ISOLATION AND CHARACTERIZATION OF FUNGAL LACCASE FROM *TRAMETES VERSICOLOR*

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

Abstract

The present work focuses on screening, isolation, and partial characterization of the extracellular laccases from mushroom *Trametes versicolor*. Laccase-producing fungi were screened and isolated from the selected samples on Malt Extract Agar (MEA) medium. Laccase-producing fungi were detected by qualitative examination with guaiacol and tannic acid. The isolated fungal strain was identified by Lactophenol cotton blue dye method and microscopic examination. Laccase producing fungal strain was cultured under solid state fermentation using banana skin as support-substrate. The highest laccase activity was found on 12 days of incubation. Laccase was partially purified by ammonium sulphate fractionation (20 % and 70 %) and its activity was monitored by guaiacol assay method. Laccase was purified 6.4 fold over crude extract. The optimum pH and optimum temperature of fungal laccase was 5 and 40 ^{o}C, respectively.

Keywords: laccase, *Trametes versicolor,* guaiacol, solid state fermentation

Introduction

Laccases (benzenediol; oxygen oxidoreductase E.C. 1.10.3.2) are extracellular,multicopper enzymes that catalyze the oxidation of a variety of phenolic and inorganic compounds, with the concomitant reduction of oxygen to water (Stoilova *et al*., 2010). Laccase contains four copper atoms and is able to oxidize its substrates by using molecular oxygen as an electron acceptor (Thurston, 1994).These oxidative enzymes are particularly abundant in white-rot fungi. They have potential applications and used in many fields, including the environmental and industrial sectors such as pharmaceutical, nano-biotech, textile, paper and pulp, food-chemistry, cosmetic due to their low substrate specificities and high redox potentials (Madhavi and Lele, 2009; Upadhyay *et al.,* 2016; Akpinar and Urek, 2017).Fungal laccases have ability in the degradation of toxic fungal metabolite, such as aflatoxin B1, in ethanol production, manufacturing of cream and wine clarification. These

[1.] 3PhD Candidate, Department of Chemistry, University of Yangon
[2.] Dr, Associate Professor, Department of Chemistry, Loikaw University
[3.] Dr, Professor and Head (Retired), Department of Chemistry, University of Yangon

characteristics have led to laccases being qualified as ‘‘eco-friendly’’ enzymes (Alberts *et al*., 2009; Lu *et al*., 2007).

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; Lui*et al*., 2017). Among them, white-rot fungi is the major laccase producer, and *Trametes versicolor* is an important representative of white-rot fungi. Many fungal laccases have been purified and characterized, such as *Trametes* sp. LAC-01, *Pleurotus* sp. MAK-II laccase and *Cerrena* sp. laccase (Manavalan *et al*., 2015 ; Ling *et al*., 2015; Yang *et al*., 2015).The genus *Trametes*, which belongs to the white-rot fungi, is assumed to be one of the main producers of laccases.

Two types of culture techniques are used for white-rot fungi to produce : solid-state fermentation (SSF) and submerged fermentation (SmF). The SSF occurs in absence or near absence of free liquid, using an inert substrate or a natural substrate as a solid support (Pandey *et al*., 1999). The former works as an attachment place for the microorganism, whereas the latter also acts as a carbon source, which considerably reduces the production costs (Ramana Murthy *et al*., 1999). SSF is advantageous in obtaining concentrated metabolites and subsequent purification procedures are economical.

This study is aimed to isolate laccases from white-rot fungi, *Trametes versicolor* using solid state fermentation and to partially characterize the enzyme.

Materials and Methods

Sample Collection

The mushroom (Figure 1) samples were collected in sterile plastic bags from the timber industry compound, Insein Township, Yangon Region, Myanmar. It was verified at Department of Agriculture, Ministry of Agriculture, Livestock and Arrigation.

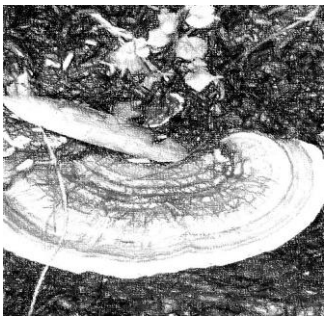


Figure 1: Mushroom (*Trametes versicolor*)

Screening of Fungal Strain

Mushroom sample (1 g) was extracted with 100 mL of sterile distilled water and then the aqueous extract was serially diluted (10^2, 10^3, 10^4, 10^5). After plating on Potato Dextrose Agar (PDA) and malt extract agar (MEA) media the petri dishes were incubated at 30°C for 5 days. The plates were marked as DMP-1, DMP-2, DMP-3 and DMP-4 for serially diluted mushroom samples in PDA media and DMM-1, DMM -2, DMM -3 and DMM -4 for diluted mushroom samples in MEA media (10^2, 10^3,10^4 and 10^5 dilutions respectively). Then white fungi were selected and sub-cultured on the PDA and MEA slant . Based on the heavy growth on MEA media it was chosen for further experiment. Then white fungi were selected and sub-cultured on the MEA media supplemented with 0.1% guaiacol and 0.1% tannic acid (Kiiskinen *et al.,* 2004). The fungi showing laccase activity were selected and then sub-cultured on the MEA slant. These strains were identified by Lactophenol dye method and observed under microscope. The fungal cultures were maintained by periodical sub-culturing on MEA slant at 30 °C and then stored at 4 °C.

Solid State Fermentation for Laccase Production

(a) Pretreatment of support – substrate, banana skin

Chopped banana skin (1 cm×1 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 thoroughly

washed with distilled water and dried at room temperature for one day. In this way the pretreated banana skin were obtained to be used as substrate for fermentation.

(b) Preparation of culture medium for solid state fermentation (SFF)

The composition of culture medium consisted of 3 g of peptone, 10 g of glucose, 0.6 g of potassium dihydrogenphosphate, 0.001 g of zinc sulphate, 0.4 g of dipotassium hydrogenphosphate, 0.0005 g of iron(II) sulphate, 0.05 g of manganese(II) sulphate and 0.5 g of 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 above culture medium.

(c)Preparation of crude laccase enzyme solution by solid state fermentation

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 the determination of laccase activity.

(d) Purification of laccase by ammonium sulphate precipitation method

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.

(e) Determination of laccase activity, protein content and specific activity in different purification steps

The laccase activities (guaiacol assay method) and protein contents (Biuret method) of the crude enzyme extract and the enzyme extract obtained after successive precipitation with 20 % and 70 % satuation of ammonium sulphate solutions.

(i) Determination of laccase activity of the crude enzyme extract by Guaiacol assay method

Guaiacol assay method was carried out according to the method of Desai *et al.* (2011).Guaiacol (2 mM) in sodium acetate buffer (10 mM pH 5.0) was used as substrate. The reaction mixture contained 3 mL of acetate buffer, 1 mL of guaiacol and 1 mL of the crude enzyme solution. For blank solution, 1 mL of distilled water was used instead of enzyme solution. The mixture was incubated at 30 ºC for 15 min and the absorbance was read at 450 nm. The laccase activity was calculated using the extinction coefficient of guaiacol (12,100 M^{-1} cm^{-1}) at 450 nm by the formula as shown below. E.A = (A x V) / (t x e x v) (Savitha *et al.,* 2011), where E.A = enzyme activity (U/mL), A = absorbance at 450nm, V = total volume of reaction mixture (mL), v = enzyme volume (mL), t = incubation time (min) and e = extinction coefficient (12100 M^{-1} cm^{-1}). One unit of enzyme was defined as the amount of enzyme required to oxidize 1 micromole of guaiacol per min.

(ii) Determination of protein content

Protein content was determined by Biuret method using Bovine Serum Albumin (BSA) as standard at 560 nm. Firstly, the calibration curve of BSA was constructed to determine the concentration of protein in the enzymes. Then 1 mL of standard protein solutions with concentrations of 1, 2, 3, 4 and 5 mg mL^{-1} was individually added to five test tubes, each containing 4 mL of Biuret reagent solution and the contents were mixed well. The solution mixture was kept for 30 min at room temperature. After that the absorbance values of the standard protein solutions were measured and the calibration curve was constructed. A blank solution and enzyme solution were prepared

by using 1 mL each of distilled water and enzyme solution, respectively, instead of standard protein solution.

(iii) Determination of specific activity

Specific activity was determined by the ratio of total activity to the total protein content. Specific activity is a measure of enzyme efficiency.

(f) Characterization of laccase

The optimum pH and optimum temperature of laccase- catalyzed reaction for the partially purified laccase enzyme obtained by 70 % ammonium sulphate precipitation were investigated by spectrophotometric method.

For the study on the effect of pH, the reaction mixtures contained 1mL each of guaiacol (2 mM) as substrate dissolved in 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.

Similarly, for the study on the effect of temperature, the mixtures contained 1 mL each of guaiacol (2 mM) as substrate dissolved in 1 mL each of enzyme solution and 3 mL each of 10 mM acetate buffer (pH 5). The mixtures were then incubated at different temperatures of 25 ºC, 30 ºC, 35 ºC, 40 ºC, 45 ºC, 50 ºC, 55 ºC, and 60 ºC for 15 min and absorbance values were recorded at 450 nm.

Results and Discussion

Isolation of Laccase-producing Fungi from Mushroom, *Trametes versicolor*

The mushroom sample (1 g) (*T. versicolor*) collected from Timber Industry Compound near Gyogone, Insein Township, Yangon Region. It was firstly extracted with sterilized distilled water (100mL).And that extracted was then serially diluted($10^{2,}$ 10^3, 10^4, 10^5) and then the diluted solutions were inoculated in fungal selected Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) media. After 5 days of incubation, growth of fungi were seen on

the PDA and MEA media (Figure 2). Among 8 petridishes, 4 petridishes (DMP-3, DMP-4, DMM-3 and DMM-4) were selected due to the appearance of white fungi. Then the white fungi were sub-cultured in PDA and MEA slants and it was found that MEA slants gave purified fungi compared to PDA slants (Figure 3). Laccase – producing fungi was examined in the presence of guaiacol and tannic acid supplemented MEA media and the formation of reddish brown zones around the fungal colonies was observed after 6 days of incubation (Figure 4). The pure culture on MEA medium was rich with white aerial mycelium (Figure 5). The reverse of the mycelia was colourless, pigmentation was not found even after two weeks of cultivation. In the lactophenol cotton blue staining, spores were cylindrical to sub cylindrical and thin-walled (Figure 6). Hyphae (threadlike elements of the mycelium) have cross-walls called septa between cells. Therefore, Figures 4, 5, 6 stated the laccase - producing fungal character and showed isolated fungi.

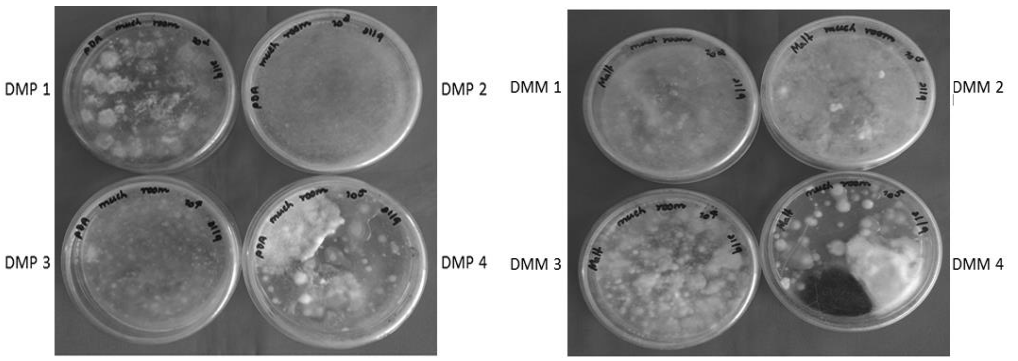
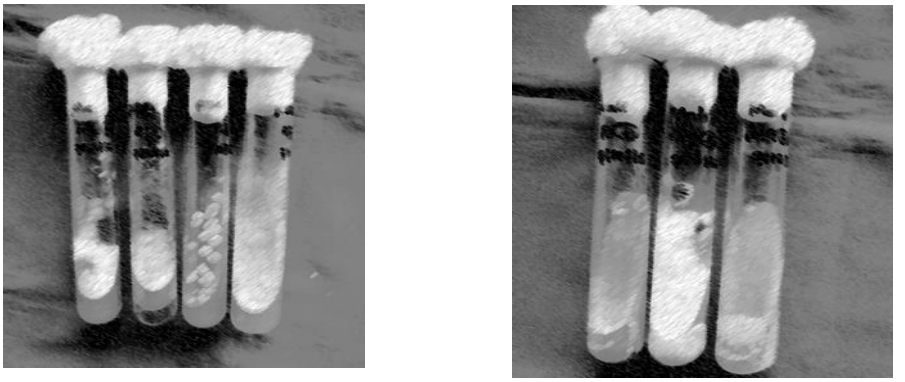
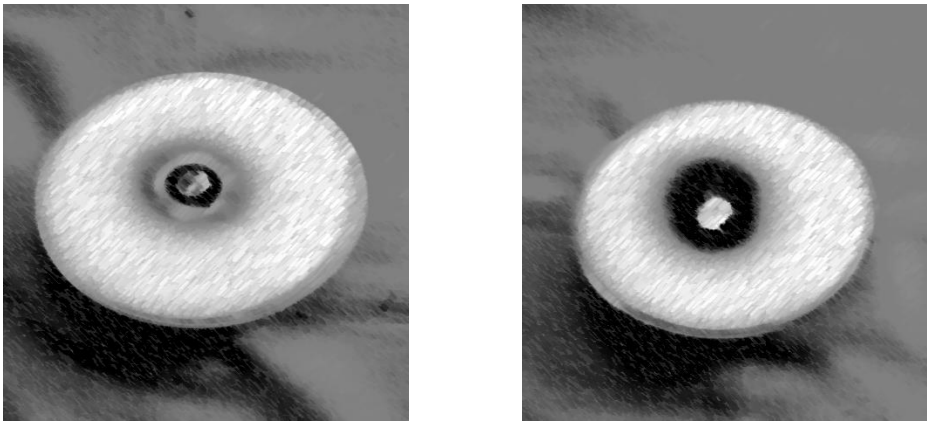


Figure 2: Growth of fungi from mushroom on (a) PDA media and (b) MEA media by serial dilution method



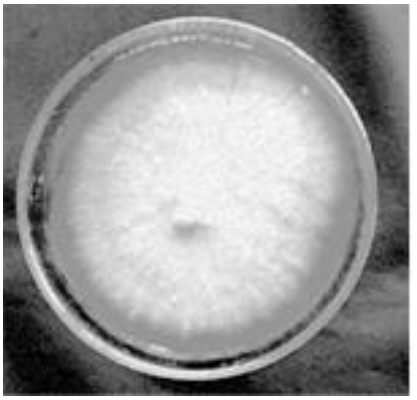
(a) (b)

Figure 3: Sub- culture of fungi in (a) PDA and (b) MEA slants



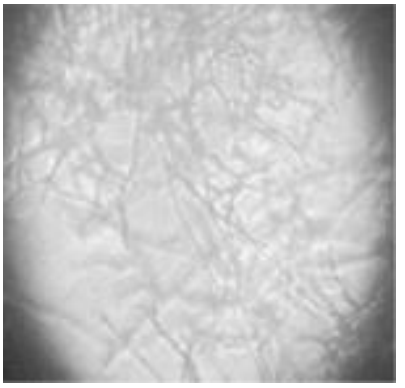
(a) (b)

Figure 4: Reddish-brown colonies zone on MEA media supplemented with (a) guaiacol and (b) tannic acid



(a)

Figure 5: Pure culture of the isolated fungal strain



(b)

Figure 6: Mycelium structure of the strain under microscope at 40 X

Production of Laccase from the Isolated Fungus from *T. versicolor* under Solid State Condition

The result of the time course study for laccase production by using the pretreated banana skin as substrate by the isolated fungus is shown in Table 1 and Figure 7. In this study,Tween 80 was used as a surfactant which could modify the fungal membrane and promote laccase secretion by many fungal strains (Dekker *et al.*,2007). The laccase activity in the culture broth increased with increase in cultivation time and the production of laccase peaked at the late stage of cultivation,i.e., 12 days. However, the laccase activity decreased after further cultivation time. Maximum laccase activity was found after 12 days of solid state fermentation in this study.

Table 1: Activity of Laccase Produced at Various Incubation Times under solid State Fermentation (SSF)

No.	Incubation time (day)	Laccase activity ($\mu mol\ mL^{-1}\ min^{-1}$)
1	3	10.2
2	6	11.5
3	9	12.3
4	12	13.9
5	15	11.1
6	18	9.5

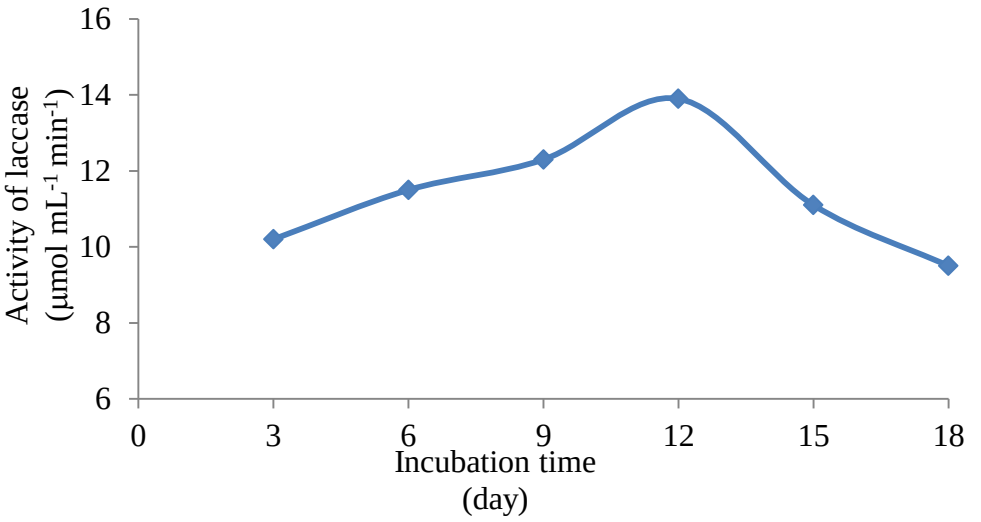


Figure 7: Activity of Laccase Produced at Various Incubation Times under solid State Fermentation (SSF)

Partial Purification of Laccase from*T. versicolor*

Laccase from *T. vesicolor* was partially purified by fractional ammonium sulphate precipitation method. Laccase activity in each purification step was determined by guaiacol assay method at 450 nm.For determination of specific activity of laccase, protein contents in enzyme extracts were determined by Biuret method using calibration curve of standard bovine serum albumin solution.

After 20 % ammonium sulphate precipitation, the specific activity of laccase increased from 0.25 µ mol $min^{-1}mg^{-1}$ in crude extract to 0.50 µ mol $min^{-1}mg^{-1}$ (Table 2). Furthermore, specific activity increased to 1.60 µ mol $min^{-1}mg^{-1}$ after 70 % ammonium sulphate precipitation and purification fold also increased to 6.4 fold over crude extract.

Table 2: Total Enzyme Activity, Total Protein and Specific Laccase Enzyme Activity Obtained at Different Purification Steps

Purification steps	Total activity (μ mol min^{-1})	Total protein (mg)	Specific activity (μ mol $min^{-1}mg^{-1}$)	Purification (fold)
Crude extract	6720	30000	0.25	1
After 20 %$(NH_4)_2SO_4$ precipitation	2590	5000	0.50	2
After 70 %$(NH_4)_2SO_4$ precipitation	3770	1600	1.60	6.4

Characterization of Purified laccase Enzyme

(a) Optimum pH

The pH optimum was determined over a range of pH 3–6 at 30 °C. The pH optimum of laccase was determined by using two different buffer systems comprising of 10 mM sodium acetate buffer (pH 3.0–5.0) and phosphate – citrate buffer (pH 6.0–7.0). Activity of laccase gradually increased from pH 3 to 5 and then abruptly increased when pH was reached at 5 (Table 3 and Figure 8). Beyond pH 5,laccase activity were found to decrease. In this study, the highest laccase activity was achieved at pH 5.Optimum pH value of laccase from both *Pleurotus ostreatus* and *Coprinusfriesii* was found as5.0 (Palmieri *et al*., 1997; Heinzkill *et al*., 1998).Stoilova *et al*. (2010) reported the optimum pH of laccase from *Trametes versicolor* as 4.5.The result in this study is in line with Holker *et al.* (2002) and Robles *et al.* (2002) who revealed that the optimal pH for fungal laccase was ranged from 4.0 to 6.0.

Table 3: Relationship between Laccase Activity and pH of Solutions

No.	pH	Buffer	Laccase activity (μmol mL^{-1} min^{-1})
1	3	Acetate	10.79
2	4	Acetate	12.40
3	5	Acetate	13.88
4	6	Phosphate - Citrate	11.75
5	7	Phosphate -Citrate	10.08

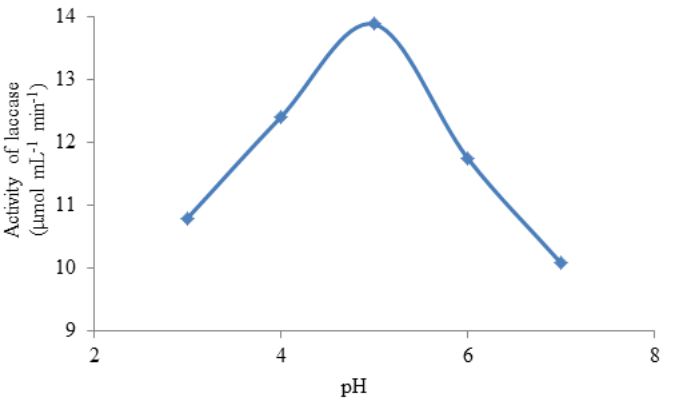


Figure 8: Plot of laccase activity as a function of pH of the solution

Optimum temperature

The effect of temperature on laccase activity was determined by oxidation of guaiacol for 30 min at temperature ranging from 25- 60 °C with an interval of 5 °C (Table 4 and Figure 9). Sodium acetate buffer (pH 5) was used for all the reactions, as optimum pH of laccase. The enzyme activity increased with increasing the temperature from 25 to 40 °C with the maximum activity at 40 °C and then gradually decreased from 40 °C to 60 °C. Rapid inactivation of the enzyme was observed at 70 °C.Thus the temperature optimum of the laccase from *Trametes versicolor* was 40^{o}C with guaiacol as a substrate in acetate buffer of pH 5.0. This result is in agreement with Kalra *et al.* (2013)who found that the optimum temperature for enzyme activity was

45-50°C.In general, laccases are stable at 30–50 °C and rapidly lose activity at temperatures above 60 °C.(Palonen *et al.*, 2003; and Xu *et al.*,1996).

Table 4: Relationship between Laccase Activity and Temperature

No.	Temperature (^{o}C)	Laccase activity (μmol mL^{-1} min^{-1})
1	25	9.45
2	30	10.03
3	35	11.57
4	40	13.30
5	45	13.80
6	50	12.85
7	55	10.10
8	60	8.81

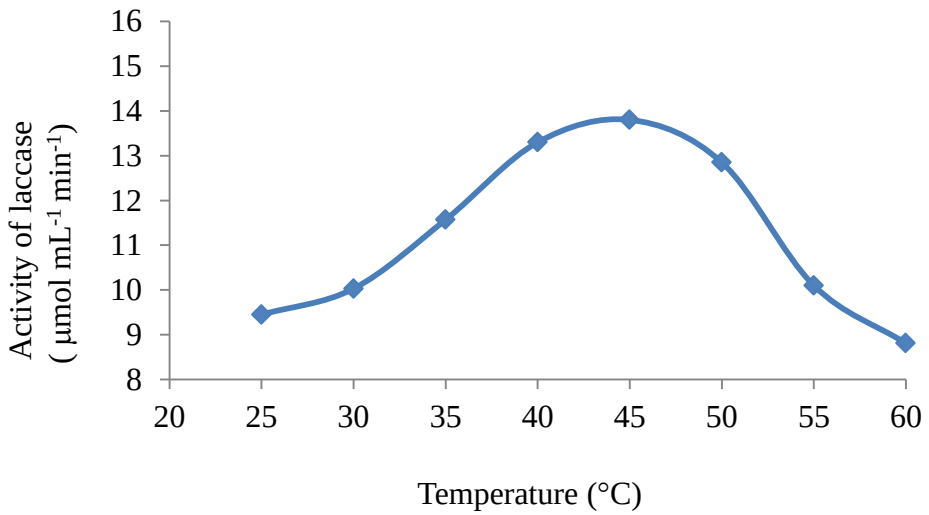


Figure 9: Plot of laccase activity as a function of temperature of the solution

Conclusion

This study revealed that interesting novel laccase producers can be discovered from the environments by very simple plate test methods using

Malt Extract Agar (MEA) as a screening medium. Banana skin , the main waste of banana plant, can be used as support-substrate for production of laccase at low cost by *Tramatese vesicolor* under solid state condition. Production of laccase under solid state condition showed that maximum laccase activity was attained after 12 days of fermentation. Partial purification of laccase by ammonium sulphate precipitation revealed that the specific activity of laccase was 1.60 μ mol $min^{-1}mg^{-1}$and it was purified by 6.4 fold over crude extract. The optimum pH and optimum temperature of fungal laccase was 5 and 40 ^{o}C, respectively.

Acknowledgements

The authors would like to express their profound gratitude to the Department of Higher Education, Ministry of Education, Myanmar for provision of opportunity to do this research and to the Myanmar Academy of Arts and Science for allowing to present this research paper.

References

Akpinar, M. and Urek, R. O.(2017).“Induction of Fungal Laccase Production under Solid State Bioprocessing of New Agroindustrial Waste and its Application on Dye Decolorization”. 3 Biotech.*,vol.7(2), p.98*

Alberts, J.F., Gelderbolm, W.C.A., Botha, A.,andVanzyl,W.H. (2009).“Degradation of Aflatoxin B1 by Fungal Laccase Enzymes”. *Int J Food Microbiol.*,vol.135,pp. 47-52

Dekker, R.F.H., Barbosa, A.M., Giese, E.C., Godoy, S.D.S. and Covizzi, L.G. (2007).“ Influence of Nutrients on Enhancing Laccase Production by *Botryosphaeriarhodina* MAMB-05”.*IntMicrobiol.*,vol.10,pp.177–185

Desai,S.S.,Tennali, G.B.,Channur, N.,Anup, A.C. ,Deshpande, D. and Murtuza, B.P.A .(2011). “Isolation of Laccase Producing Fungi and Partial Characterization of Laccase”. *Biotechnol. Bioinf. Bioeng.*,vol.1(4), pp.543-549

Heinzkill, M., Bech, L.,Halkier, T., Schneider, P. and Anke, T. (1998). “Characterization of Laccases from Wood - rotting Fungi (Family Coprinaceae)”. *Applied Environmental Microbiology*,vol.64 ,pp.1601 -1606

Holker, U., Dohse, J.,and Hofer, M., (2002). Extracellular Laccase in ascomycetes *Trichderma atroviride* and Trichoderma harziaman.Folia Microbiol.47, 423.

Kalra, K., Chauhan, R., Shavez, M. and Sachdeva, S. (2013).“Isolation of Laccase Producing *Trichoderma* spp. and Effect of pH and Temperature on its Activity”.*Int. J. Chem. Environ. Technol.*,vol. 5 (5), pp. 2229-2235.

Kiiskinen, L.L.,Rättö, M. and Kruus**,**K. (2004). “Screening for Novel Laccase-producing Microbes”. *Journal of Applied Microbiology*,vol.97 (3),pp. 640-646

Ling, Z.R., Wang, S.S., Zhu, M.J., Ning, Y.J., Wang, S.N., Li, B., Yang, A.Z., Zhang, G.Q. and Zhao, X.M.(2015).“An Extracellular Laccase with Potent Dye Decolorizing Ability from White Rot Fungus Trametes sp. Lac-01”. *International Journal of Biological Macromolecules*,vol.81 ,pp.785-793

Liu, L., Qin, Z.,Yue, Q. and Borras-Hidalgo, O. (2017). “Laccase Enzyme from *Trametesversicolor* with a High Decolorizing Ability on Malachite Green”. *EC Microbiology* ,vol.10 (3),pp.127-133

Lu, L., Zhao, M., Zhang, B. B., Yu, S. Y., Bain, X. J. and Wang, Y. (2017). “Purification and Characterization of Laccase from *Pycnoporussanguineus* and Decolorization of an Anthraquinone Dye by the Enzyme”. *Appl Microbiol Biotechnol.*, vol.74,pp.1232–1239.

Madhavi, V. and Lele, S.S. (2009).“Laccase: Properties and Applications ”. *Bioresources*,vol.4,pp.1694- 1717

Majeau, J. A., Brar, S. K. and Tyagi, R. D. (2010). “Laccases for Removal of Recalcitrant and Emerging Pollutants”. *Bioresour. Technol*.,vol. 101, pp. 2331–2350

Manavalan, A., Manavalan, T.,Murugesan, K.,Kutzner, A.,Thangavelu, K. P. and Heese, K. (2015).“Characterization of a Solvent, Surfactant and Temperature-Tolerant Laccase from Pleurotus Sp. Mak-Ii and Its Dye Decolorizing Property”.*Biotechnology Letters*,vol.37(12),pp.2403-2409

Murthy, M.V.R., Karanth, N.G. and RaghavaRao, K.S.M.S. (1993). “Biochemical Engineering Aspects of Solid-state Fermentation”. *Advances in Applied Microbiology*, vol.38, pp. 99-147

Palmieri, G., Giardina, P.,Bianco, C.,Scaloni , A.,Capasso, A. and Sannia, G. (1997). “A Novel White Laccase from *Pleurotusostreatus”. Journal Biochemistry* ,vol.272,pp.31301-31307

Palonen, H., Saloheimo, M., Viikariand, L. and Kruus, K. (2003). “Purification, Characterization and Sequence Analysis of a Laccase from the Ascomycetes *Mauginiella* sp.”.*Enzyme Microbiol. Technol.*vol. 33, pp.854–862

Pandey, A., Selvakumar, P., Soccol, C.R. and Nigam, P. (1999). “Solid State Fermentation for the Production of Industrial Enzymes”. *Current Science*, vol.77(1), pp.149-162

Robles, A., lucas, R., Martinez-Canamero, M., Omar, N.B., Perez, R.and Galvez, A..(2002). Characterization of laccase activity produced by hyphomycetes Chalara (syn. Thielaviopsis) paradoxa CH 32.Enzyme Microbiol. Technol.31, 516.

Savitha, S. and Gururaj, B. T.(2011). "Studies on Isolation of Laccase Producing Fungi and Partial Characterization of Laccase".*Society of Applied Biotechnology*, vol.1(4), pp.543-549

Stoilova, I.,Krastanov, A. and Stanchev.V. (2010)."Properties of Crude Laccase from *Trametes versicolor* Produced by Solid-substrate Fermentation". *Advances in Bioscience and Biotechnology*, vol.1, pp. 208-215

Thurston , C.F. (1994)."The Structure and Function of Fungal Laccase".*Microbiol.*,vol. 140, pp.19-26

Upadhyay, P., Shrivastava, R., and Agrawal, P.K. (2016).Bioprospectingand Biotechnological Applications of Fungal Laccase". *3 Biotech*.,vol.6,pp.189–199

Xu, F., Shin, W., Brown, S.H., Wahleitner, J.A., Sundaram, U.M. and Solomon, E.I.A. (1996). "Study of Recombinant Fungal Laccases and Bilirubin Oxidase that Exhibit Significant Differences in Redox Potential, Substrate Specificity, and Stability". *Biochem. Biophys. Acta*,vol.1292, pp.303-311

Yang, J., Yang, X.,Lin,Y., Ng, T. Z., Lin, J., Ye, X. and Hildén, K. (2015). " Laccase-Catalyzed Decolorization of Malachite Green: Performance Optimization and Degradation Mechanism". *PLoS One*,vol.10(5) , p. e 0127714