, with average standard deviation <5%.

Figure 2
Effect of pH on the activity of free and immobilized enzyme.



Free and immobilized enzymes were incubated at 40°C in 50mM buffers to see the effect from pH3.5-5.0 (sodium acetate buffers) and from pH 6-8 (sodium phosphate buffers). Plots were drawn for the same. Each value represents the mean of three independent experiments performed in duplicate, with average standard deviation <5%.

Figure 3
Kinetics of free and immobilized Sapindus peroxidase.

Fig. 3A

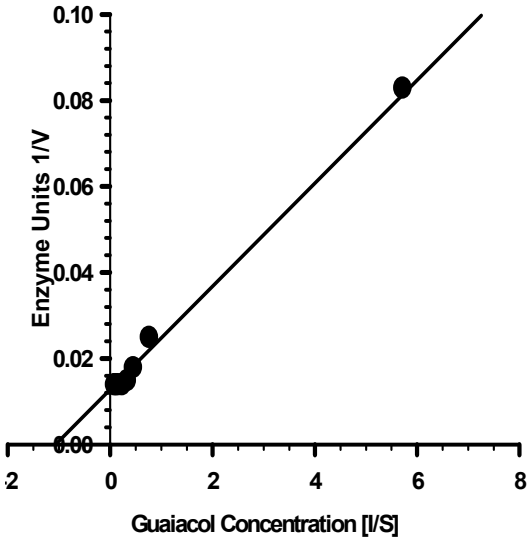


Fig. 3B

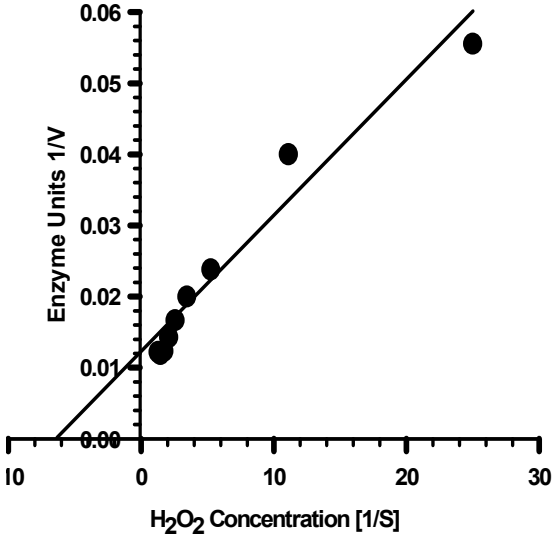


Fig. 3C

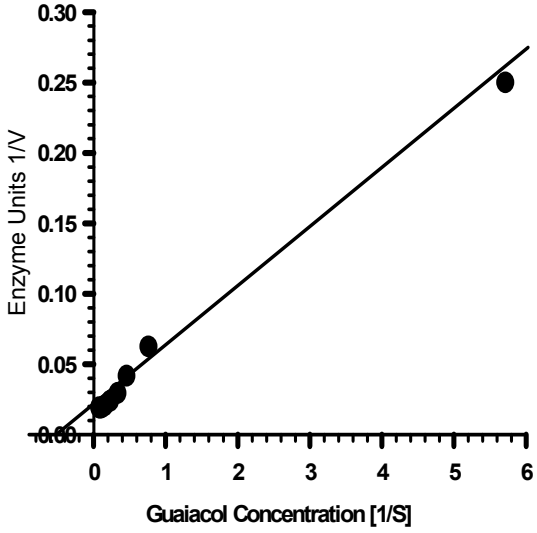
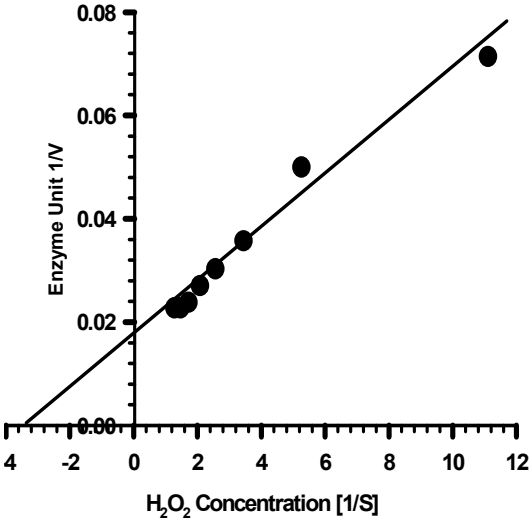


Fig. 3D



Free/immobilized enzyme (3A,3B/3C,3D) was taken in excess and the amount of H_2O_2 and guaiacol was varied for each reaction. The enzyme activity was recorded and Lineweaver-Burk plots were prepared to determine the values of K_m and V_{max}. Each value represents the mean of three independent experiments performed in duplicate, with average standard deviation <5%.

Figure 4

Optimization of batch reactions for the removal of 4-CP from aqueous solutions.

Fig. 4A

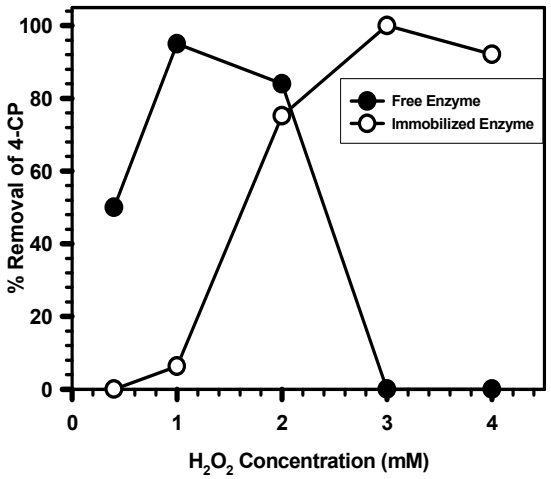


Fig. 4B

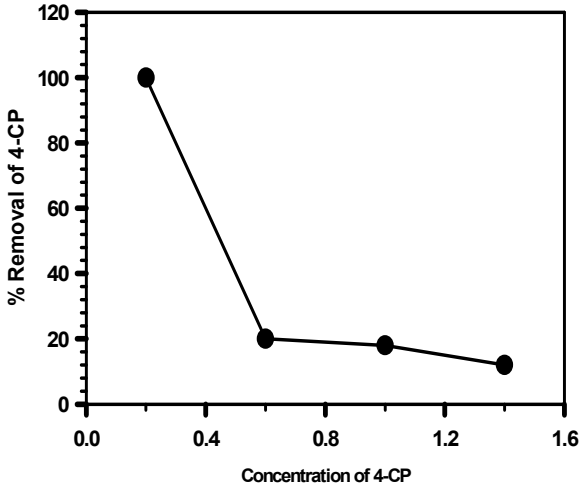


Fig. 4C

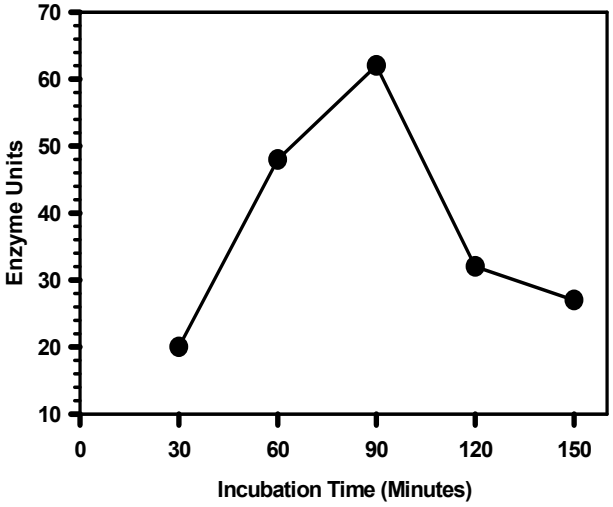
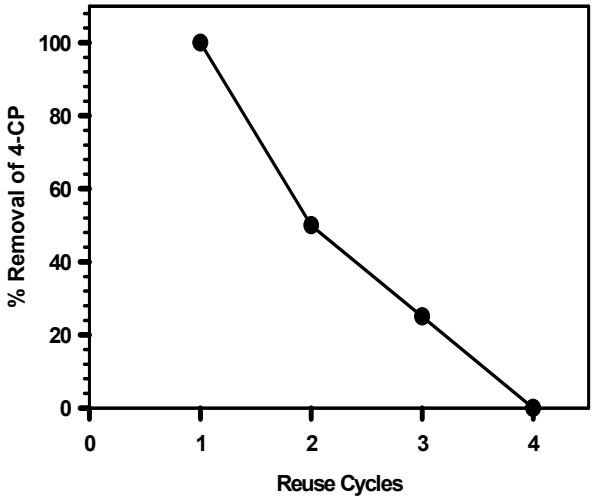


Fig. 4D



4A). Effect of H_2O_2 concentration for the removal of 4-CP.
A batch reaction consisting of 4-CP, immobilized enzyme and various concentrations of H_2O_2 was prepared and allowed to react at 40°C for one hour. The samples were withdrawn to determine the percentage removal of 4-CP.Each value represents the mean of three independent experiments performed in duplicate, with average standard deviation <5%.

4B). Efficiency of immobilized enzyme for the removal of different concentrations of 4-CP.
The removal of 4-CP was studied at different concentrations by incubating the immobilized enzyme for one hour in the presence of H_2O_2 at 40°C.The samples were withdrawn to determine the percentage removal of 4-CP.Each value represents the mean of three independent experiments performed in duplicate, with average standard deviation <5%.

4C). To study the contact time for the removal of 4-CP.
A batch reaction was set up by mixing the immobilized enzyme, 4-CP and H_2O_2 at 40°C. The samples were withdrawn after 30 minutes of intervals and the percentage removal of 4-CP was calculated. Each value represents the mean of three independent experiments performed in duplicate, with average standard deviation <5%.

4D). Reuse efficiency of the immobilized enzyme.
The re-usability of immobilized enzyme was tested by washing the used enzyme with acetate buffer and using it again for removal of 4-CP from aqueous solution. The re-use efficiency of the immobilized enzyme was determined by plotting the percentage removal of 4-CP in the subsequent cycles. Each value represents the mean of three independent experiments performed in duplicate, with average standard deviation <5%.

CONCLUSION

A novel method of covalent immobilization of Sapindus peroxidase on polyaniline was presented in this study. This process improved the enzyme stability over a wider range of pH and temperature than the free form and also protects the enzyme from its inhibition by products or substrates.It also takes less time to efficiently remove the phenolic compound from water than the free enzyme and can be re-use upto 3rd cycle. So, it seemed to be advantageous for the industrial wastewater treatment at a lower cost than other peroxidases.

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