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Partial purification and immobilization of laccase isolated from medicinal mushroom (*Ganoderma lucidum)*

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Ganoderma lucidum, a white rot fungus is used as a source for laccase isolation. The production of laccase has been carried out using by submerged fermentation and the crude enzyme obtained showed activity of 0.42 µg/mL/min. Laccase is partially purified using ammonium sulphate precipitation which showed activity of 0.71 µg/mL/min and it was further purified by DEAE cellulose chromatography with an activity of 0.95 µg/mL/min. Partially purified laccase is immobilized by covalent attachment onto a polyvinyl alcohol (PVA) membrane with retention of 65.71% of initial activity of the enzyme. After immobilization the optimum *p*H of the enzyme increases from 4.5 to 5.0 and temperature for maximum activity increases from 45ºC to 55ºC. The immobilized enzyme show its maximum activity at 10 mM of guaiacol concentration which is more than that of free enzyme (8 mM). Compared with the free enzyme, the immobilized enzymes display a higher activity and affinity, also improved thermal and operational stabilities. Finally, covalently immobilized laccase on PVA has been found to exhibit significant advantages such as reusability, economic benefits, and the cheap support, all these interesting properties, show the suitability of these biocatalysts for industrial applications.

Keywords: *Ganoderma lucidum*, Immobilization, Laccase, Submerged fermentation, White rot fungus

Laccase (E.C. 1.10.3.2, *p*-benzenediol: oxygen oxidoreductase) is an oxidoreductase that catalyzes the oxidation of aromatic compounds (particularly phenols), reducing oxygen to water in the process[1]. Laccases are common enzymes in nature, especially in plants and fungi and also found in insects and bacteria. Most of the laccase studied are of fungal origin especially from the classes of white-rot fungi. White rot basidiomycetes are able to degrade lignin efficiently[2]. *Ganoderma lucidum* is a white rot fungus with great industrial importance due to its potential of producing laccase in a considerable amount.

Laccase is an extracellular enzyme that can be easily isolated, purified and characterized by conventional methods. The immobilization of enzymes has been done by several procedures, including covalent attachment to various matrices, adsorption to insoluble substances, or entrapment within a matrix. For large-scale applications, some properties of free laccase viz. sensitivity to denaturing processes and the non-reusability are not desirable. These constraints could be eliminated by the immobilization process. Immobilization of laccase on different solid carriers improved their stability and made possible the reuse of the biocatalyst, leading to more economic processes[3]. Immobilized laccase has different application in lignin degradation, detoxification, textile-dye decolourization[4], pulp bleaching[5], food improvement[6], polymer synthesis[7], the development of biosensors and biofuel cells[8]. Recently laccase have been efficiently applied to nanobiotechnology due to their ability to catalyze electron transfer reactions without any extra cofactor[9,10].

Keeping in view the industrial importance as well as applications, a laccase has been partially purified from *G. lucidum* culture and immobilized covalently onto polyvinyl alcohol (PVA) membrane by cross linking with glutaraldehyde. The advantage of this support is that it is reusable and hence reduces the cost of production and can also be used for the preparation of ampereometeric biosensor.

Experimental Section

Sources of Laccase

Ganoderma samples were collected from different trees from the University Campus, D.C.R.U.S.T., Murthal viz. sample-1 and sample-2 from *Dalbergia sissoo* (Seesam), sample-3 from *Cassia fistula* (Amaltas) and sample- 4 from *Acacia Arabica* (Keekar).

Preparation of mushroom sample

The white rot fungus *G. lucidum* was maintained on Potato dextrose agar (PDA) plates to obtain mycelial culture. Plates were incubated at 27^{o}C for 5 days. From the developing mycelium, mycelial tissues were transferred using inoculation needle to fresh PDA plates and sub-cultured routinely. Each 250 mL Erlenmeyer flasks containing 50 mL of Potato dextrose broth (PDB) was inoculated with four mycelial discs (5 mm diameter each), taken

from growing edges of 5days old Potato dextrose agar (PDA) plates of *G. lucidum*. The flasks were incubated at 27°C for 12 days under static cultivation conditions. The flasks were harvested by filtering the contents through a pre-weighed Whattman filter paper No. 1 with the help of a vacuum filter. The filtrate obtained was further used for the analysis of laccase enzyme.

Production and extraction of laccase

Potent strain was cultivated in Olga liquid medium containing 3 g peptone, 10 g glucose, and 0.6 g KH_2PO_4, 0.001 g $ZnSO_4$, 0.4 g K_2HPO_4, 0.0005 g $FeSO_4$, 0.05 g $MnSO_4$ and 0.5 g $MgSO_4$ per litre and kept in incubator shaker at 200 rpm, 27ºC for 12 days. Then 3 mL of broth was collected on fifth day and fungal mycelium was separated from the broth by filtering it through Whattman No. 1 filter paper. The filtrate collected was used for enzyme assay and all the experiments were carried out in duplicates.

Assay of laccase

Laccase activity was assayed using guaiacol as a substrate[11]. The reaction mixture (1.0 mL) containing 0.1 mL of cell free culture filtrate and 0.9 mL of 10.0 mM guaiacol reagent prepared in 100.0 mM citrate phosphate buffer (*p*H 5.0). One unit of laccase was defined as the change in absorbance of 0.01/mL/min at 470 nm. Molar extinction coefficient at 470 nm was 26.6×10^3 $M^{-1}cm^{-1}$. The protein content of various enzyme preparations was determined using bovine albumin serum (BSA) as standard protein[12]. A standard curve for protein was plotted with BSA conc. *vs* A_{660}. The amount of protein in the enzyme preparation was calculated from standard curve.

Partial purification of enzyme

Crude extract of *G. lucidum* laccase was fractionated in a sequence saturation of ammonium sulphate ($[NH_4]_2SO_4$) from 0-20, 20-40, 40-60, 60-80 and 80-100% saturations. Each precipitate fraction was resuspended in a small volume of 0.1 M sodium acetate buffer (*p*H 4.5) and subjected to dialysis to remove salt before laccase activity assay and protein determination. Then the dissolved ammonium sulphate precipitate was placed on Diethyl amino ethyl (DEAE) cellulose column pre-treated with 2% HCl and then with 2% NaOH for 30 min followed by washing with 0.01 M sodium phosphate buffer of *p*H 6.8 for 24 hr. The column was allowed to run in 0.1 M sodium acetate buffer (*p*H 5.6) at a flow rate of 0.5 ml/min. Fractions of 3 mL were collected and tested for the enzyme activity and protein concentration. The active fractions were pooled and treated as partially purified enzyme.

Immobilization of laccase enzyme

The laccase enzyme was immobilized on Poly vinyl alcohol (PVA) membrane prepared with suitable modifications[13]. For this the mixture of the enzyme (0.3 mL) and 0.5 mL of 2.5% glutaraldehyde in 0.02 M sodium phosphate buffer (*p*H 7.0), was spread evenly onto PVA membrane and kept overnight at room temperature for co-immobilization. The membrane was washed with 0.05 M sodium phosphate buffer (*p*H 7) and tested for activity.

Kinetic properties of Free and Immobilized laccase enzyme

In order to determine the optimum *p*H of free and immobilized enzyme various reaction buffers with *p*H range of 3.0 to 9.0 were used. The buffer systems used were citrate buffer for *p*H 2.0-3.5; acetate buffer for *p*H 4.0-5.5; phosphate buffer for *p*H 6.0-7.5; Tris-HCl buffer for *p*H 7.5-9.0. To determine the effect of temperature on the activity of free and immobilized enzyme, the reaction mixture was incubated at different temperatures ranging from 30-80ºC for 90 min. The activity of Free and immobilized laccase was determined by the standard enzyme assay (100 mM citrate phosphate buffer, *p*H 5.0). Thermal stability of free and immobilized enzyme was determined with different pre-incubation periods (15 min., 30 min, 45 min, 60 min, 75 min, 90 min) as the residual activity detectable with Guaiacol in 100mM citrate phosphate buffer, *p*H 5.0.

Laccase activity was measured by using guaiacol as substrate to determine kinetic parameters, maximum reaction rate (V_{max}) and Michaelis-Menton constant (K_m) for free and immobilized laccase. The kinetic parameters were estimated from the Lineweaver- Burk plot and K_m and V_{max} were determined from the intercepts at X- and Y- axis respectively. To determine the effect of substrate concentration on the free and immobilized laccase, different concentrations of guaiacol (2 mM, 4 mM, 6 mM, 8 mM, and 10 mM) were used.

Results and Discussion

Out of the four samples collected Sample 2 from *Dalbergia sissoo* shows highest laccase activity. So the sample 2 was used for further partial purification, immobilization and estimation of laccase activity.

Enzyme production and extraction

Laccase production from *G. lucidum* was carried out by submerged fermentation. The maximum laccase activity *i.e.* 0.42 µg/mL/min was detected on 5th day after that a decrease in enzyme level was observed. Similar results were obtained as they also used submerged fermentation of *G. lucidum* and obtained highest activity after 7 days[14]. Laccase activity was checked after each steps of purification by using different concentration (2 mM, 4 mM, 6 mM, 8 mM and 10 mM) of guaiacol. It was observed that free enzyme was saturated at a concentration of 8 mM guaiacol.

Partial purification of laccase

The crude enzyme obtained through submerged fermentation was assayed and then partially purified by ammonium sulphate precipitation followed by dialysis which gave 1.7 fold purification with 0.55 % recovery of enzyme (Table 1). This also helped in concentrating the enzyme for loading onto cellulose column. A standard curve of protein was plotted by Lowry method using Bovine serum albumin.

DEAE-cellulose column chromatography

The chromatography of ammonium sulphate fraction of crude enzyme on diethylamino ethyl (DEAE) cellulose column using a gradient of acetate buffer (*p*H 5.6) from 0.05 M to 1 M gave various fractions out of these, 21 fractions showing the highest specific activity (Fig. 1) were pooled and considered as purified enzyme. This enzyme showed 0.67% field and 3.06 purification fold Table 1. Similarly laccase from *Stereumostrea* was purified with ammonium sulphate precipitation followed by Sephadex G-100 column chromatography with 70-fold purification[15]. Laccase from fruiting bodies was purified with ammonium sulphate precipitation with 40-70% saturation and DEAE cellulose chromatography resulting in 1.34 and 3.07 fold purification respectively[16].

Immobilization of laccase

The partially purified laccase from *G. lucidum* with an activity of 0.95 µg/mL/min was further used for immobilization onto polyvinyl alcohol membrane with 65.71% retention. This increased stability is probably due to the enzyme binding to the support that contributes to the stabilization of three-dimensional

Table 1 — Partial purification of laccase from *Ganoderma lucidum*

Purification steps	Total volume (mL)	Protein (mg/mL)	Activity (µg/mL/mi)	Total activity (Units *mL)	Specific activity	Purification Fold	% yield
Crude enzyme	100	0.95	0.42	42.0	0.44	1	100
Ammonium sulphate precipitation	30	0.9	0.71	23.4	0.78	1.7	0.55
DEAE cellulose	21	0.70	0.95	28.3	1.35	3.06	0.67

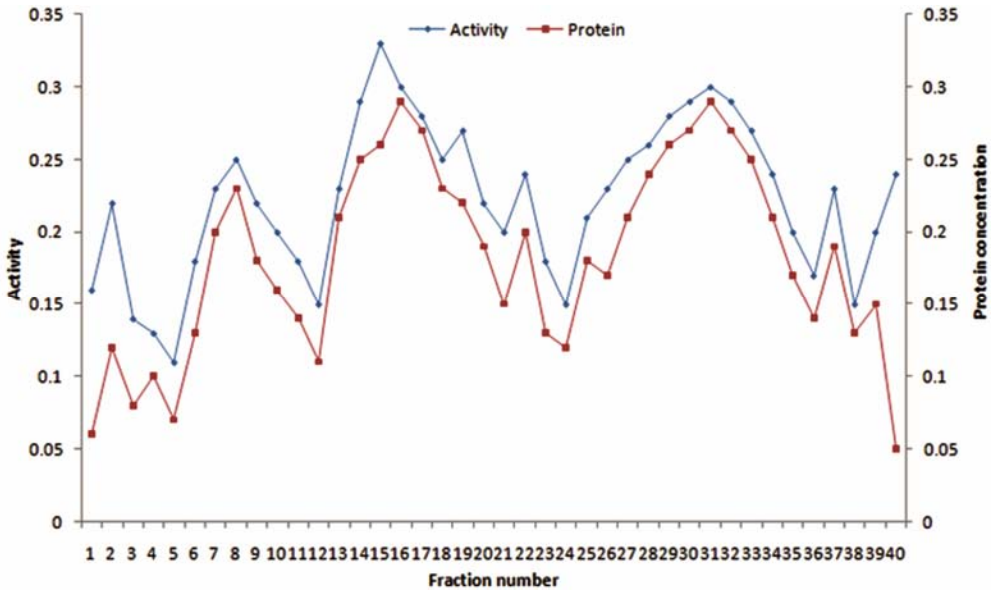

Fig. 1 —Column chromatography of *Ganoderma* laccase on DEAE-Cellulose

structure of the immobilized enzyme[17,18]. The immobilization by covalent attachment promotes stronger bonds that help to prevent the loss of enzyme[19].

Kinetic properties of immobilized laccase

K_m and V_{max} for guaiacol were calculated from L.B plot (Fig. 2). The apparent K_m values were found to be 6.08 mM and 3.82 mM for the free and immobilized laccase respectively while the apparent V_{max} value of the immobilized was 0.617 mM/min and for free laccase was 0.323 mM/min (Table 2). The apparent K_m for the immobilized enzyme was decreased about 1.57 fold compared to the free enzyme. On the other hand, the V_{max} value of the immobilized increased about 1.91 fold compared to the free enzyme. Several factors *viz*. change in enzyme conformation, stearic effect, change in microenvironment, can account for the variations of the K_m and V_{max} values of the enzyme upon immobilization[20].

The ratio of V_{max}/K_m defines a measure of the catalytic efficiency of an enzyme-substrate pair. In this study, the catalytic efficiencies (Vmax/Km) of the free and immobilized laccase onto PVA membrane were found to be 0.05 and 0.16 respectively. The catalytic efficiency of laccase was decreased about 3.2 fold upon immobilization.

Effect of *p*H

The activity of immobilized enzyme was determined in *p*H range of *p*H 3.0 to 9.0. The results revealed that the immobilized enzyme had an optimum *p*H of 5.0. The optimum *p*H of laccase was increased from 4.5 to 5.0 after immobilization which might be due to the loss of NH_2 group in diazo coupling for covalent bonding of enzyme[21]. Fungal laccase typically exhibit *p*H optima in the acidic range. The *p*H optima for the oxidation of phenolic compounds like guaiacol exhibits higher values i.e. 4.0-7.0. The enzyme activity at higher *p*H is decreased by the binding of a hydroxide anion to the T2/T3 coppers of laccase, which blocks the internal electron transfer from T1 to T2/T3 centers. Not only the rate of oxidation but also the reaction products can differ according to *p*H. This may be due to abiotic follow-up reactions of primary radicals formed by laccase. The stability of fungal laccases is generally higher at acidic *p*H[22].

Effect of incubation temperature

To study the effect of incubation temperature on maximum activity of immobilized enzyme, the

Table 2 — Kinetic properties of free and immobilized laccase bound onto PVA membrane

Parameters	Free laccase	Laccase bound onto PVA membrane
Optimum *p*H	4.5	5.0
Optimum temperature	45 ºC	55 ºC
Thermal stability	30-55ºC	30-80ºC
Saturation conc. of Guaiacol	8 mM	10 mM
Km for Guaiacol	6.08 mM	3.82 Mm
Vmax	0.323 mM/min	0.617 mM/min

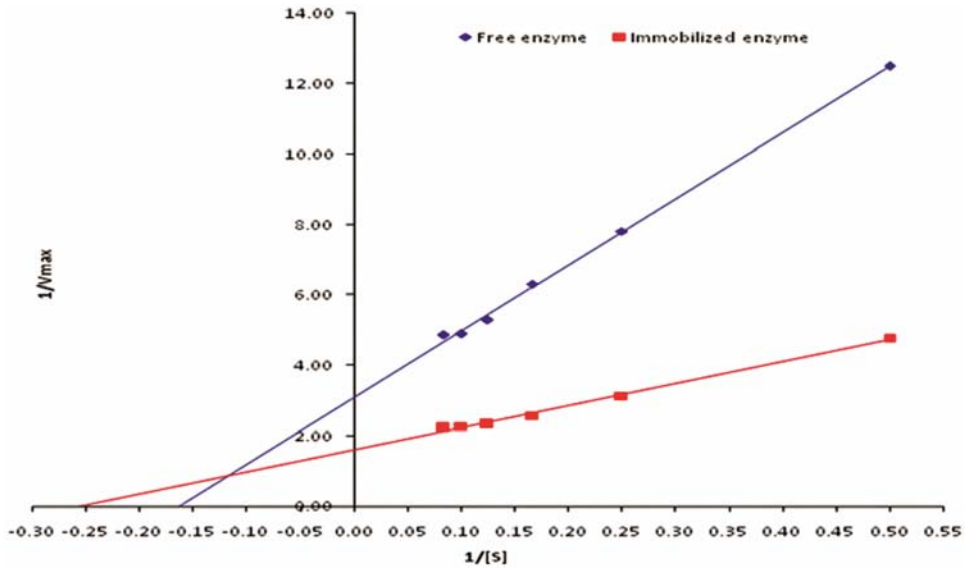

Fig. 2 — A comparison of the Michaelis-Menten constants with guaiacol in citrate phosphate buffer, *p*H 5.0

reaction mixture was incubated at different temperatures ranging from 30ºC to 80ºC at an interval of 10ºC. The immobilized laccase showed maximum activity at 55ºC which is higher than that of the free enzyme (45ºC). Compared with the free laccase, the immobilized preparation gave a significantly broader temperature profile. This indicated an increase in the thermal stability of laccase enzyme after immobilization. Enzyme immobilization is known to increase enzymes stability against their denaturation, thereby limiting its freedom to undergo drastic conformational changes[23] as it was reported by other authors[17,18]. Thermal stability of free and immobilized enzyme was analyzed by incubating the same in the absence of substrate at various temperatures. The immobilized laccase was inactivated at a much slower rate than that of the free form. The immobilized enzyme was more stable than free enzyme in the temperature range of 30-80ºC. The laccase immobilization by covalent attachment led to an increment of the enzyme stability towards heat denaturation in relation to free enzyme.

Effect of substrate concentration

The activity of the free and the immobilized laccase was determined at different concentration of Guaiacol (substrate). The result showed that the activity of enzyme was increased as the substrate concentration was increased up to 8 mM after it became constant. The guaiacol (substrate) conc. required for the maximum activity or saturation of laccase was increased from 8 mM to 10 mM after immobilization.

Storage and stability

The immobilized laccase on PVA membrane containing sodium phosphate buffer (0.05 M, *p*H 7.0) was stored at 4ºC. The enzyme showed no noticeable loss of activity during its regular use for about 15 days. The immobilization by covalent attachment is more promising in terms of operational stability and showed better results. The laccase immobilized by covalent attachment on PVA membrane proved to be a suitable biocatalyst for different industrial applications.

Conclusion

From the present study it can be concluded that *G. lucidum* is a promising source for laccase production by submerged fermentation. The crude enzyme is partially purified by ammonium sulfate precipitation followed by DEAE cellulose chromatography. Partially purified laccase is successfully immobilized by covalent attachment on PVA membrane. Compared with the free enzyme, the immobilized enzyme display a higher activity and affinity, also improved thermal and operational stabilities. Finally, covalently immobilized laccase on PVA exhibit significant advantages such as reusability, economic benefits, and the cheap support, all these interesting properties, shows the suitability of these biocatalysts for industrial applications.

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