PURIFICATION, IMMOBILIZATION AND BIOCHEMICAL CHARACTERIZATION OF FUNGAL LACCASE FROM MUSHROOM, *TRAMETES VERSICOLOR*

Khaing Khaing Myint[1], Ohn Ohn Soe[2], Daw Hla Ngwe[3]

Abstract

In this research, laccase isolated from fungal source of mushroom, *Trametes versicolor*, white rot fungi in solid state fermentation. Laccase was purified by ammonium sulphate fractionation (20 % and 70 %) and dialysis followed by Sephadex G-100 gel filtration chromatography. In each purification step protein content was determined by Biuret method using Bovine Serum Albumin as standard at 560 nm and laccase activity was determined by gauaicol assay method at 450 nm. The extracellular laccase from *T. vesicolor* was purified to 12.72 fold. The purity of laccase was confirmed by SDS-PAGE as single band. The molecular weight of purified laccase was found to be 60.26 kDa. The immobilization of laccase was carried out by gel entrapment technique using sodium alginate- gelatin- agar mixed gel. The highest laccase activities were found at pH 5 for free laccase at 40 ^{o}C and pH 6 for the immobilized laccase. The optimal temperatures of free laccase and the immobilized laccase were 40 ^{o}C and 45 ^{o}C, respectively, showing the improvement in thermal stability of immobilized laccase. The reaction order of both free and immobilized laccase catalyzed reactions was first order reaction.

Keywords: Laccase, *Trametes versicolor*, guaiacol, immobilization, gel entrapment technique

Introduction

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belongs to a group of polyphenol oxidases containing copper atoms in the catalytic centre and usually called multicopper oxidases (Baldrian, 2005). They are extracellular, multicopper enzymes that use molecular oxygen to oxidize various aromatic and non-aromatic compounds by a radical-catalysed reaction mechanism (Thurston, 1994). Laccases can oxidize *o*-and *p*-diphenols, aminophenols, methoxy-substituted phenols, benzenethiols, polyphenols, polyamines, hydroxyindols, some aryl diamines and a considerable range of other compounds but do not oxidize tyrosine. Its rather low specificity makes laccase a promising tool in transforming many toxic substituted phenols or even non-phenolic compounds such as polycyclic aromatic hydrocarbons. Due to its high efficiency, low cost, and good availability, it has been widely used in various applications including environmental bioremediation (Ashrafi *et al*., 2013; Zhang *et al*., 2012), the food industry (Dhillon *et al*., 2012) and textile engineering (Basto *et al*., 2007).

However, free enzymes have the weakness of non-recyclability, and reusability in industrial applications. These problems can be well solved by enzyme immobilization. Among the various immobilization techniques such as adsorption, crosslinking, entrapment and so on, the entrapment method may be a good choice for enzyme immobilization, as the process of entrapment is mild and causes relatively little damage to the enzyme native structure (Dura ´n *et al*., 2002).

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Laccases are widely distributed in nature and also been detected in plants (lacquer, mango, mung bean, peach, pin etc.), bacteria (*Bacillus subtilis*, *Escherichia coli*, *Pseudomonas syringae*) and especially in fungi (Majeau *et al*., 2010; Lu *et al*., 2017). Among them, white-rot fungi is the major laccase producer, and *Trametes versicolor* is an important representative of white-rot fungi.

Submerged and solid-state modes of fermentation are used intensely for the production of laccase. Submerged fermentation involves the nurturing of microorganisms in high oxygen concentrated liquid nutrient medium. Solid state fermentation is suitable for the production of enzymes by using natural substrates such as agricultural residues because they mimic the conditions under which the fungi grow naturally (Brijwani *et al*., 2010; Couto and Sanromán *et al.,* 2005; Pandey *et al.,* 1999). The lignin, cellulose and hemicelluloses are rich in sugar and promote fungal growth in fermenter and make the process more economical (Couto and Toca-Herrera, 2007).

This study is aimed to isolate laccases from white-rot fungi, *Trametes versicolor* using banana skin under solid state fermentation and to compare the kinetic properties of free laccase and laccase immobilized by alginate-gelatin-agar mixed gel.

Materials and Methods

Chemicals

Potassium hydroxide, guaiacol, sodium acetate buffer, phosphate-citrate buffer, Bovine Serum Albumin(BSA), culture medium, Tween-80, ammonium sulphate, Sephadex G- 100, sodium dodecylsulphate polyacrylamide gel, Coomassie brilliant blue, and a standard high molecular weight protein markers, sodium alginate, gelatin, agar, calcium chloride were used.

Apparatus

Conical flasks, digital pH meter, a constant temperature water bath, UV visible spectrophotometer, glass column (2.0 × 40 cm), an incubator, a shaker, a centrifuge, voltage current stabilizer, air compressor, digital balance, slab gel mould and electrophoresis apparatus, 5 mL syringe were used.

Sample Collection

The mushroom samples were collected in sterile plastic bags from the timber industry compound, Insein Township, Yangon Region, Myanmar.

Isolation and Purification of Laccase Enzyme Solution by Solid State Fermentation

Chopped banana skin (1.0 cm × 1.0 cm) (70 g) was autoclaved at 120 °C for 20 min and then soaked in 200 mL of 83.17 mM potassium hydroxide solution for 1 h to neutralize the organic acid. The samples were dried at room temperature for one day after washing thoroughly with distilled water.

The composition of culture medium consisted of 3 g of peptone, 10 g of glucose, 0.6 g of potassium dihydrogen phosphate, 0.001 g zinc sulphate, 0.4 g dipotassium hydrogen phosphate, 0.0005 g of iron(II) sulphate, 0.05 g manganese(II) sulphate and 0.5 g magnesium sulphate in

1 L of distilled water . An inducer , copper (II) sulphate pentahydrate (0.0001 g) and one drop of Tween - 80 were added to the culture medium.

Laccase was extracellularly excreted by *Trametes versicolor* during solid state fermentation. Three loops of fungal strain, 5 mL of culture medium and 30 g of the pretreated banana skin were inoculated in a 500 mL conical flask. This flask was incubated at 30 °C for 18 days. Fungal growth and enzyme activity were assayed periodically. To optimize the time for fungal growth, the fermented matter (2 g) of the specified period (3, 6, 9, 12, 15, 18 days) was obtained by adding 10 mL of distilled water to it. The flasks were mixed for 30 min at room temperature using a shaker (180 rpm). Solids were removed first by filtering and then by centrifuging at 2000 rpm for 20 min. The cell free supernatant obtained was used as crude enzyme extract for purification. (Khaing Khaing Myint *et al.,* 2018).

Purification of Laccase

After 12 days of fermentation, the fermented matter of *Trametes versicolor* was dissolved in acetate buffer (pH 5) with 1:10 ratio and shaken on the shaker for 20 min. It was filtered to remove mycelia, followed by centrifugation at 2000 rpm for 30 min. The supernatant thus obtained was subjected to the total protein precipitation with ammonium sulphate in the range of 20–70 % saturation to obtain partially purified enzyme extract. It was concentrated and dialysed overnight against sodium acetate buffer at pH 5. Gel filtration chromatography was performed using Sephadex G-100 column (2.0 × 40 cm). The dialyzed sample was then applied to Sephadex G- 100 for further purification. Active fractions with high laccase activity and high protein content were pooled together and the resulting solution was subjected to sodium dodecylsulphate polyacrylamide gel electrophoresis. To determine the purity of the purified laccase and its molecular weight, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed and protein was visualized by staining the gel with Coomassie brilliant blue. The molecular mass of laccase was determined by comparing with standard high molecular weight protein markers.

Immobilization of Purified Laccase

The purified laccase was immobilized with alginate-gelatin-agar mixed gel by gel entrapment method. A mixture of 3g of sodium alginate and 3 g of gelatin were mixed with 10 mL of distilled water and stirred for 30min and then added 0.5 g of agar. A mixed gel solution obtained was added to 1 mL of purified enzyme solution. It was taken up with 5mL syringe and injected drop-wise into 250 mL of 1 % $CaCl_2$ solution.

Determination of Laccase Activity

Activity of the free and immobilized laccase was determined by Guaiacol assay method according to Desai *et al*. (2011). Guaiacol (2 mM) in sodium acetate buffer (10 mM pH 5.0) was used as substrate. The assay mixture contained 3 mL acetate buffer, 1 mL guaiacol and 1 mL enzyme solution. For blank solution, 1 mL of distilled water was used instead of enzyme solution. The oxidation of guaiacol was monitored spectrophotometrically after incubation of the mixture at 30 ºC for 15 min by measuring the increase of absorbance at 450 nm. The laccase activity was calculated using the extinction coefficient of guaiacol (12,100 M^{-1} cm^{-1}) at 450 nm. One unit of enzyme was defined as amount of enzyme required to oxidize 1 micromole of guaiacol per minute.

Determination of Protein Content

Protein content of the free and immobilized enzyme was determined by Biuret method using Bovine Serum Albumin (BSA) as standard at 560 nm. The calibration curve of BSA was constructed to determine the concentration of protein in the enzymes (Khaing Khaing Myint *et al.,* 2018).

Characterization of Laccase

Effect of pH

The optimum pH of laccase- catalyzed reaction for the free and immobilized laccase enzymes were investigated by spectrophotometric method. For the study on the effect of pH, the reaction mixtures contained 1 mL each of guaiacol (2 mM) as substrate, 1 mL each of enzyme solution and 3 mL each of buffers of different pH values (acetate buffer pH 3, pH 4, pH 5, phosphate-citrate buffer pH 6 and pH 7). The mixtures were incubated at 40 ºC for 15 min and the absorbance values were recorded at 450 nm. For immobilized laccase, number of beads equivalent to 1 mL of enzyme was used instead of enzyme solution.

Effect of Temperature

The free and immobilized laccase activities were determined at the range of temperature 25-60 ^{o}C with difference of 5 degrees between each temperature values while other conditions were kept constant.

Kinetic pararmeters

The reaction rate was determined with different concentrations of guaiacol in the range of 0.4 mM to 3.6 mM while other conditions were kept constant. The kinetic constant K_m and V_{max} were calculated for the free and immobilized laccase using guaiacol as substrate.

Results and Discussion

Purification of the Isolated Laccase

In this study, laccase was extracted from *Trametes versicolor* by solid state fermentation and the extract was purified by using ammonium sulphate precipitation method followed by Sephadex G -100 gel filtration chromatography. Figure 1 shows the variation of absorbance of protein at 280 nm and the variation of activity of laccase with fraction number. The fraction numbers from 23 to 43 were pooled.

Total Activity, Total Protein and Specific Activity of Crude Laccase Enzyme Obtained in each Purification Step

Figure 1 shows the chromatogram of variation of absorbance of protein at 280 nm and activity of laccase with fraction number. The fraction numbers from 23 to 43 with high protein content and high laccase activity were pooled.

The crude extract having specific activity of 0.22 μmol min^{-1} mg^{-1} was subjected to ammonium sulphate precipitation followed by gel filtration and resulted in specific activity of 2.80 μmol min^{-1} ml^{-1} mg^{-1} at the final purification step. So 12.72 fold purification was achieved (Table 1).

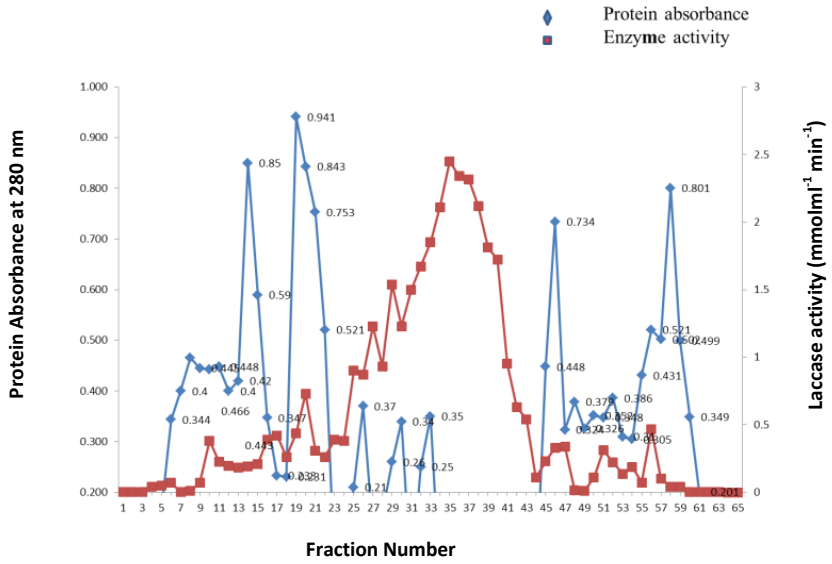


Figure 1 Chromatogram of laccase extract on Sephadex G-100 gel chromatography

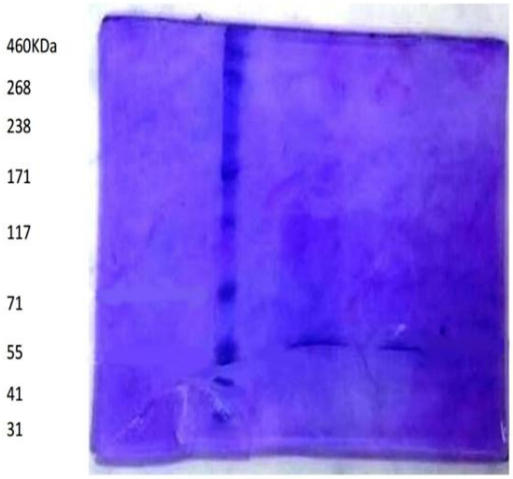
Table 1 Total Activity, Total Protein and Specific Activity of Laccase Enzyme Obtained in each Purification Step

Purification steps	Total activity (µmol min^{-1})	Total protein (mg)	Specific activity (µmol min^{-1} mg^{-1})	Purification (fold)
Crude enzyme solution	6720	30000	0.22	1
After 20 % $(NH_4)_2SO_4$ precipitation	2590	5000	0.52	2.36
After 70 % $(NH_4)_2SO_4$ precipitation	3770	1600	2.36	10.72
After dialysis	999	400	2.50	11.36
Gel filtration	22.4	8	2.80	12.72

Determination of Molecular Weight of the Purified Laccase

The purified laccase was homogenous showing a single-protein band on SDS-PAGE. Thus, activity staining of purified enzyme showed that only one extracellular laccase was secreted. The molecular weight of purified laccase was further confirmed by comparing its electrophoretic mobility with those of protein standards of known molecular weights.

Figure 2 shows the zymogram of sodium dodecylsulphate polyacrylamide gel electrophoresis, lane (a) for a standard high molecular weight protein markers and lanes (b) and (c) for the purified laccases. The molecular weight of purified laccase was observed to be 60.26 kDa from the plot of relative molecular mass versus log molecular weight of standard proteins (Table 2 and Figure 3).



(a) (b) (c)

Figure 2 Photograph of sodium dodecylsulphate polyacrylamide gel electrophoresis: Lane (a) Standard high molecular weight marker proteins Lanes (b) purified laccase and (c) purified laccase

Table 2 Relationship between Molecular Weight of Standard High Protein Markers (HMW) and Relative Mobility (R_f) Values Obtained from SDS – PAGE

No	HMW marker protein	Molecular weight (Da)	Log molecular weight	R_f
1	Standard I (kinase)	460000	5.6628	0.08
2	Standard II	268000	5.4281	0.14
3	Standard III	238000	5.3766	0.28
4	Standard IV (phosphorylase B)	171000	5.2330	0.36
5	StandardV (galactosidase)	117000	5.0682	0.48
6	Standard VI (bovine serum albumin)	71000	4.8513	0.65
7	Standard VII (glutamic dehydrogenase)	55000	4.7404	0.82
8	Standard VIII(turkey albumin)	41000	4.6128	0.86
9	Standard IX (carbonic anhydrase)	31000	4.4914	0.95

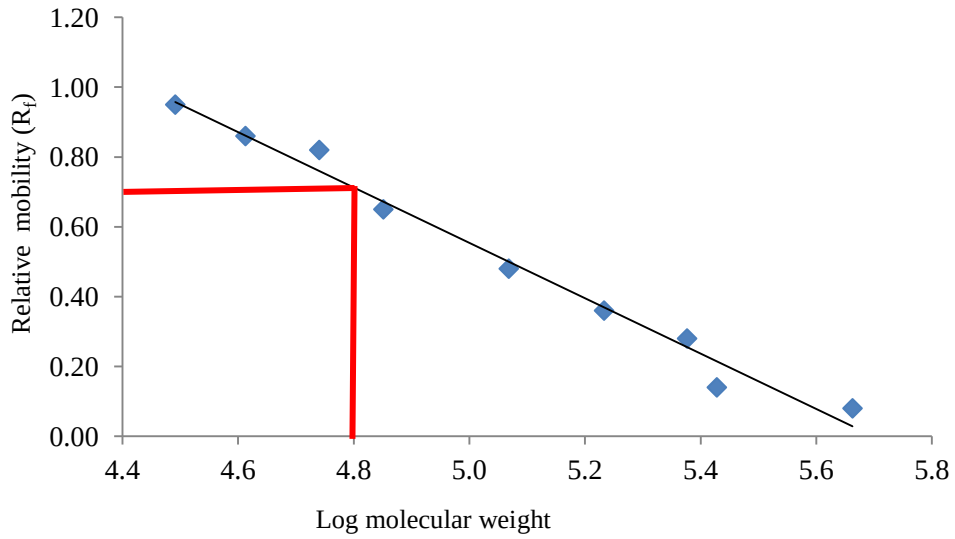


Figure 3 A plot of log molecular weight of standard protein markers as a function of R_f values

Optimum pH of the Free and Immobilized Laccase

The effect of pH on the activity of the free and immobilized laccase was studied in the pH range from 3 to 7 in which pH 3 to 5 using acetate buffer and pH 6 to 7 using citrate -phosphate buffer. Table 3 and **Figure 4** show that the optimum pH value was 5 for free laccase and 6 for the immobilized laccase The free laccase showed the highest activity at pH 5 with guaiacol as substrate, which is consistent with other reports (Palmieri *et al*., 1997; Heinzkill *et al*., 1998). However, after immobilization by physical adsorption, the optimal pH was shifted from pH 5 to pH 6. Ghorbani *et al*. (2018) reported that the optimum pH of the laccase immobilized on porous zinc oxide nanoparticles was 6. In comparison with the free laccase, the immobilized laccase exhibited higher activity than the free laccase between pH 3 to 7. Thus the laccase immobilization by gel entrapment could increase the pH resistance of high catalytic activity and this property is crucial in practical applications, e.g., in the treatment of textile industrial waste effluent.

Table 3 Relationship between Activities of the Free and Immobilized Laccase and pH of Solutions at 40 ^{o}C

No.	pH	Buffer	Laccase activity (μmol mL^{-1} min^{-1})	
			Free laccase	Immobilized laccase
1	3	Acetate	1.074	2.694
2	4	Acetate	1.260	3.471
3	5	Acetate	5.619	4.320
4	6	Citrate - Phosphate	4.269	5.895
5	7	Citrate - Phosphate	1.810	2.520

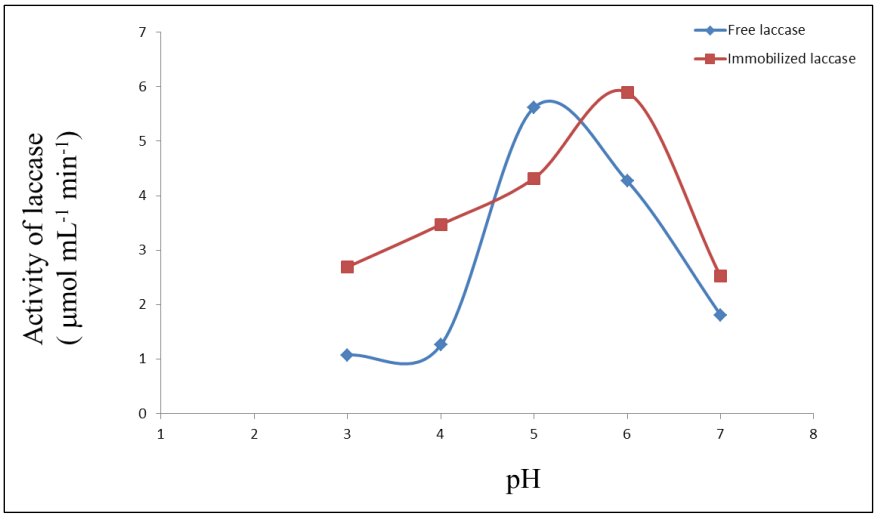


Figure 4 Plot of laccase activity as a function of pH of the solution

Optimum Temperature of the Free and Immobilized Laccase

The effect of temperature on the activity of the free and immobilized laccases was also studied in the temperature range of 25–60 °C (Table 4 and Figure 5). The activities of either free or immobilized laccase increased with increasing temperature until optima and thereafter decreased with further increase of temperature. The optimum temperature of free laccase was 40 ^{o}C. Optimum temperature of laccase from *Trametes versicolor* was reported as 45 ^{o}C (Stoilova *et al.,* 2010). The optimum temperature of immobilized laccases was found to be higher than that of free laccase and shifted from 40 °C to 45 °C.The shift of the optimum temperature indicates an increase in the thermal stability of the immobilized laccases.

Table 4 Relationship between Activities of Laccase and Temperature at pH 5

No	Temperature (^{o}C)	Laccase activity (µmol mL^{-1} min^{-1})	
		Free laccase	Immobilized laccase
1	25	4.358	5.147
2	30	5.279	5.976
3	35	5.509	6.144
4	40	6.152	7.174
5	45	5.399	7.688
6	50	4.127	7.234
7	55	3.644	5.901
8	60	2.550	4.940

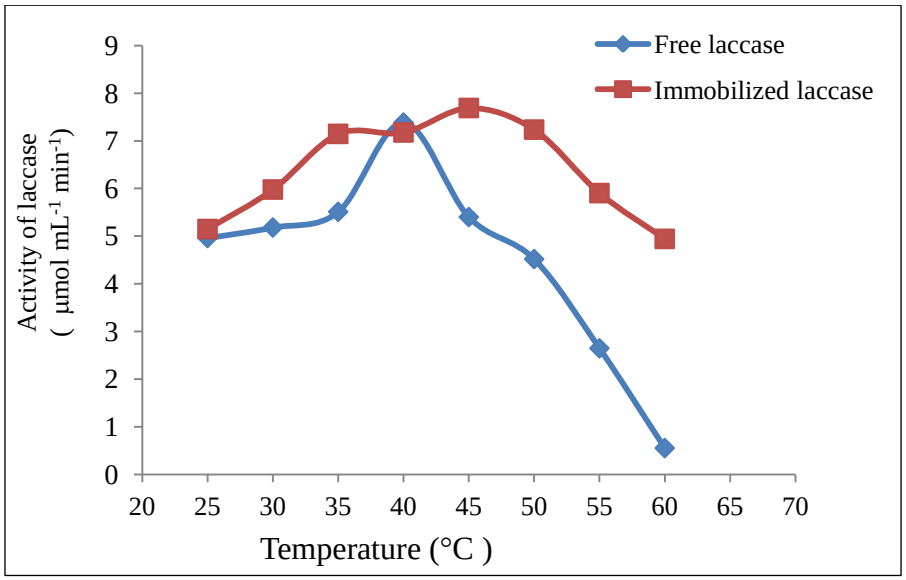


Figure 5 Plot of laccase activity as a function of temperature at pH 5

Kinetic Parameters

Relationship between velocities of free and immobilized laccase catalyzed reaction and guaiacol substrate concentration is shown in Table 5 and Figure 6 (Michaelis-Menten plot). At relatively low guaiacol concentration, initial velocities of both free and immobilized laccase increased almost linearly with increase in concentration of guaiacol. At higher guaiacol concentration, the initial velocities increased by smaller and smaller extent in response to increase in concentration of guaiacol. Michaelis-Menten equation, $v = \frac{V_{max}[S]}{K_m} + [S]$ explains kinetics, but because it is nonlinear, is a little hard to deal with real practical data. From Michaelis-Menten plot K_m values were found to be 0.55 mM and 0.70 mM for guaiacol substrate and V_{max} values were 13.81 µM min^{-1} and 17.47 µM min^{-1}, respectively, for the free and immobilized laccase- catalyzed reactions.

Lineweaver-Burk plot or double reciprocal ($\frac{1}{v}$ vs $\frac{1}{[S]}$ plot) transformation distorts the error in the measurements. As shown in Figure 7 , the noisiest data are too heavily weighted when linear regression is used to determine the best straight line. From this Lineweaver-Burk plot K_m values were found to be 0.68 mM and 1.00 mM for guaiacol substrate and V_{max} values were 16.86 µM min^{-1} and 22.49 µM min^{-1}, respectively, for free and immobilized laccase- catalyzed reactions.

Eadie-Hofstee plot (v vs $\frac{v}{[S]}$ plot) not only yields K_m and V_{max} magnifies departures from linearity which may not be apparent in a double reciprocal plot. From Eadie-Hofstee plot (Figure 8) of free and immobilized laccase- catalyzed reactions K_m values were found to be 0.65 mM and 0.98 mM for guaiacol substrate and V_{max} values were 16.64 µM min^{-1} and 23.22 µM min^{-1}, respectively.

Table 5 Relationship between Velocities of the Free and Immobilized Laccase Catalyzed Reactions and Guaiacol Substrate Concentration

[S] mM	-[S] mM	1/[S] mM^{-1}	V ($\mu M min^{-1}$)		1/V ($\mu M^{-1} min$)		V/[S] (x 10^{3} min^{-1})		[S]/V (x 10^{-3} min)	
			Free	Immo	Free	Immo	Free	Immo	Free	Immo
0.4	- 0.4	2.500	6.198	6.551	0.161	0.153	15.495	16.3774	0.065	0.061
0.8	- 0.8	1.250	9.217	10.099	0.108	0.099	11.521	12.6240	0.087	0.079
1.2	- 1.2	0.833	11.096	12.562	0.090	0.080	9.247	10.4683	0.108	0.096
1.6	- 1.6	0.625	11.840	14.876	0.084	0.067	7.400	9.2975	0.135	0.108
2.0	- 2.0	0.500	12.837	16.143	0.078	0.062	6.419	8.0716	0.156	0.124
2.4	- 2.4	0.417	13.030	16.694	0.077	0.060	5.429	6.9559	0.184	0.144
2.8	- 2.8	0.357	13.570	17.025	0.074	0.059	4.846	6.0803	0.206	0.164
3.2	- 3.2	0.313	13.580	17.355	0.074	0.058	4.244	5.4236	0.236	0.184
3.6	-3.6	0.278	13.810	17.466	0.072	0.057	3.836	4.8515	0.261	0.206

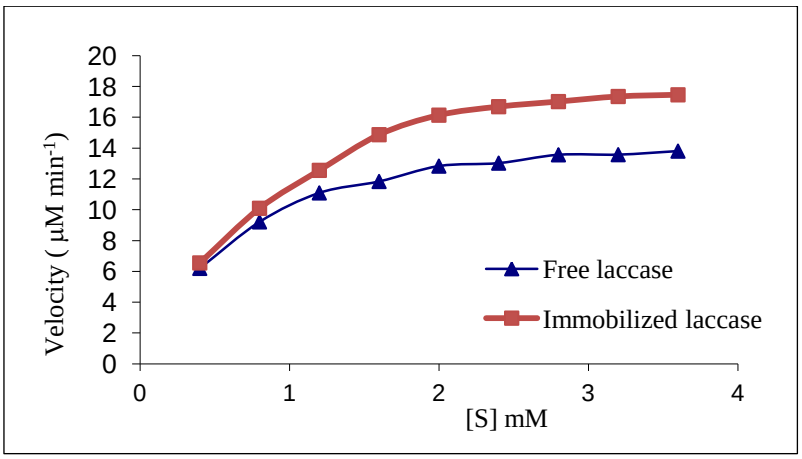


Figure 6 Plot of velocity as a function of guaiacol substrate concentration for the free and immobilized laccase- catalyzed reactions

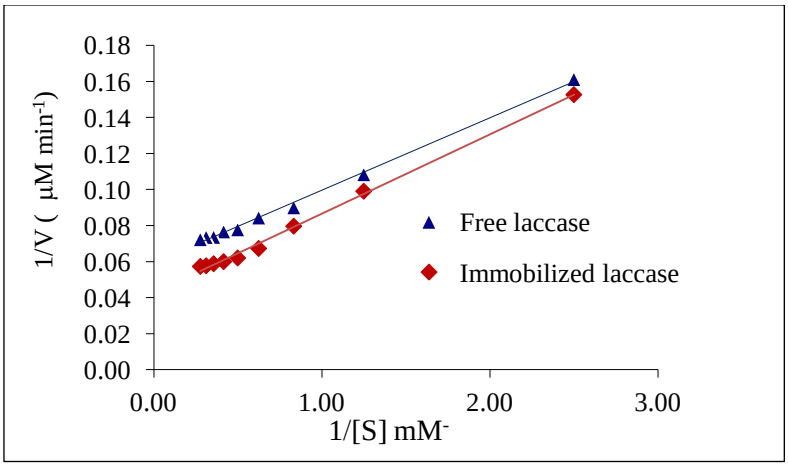


Figure 7 Lineweaver- Burk plot of 1/V vs 1/[S] used for evaluation of K_m and V_{max} for free and immobilized laccase-catalyzed reactions

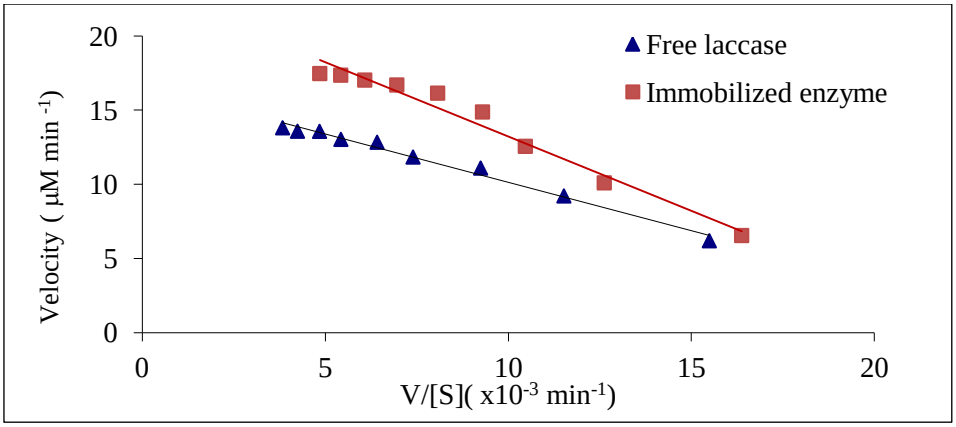


Figure 8 Eadie -Hofstee plot of V vs V/[S] used for evaluation of K_m and V_{max} for the free and immobilized laccase-catalyzed reactions

Figure 9 shows Hanes-Wilkinson plot ([S]/V vs [S] plot) of free and immobilized laccase-catalyzed reactions. From this plot, K_m values were found to be 0.60 mM and 0.91 mM for guaiacol substrate and V_{max} values were 16.25 μM min^{-1} and 22.43 μM min^{-1}, respectively, for the free and immobilized laccase-catalyzed reactions.

Figures 10 and 11 are the Eisenthal- Cornish Bowden plost or direct linear plots. [S] values are plotted on the negative X-axis and observed v values on the Y-axis. From Eisenthal-Cornish Bowden plot of free and immobilized laccase-catalyzed reactions K_m values were found to be 0.61 mM and 0.91 mM for guaiacol substrate and V_{max} values were 16.10 μM min^{-1} and 21.00 μM min^{-1}, respectively.

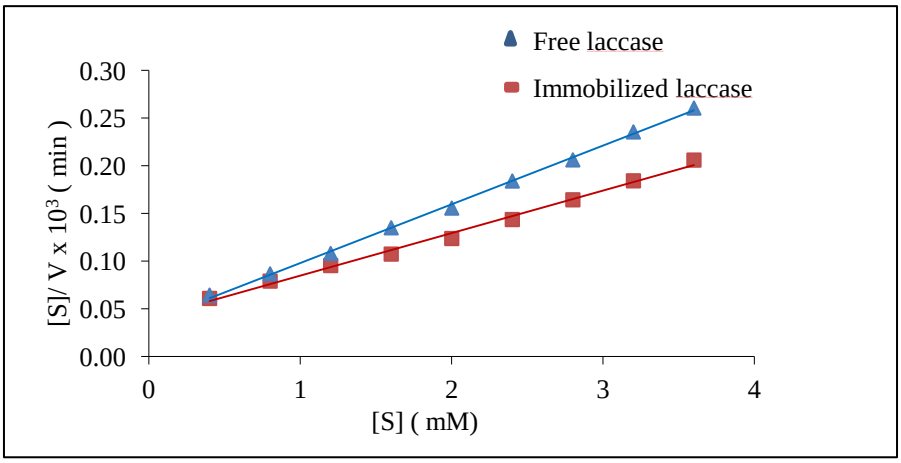


Figure 9 Hanes-Wilkinson plot of [S]/V vs [S] used for evaluation of K_m and V_{max} for the free and immobilized laccase-catalyzed reactions

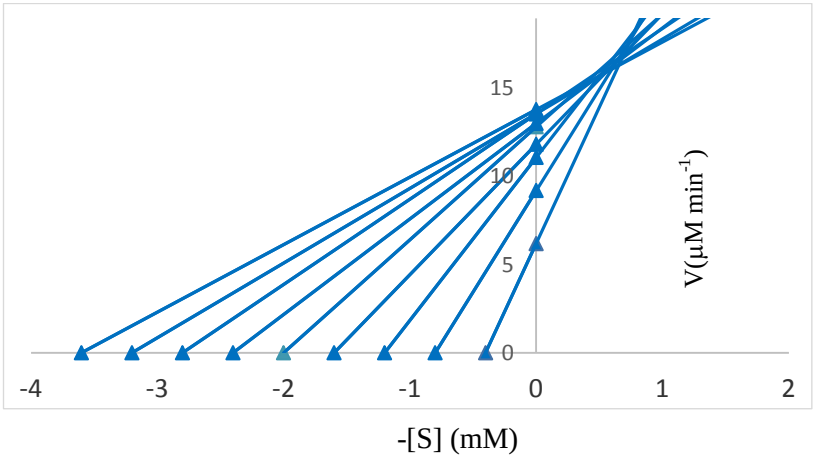


Figure 10 Eisenthal-Cornish-Bowden or direct linear plot of V vs - [S] used for evaluation of K_m and V_{max} for the free laccase-catalyzed reaction

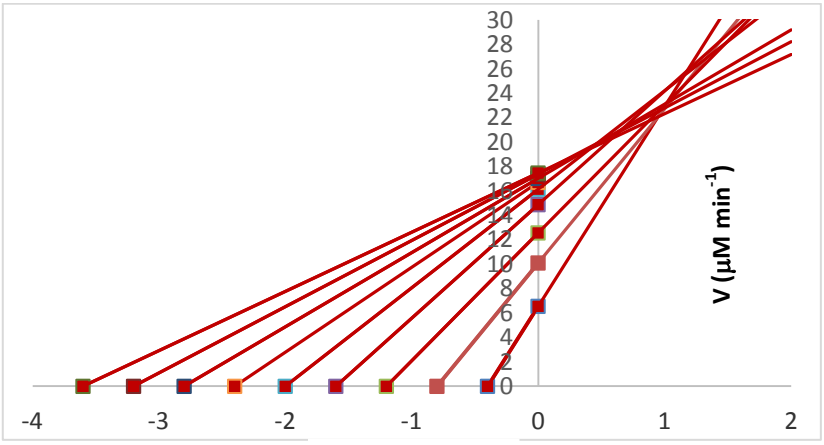


Figure 11 Eisenthal-Cornish-Bowden or direct linear plot of V vs - [S] used for evaluation of K_m and V_{max} for the immobilized laccase-catalyzed reaction

Table 6 shows the K_m and V_{max} values of free and immobilized laccase-catayzed reactions by different methods. K_m and V_{max} values obtained by different methods were found to be agreed with each other. K_m and V_{max} are significant coefficients in guiding scientific research and engineering design. The more firmly the enzyme binds to its substrate, the smaller will be the value of K_m. The results show good affinity of the enzyme for guaiacol substrate.

Table 6 Comparison of K_m and V_{max} Values of Free and Immobilized Laccase-Catayzed Reactions by Different Methods

No	Methods	K_m (mM)		V_{max} (μM min^{-1})	
		Free laccase	Immobilized laccase	Free laccase	Immobilized laccase
1	Michaelis –Menten	0.55	0.70	13.81	17.47
2	Lineweaver- Burk	0.68	1.00	16.86	22.97
3	Eadie -Hofstee	0.65	0.98	16.64	23.22
4	Hanes-Wilkinson	0.60	0.91	16.25	22.43
5	Eisenthal-Cornish-Bowden	0.61	0.91	16.10	21.00

When the Michaelis–Menten equation is written in the form of a straight line, the Hill equation (Martin, 1993),

$$\log \frac{V}{V_{max} - V} = n \log[S] - \log K_m$$

is obtained. The equation states that, when [S] is low compared to K_m, the reaction velocity increases as the n^{th} power of [S].

In the present work, n value was determined from the plot of $\log \frac{V}{V_{max} - V}$ vs. log [S] using the linear regression method (Table 7 and Figure 12) .The reaction order (n) for laccase was calculated to be 0.94 for free laccase and 0.90 for immobilized laccase proving that the reaction order is first order.

Table 7 Relationship between $\log \frac{V}{V_{max} - V}$ and log [S] for the Determination of Reaction Order of Laccase –Catalyzed Reaction

No	[S] (mM)	log [S]	V(mMmin^{-1})		$\log \frac{V}{V_{max} - V}$	
			Free	Immo	Free	Immo
1	0.4	-0.398	6.198	0.581	-0.235	-0.399
2	0.8	-0.097	9.217	1.206	0.081	-0.105
3	1.2	0.079	11.096	1.926	0.285	0.082
4	1.6	0.204	11.840	2.360	0.373	0.211
5	2.0	0.301	12.837	3.193	0.504	0.374
6	2.4	0.380	13.030	3.404	0.532	0.384
7	2.8	0.447	13.570	4.128	0.616	0.436
8	3.2	0.505	13.580	4.143	0.617	0.463
9	3.6	0.556	13.810	4.532	0.656	0.502

V_{max} = 16.86 μM min^{-1} (Free laccase)

= 22.97 μM min^{-1} (Immobilized laccase)

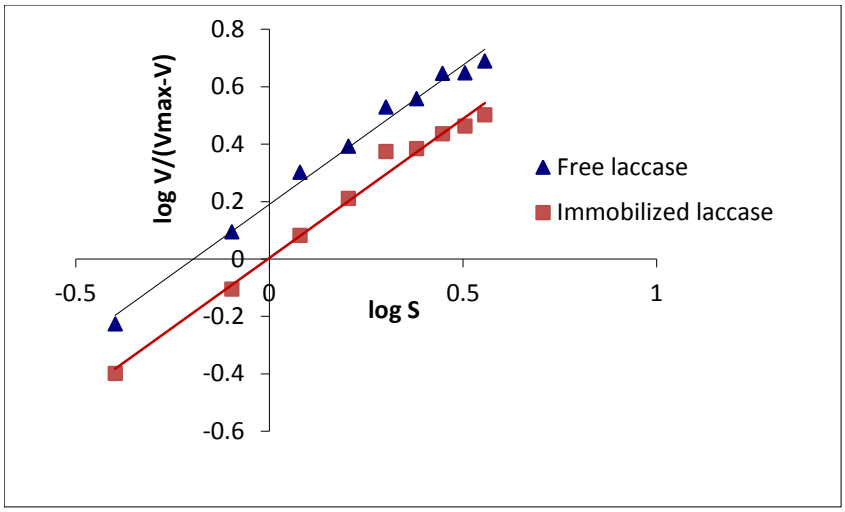


Figure 12 Plot of $\log \frac{V}{V_{max} - V}$ as a function of log [S] used for evaluation of reaction order for the free and immobilized laccase-catalyzed reactions

Conclusion

This study revealed that interesting novel laccase producers can be discovered from the environments especially mushroom. Banana skin , the main waste of banana plant, can be used as support-substrate for production of laccase at low cost by *Trametes vesicolor* under solid state condition. Purification of laccase by ammonium sulphate precipitation, dialysis and Sephadex G- 100 gel filtration chromatography revealed that the specific activity of crude and purified laccase were 0.22 μmol $min^{-1}mg^{-1}$ and 2.80 μmol $min^{-1}mg^{-1}$ respectively. Laccase was purified by 12.72 fold over crude extract. The molecular weight of Laccase from *T. vesicolor* is 60.26 kDa by gel electrophoresis using SDS-PAGE. The optimum pH of free laccase was 5 and after immobilization it was shifted to 6. The optimum temperature of free laccase was 40 ^{o}C and that of immobilized laccase was 45 ^{o}C. From Lineweaver-Burk method, Michaelis-Menten constant K_m of the free laccase was 0.68 mM and that of immobilized laccase was 1.00 mM and maximum velocity V_{max} of free laccase-catalyzed reaction was 16.86μM min^{-1} and it increased to 22.97 μM min^{-1} in the immobilized laccase-catalyzed reaction.

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