Int J Pharm Bio Sci 2012 July; 3(3): B 355 - 365

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

PRODUCTION, PURIFICATION AND PARTIAL CHARACTERIZATION OF LACCASE FROM THE MUSHROOM *HYPSIZYGUS ULMARIUS*

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ABSTRACT

The aim of the present study reported here is to examine the production, purification and characterization of the enzyme laccase from the mushroom *Hypsizygus ulmarius.* The culture conditions were optimized for the maximum production of enzymes with different parameters such as incubation time, carbon, nitrogen sources. The enzyme was purified by ammonium sulphate precipitation and partially characterized. The optimum conditions for the maximum production of enzyme were; incubation period 6^{th} day, pH 5.0, temperature 30^{o}C, inoculum 3%, carbon source (xylose 3%) and nitrogen source (KNO_3 3%). The enzyme was purified to 5.11 fold with the recovery of 53.33%. The optimum pH and temperature for the purified laccase was found to be 6.0 and 40^{o}C respectively. The enzyme activity was enhanced by the metal ions $Mn^{2+}$and Cu^{2+} whereas it was reduced by Fe^{2+}, Na^{2+} and Co^{2+}. These results suggest that *Hypsizygus ulmarius* could produce high yield of laccase. With its distinct characteristics laccase could be used for various applications.

KEYWORDS: **Laccase, *Hypsizygus ulmarius,* optimization, industrial enzyme**.

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INTRODUCTION

In the recent years, enzymes have gained great importance in industries; laccases are one among them which are widely present in the nature. These are a family of multicopper oxidases that require O_2 to oxidize organic compounds, particularly phenols and different non-phenolic substrates by one-electron transfer, resulting in the formation of reactive radicals, while reducing molecular oxygen to water[1]. One electron at a time is removed from the substrate, and molecular oxygen is used as the electron acceptor. The substrate loses a single electron and forms a free radical. The unstable radical undergoes further nonenzymatic reactions including hydration, disproportionation, and polymerization. The substrates of laccases may vary from diphenols and polyphenols to diamines, aromatic amines, benzenethiols, and substituted phenols[2].

The distribution of laccase is widespread among plants and fungi[3] and also in bacteria[4]. These enzymes are involved in various physiological functions; in plants, they seem to be involved in lignin synthesis[5], whereas in fungi, they are involved in lignin degradation, pigmentation, and pathogenesis[6]. Recently, the occurrence and properties of the fungal laccases have been reviewed comprehensively[7]. They have been isolated from Ascomyceteous, Deuteromycteous and Basidiomycetous fungi to which more than 60 fungal strains belong[8].

From a biotechnological point of view fungal laccases are receiving growing interest as potential industrial enzyme. Due to its low substrate specificity, laccase can be used in drug analysis, wine clarification, bioremediation[3], paper-pulp bleaching, and decolourization of synthetic dyes[9] and biosensors[10]. Recently laccase was even reported to show an inhibitory activity to HIV-1 reverse transcriptase[11]. The practical applications of laccase in biotechnology have resulted in the need for expanding the spectrum of microorganisms with laccase activities and isolating novel laccase with different physiochemical and catalytic properties[12].

In view of the broad biotechnological applications of laccases, there is a scientific need to identify different sources of laccases having diverse properties so that suitable laccases for various applications[13-16] could be identified. Keeping these points in view, the authors have initiated studies on the characteristics of laccase produced by indigenous lignolytic fungal strains. *Hypsizygus ulmarius* (elm oyster mushroom) is a high yielding mushroom for which commercial cultivation technology has been released and is gaining popularity. Previous reports suggests that the mushroom is rich in antioxidants and proved for its antidiabetic activity[17]. In this communication, the secretion of laccase from the mushroom *Hypsizygus ulmarius* has been reported here.

MATERIALS AND METHODS

Organism and culture conditions:

Hypsizygus ulmarius was obtained from the mushroom research centre, TNAU, Coimbatore, India. The culture was maintained on PDA and transferred to liquid medium[18]. The production medium was inoculated with a loop of culture and incubated at 30^{o}C.

Optimization of the culture conditions:

The enzyme activity was measured by altering the individual parameters such as incubation period (1-8 days), pH (3.0-8.0), temperature (20^{o}C-60^{o}C), inoculum size (1-5%), carbon (Glucose, Maltose, Ribose, Fructose and Xylose) and nitrogen sources ($NaNO_3$, NH_4NO_3, KNO_3, $(NH4)_2SO_4$, NH_4Cl) with different concentrations (1-5%) while the remaining parameters were unaltered[19].

Assay of laccase activity:

Laccase activity was assayed spectrophotometrically by measuring the oxidation of ABTS at 420 nm[20]. The assay

mixture in a total volume of 1 ml contained 0.1 ml enzyme extract and 1 mM ABTS in 100 mM citrate buffer (pH 3.4). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol ABTS per minute at 30^{o}C and the activities were expressed in units per liter.

Purification and characterization of Laccase

Crude extract was precipitated by 70% saturation with ammonium sulphate and then dialyzed against 100mM phosphate buffer (pH 7.2) for 24 hours at 30^{o}C. The filtrate was loaded onto a DEAE-Cellulose chromatographic column equilibrated with phosphate buffer, 100mM, pH 7.2 at 4^{o}C.The enzyme was eluted with a linear salt concentration gradient (NaCl, 0-0.4 M) in the same buffer and 3.0 ml fractions were collected at a flow rate of 20 ml per hour.

SDS-PAGE electrophoresis was carried out and molecular weight was determined. The protein content was estimated by the method of Lowry *et al.,* 1951[21]. The optimum pH 3.0-8.0 [The pH was adjusted using, the following buffers: 50 mM sodium citrate (pH 3.0-6.0) and 50 mM sodium phosphate (pH 7.0 & 8.0)], temperature (20-60^{o}C), and metal ions (Mn^{2+}, Cu^{2+}, Fe^{2+}, Na^{2+} and Co^{2+})on the activity of the enzyme was assayed. All experiments were conducted in triplicates and their mean values represented.

RESULTS

Enzyme production is greatly influenced by media components, especially carbon, nitrogen sources, and physical factors such as temperature, pH, incubation time, inoculum density. It is important to produce the enzyme in large scale in inexpensive manner. Hence the influence of various physico-chemical parameters such as incubation periods, inoculum size, temperature, pH, carbon, and nitrogen sources were studied on laccase production.

Laccase production time was standardized using composite medium containing different carbon and nitrogen sources. Standard time for production of laccase was found to be day 6 (Figure 1) as the maximum enzyme activity was obtained on 6th day. Hence 6th day was taken as the standard incubation for production of laccase in medium containing different carbon and nitrogen sources.

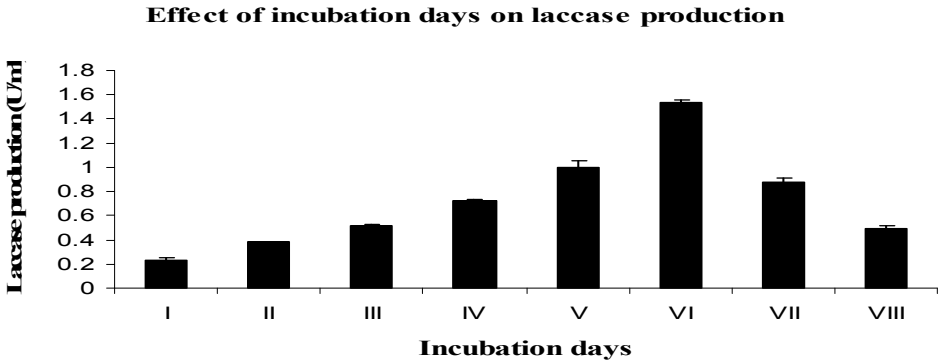

Figure 1

pH and temperature of the fermentation medium are critical factors which have insightful influence on the production of the enzyme. The effect of different pH (3.0-8.0) and temperature (20-60^{0}C) on the laccase production was studied. The maximum enzyme production was obtained in pH 5.0 (Figure 2) and temperature 30^{0}C (Figure 3) respectively.

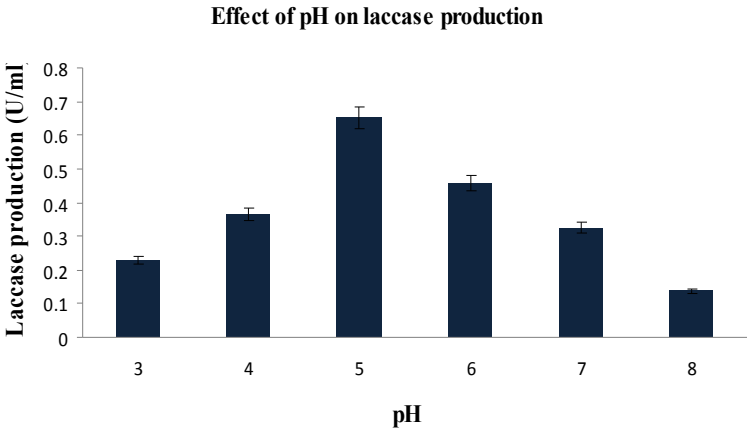

Figure 2

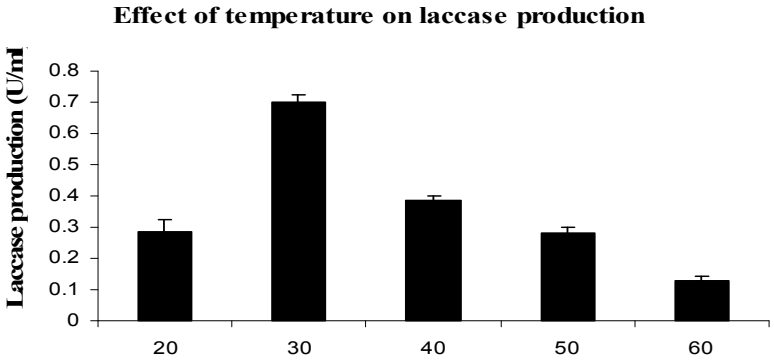

Figure 3

Temperature ^{O} C

The inoculum size is an important factor in enzyme production. The enzyme production was optimum when the medium was inoculated with 3.0 ml of inoculum (Figure 4).

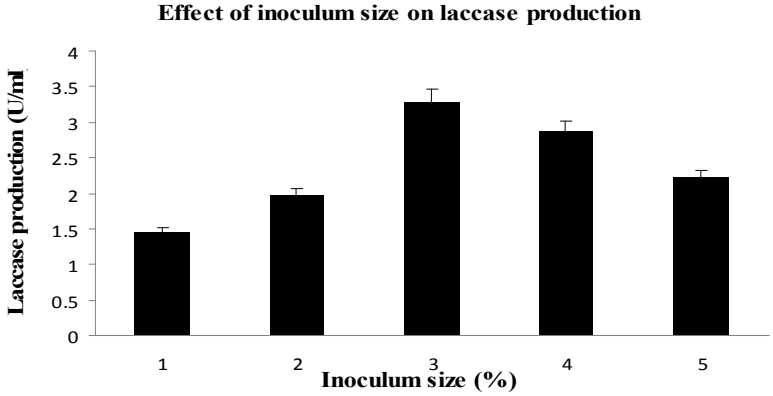

Figure 4

Laccase production and activity was measured by culturing the fungi in medium containing different carbon sources. Among different carbon sources (Glucose, Maltose, Ribose, Fructose and Xylose), Xylose produced the maximal enzyme activity (Figure 5) which was subjected to different concentration in which 3 % produced the higher activity (Figure 6).

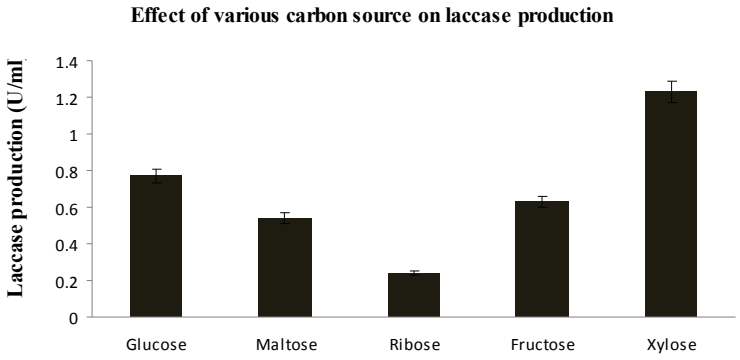

Figure 5

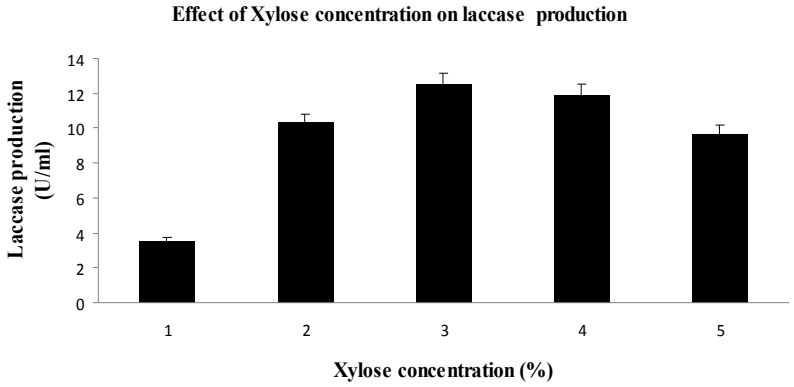

Figure 6

Likewise, among the different nitrogen sources ($NaNO_3$, NH_4NO_3, KNO_3, $(NH4)_2SO_4$, NH_4Cl), Potassium nitrate produced the maximal activity (Figure 7). The influence of various concentrations of ammonium nitrate was investigated. In that, 3% Potassium nitrate was found to be best in laccase production (Figure 8).

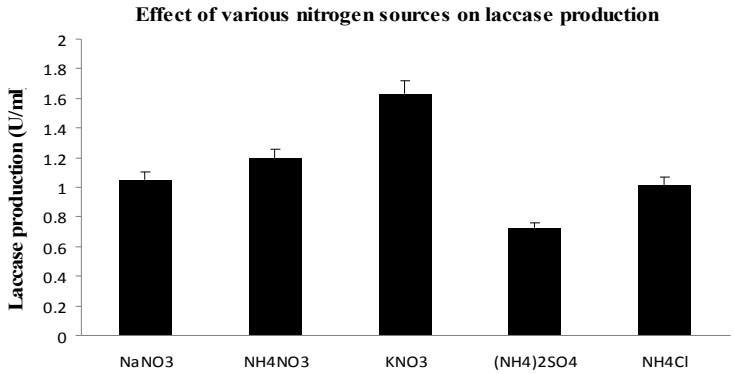

Figure 7

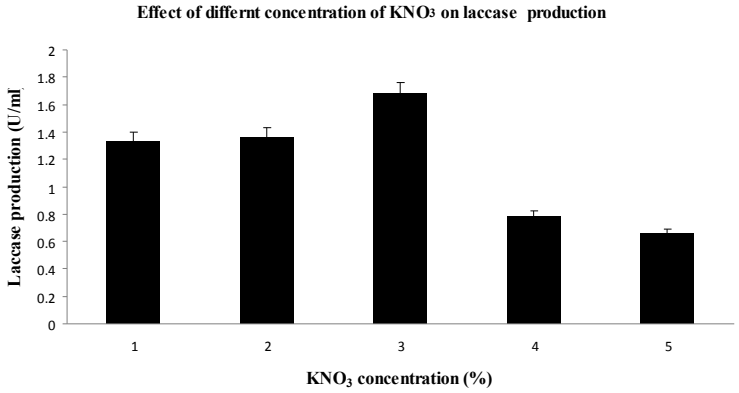

Figure 8

The extra cellular laccase from *Hypsizygus ulmarius* was purified to 5.11-fold with a yield of 53.33% (Table 1), using a series of purification steps that included ammonium sulphate precipitation, dialysis and DEAE cellulose column chromatography. The purified enzyme showed a single-protein band on SDS-PAGE with a molecular mass of 63 ± 1 kDa when compared to authentic standards.

Table 1.

Purification and recovery of laccase from Hypsizygus ulmarius

`Steps	Laccase production (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	130	31	4.19	1	100
70% Ammonium sulphate precipitation	114	13.13	8.68	2.07	87.69
Dialysis	95.34	2.76	34.50	3.97	73.33
DEAE Cellulose column chromatography	69.29	0.39	176.29	5.11	53.33

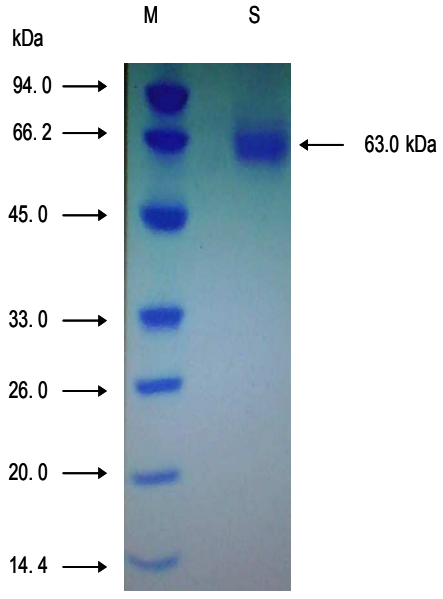

Figure 9. SDS PAGE

Lane 1 M- Marker; Lane 2 S- Laccase enzyme

The enzyme was characterized by carrying out the assay at different pH (3.0-8.0) and temperatures (20 - 60°C). The optimum pH and temperature of the purified enzyme was found to be 6.0 (Figure 10) and 40°C (Figure 11) respectively.

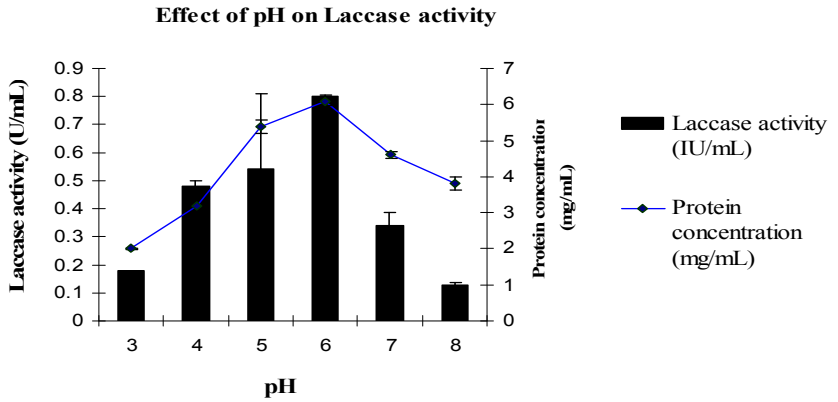

Figure 10

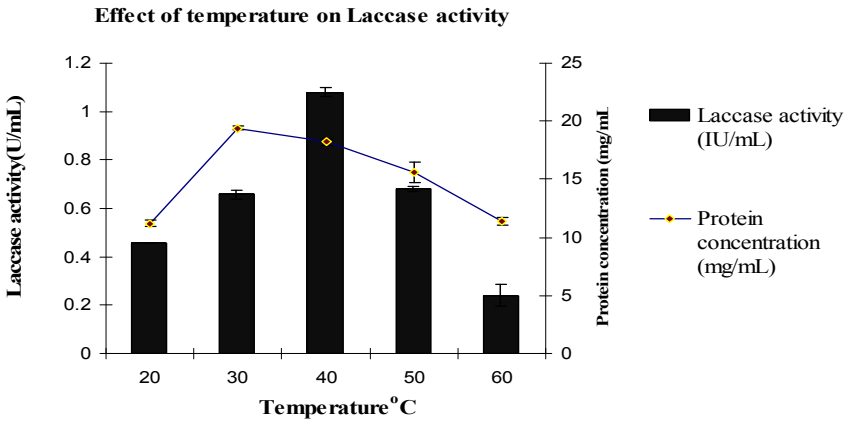

Figure 11

The effects of various ions on activity of the laccase of *Hypsizygus ulmarius* is shown in Figure. 12. Significant inactivation of the laccase of *Hypsizygus ulmarius* was observed with Fe^{2+} Na^{2+}, Co^{2+} but Cu^{2+} and Mn^{2+} enhanced the enzyme activity.

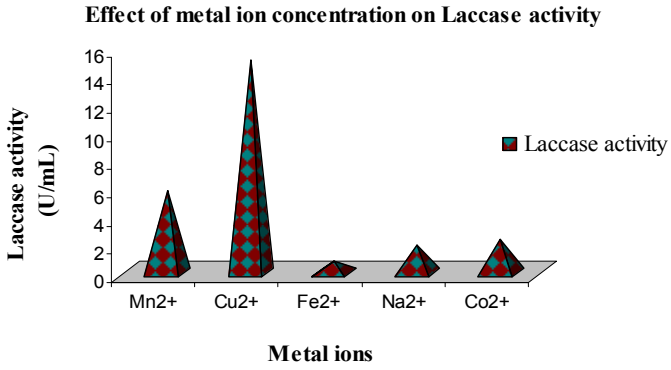

Figure 12

DISCUSSION

Laccase is a very unique enzyme capable of degrading multiple substrates. Hence it is no surprise that such an enzyme will be of considerable market value. However, as this enzyme is secreted in very low amounts by the organism a lot of work is being carried out in increasing the production output and optimization. The efficiency of laccase producing organisms can only be exploited with the aid of media designed with optimum concentrations of every component which influences the production. In this regard, the present study was aimed at optimization, purification and characterization of the enzyme laccase from *Hypsizygus ulmarius.*

The maximum enzyme activity was obtained on sixth day of incubation. This is in line with the findings of Wang *et al.,*[1] who reported that the enzyme was produced maximum on the seventh day of fermentation. When the incubation period was increased the production of laccase by *Hypsizygus ulmarius* was reduced.

pH and temperature are important factors that influence the production of the enzyme in the medium. Therefore the initial pH (3.0-8.0) and temperature (20-60°C) were studied for the optimum production of the enzyme. The optimum pH for the maximum production of the enzyme was found to be 5.0. However, when the pH was further increased the enzyme activity was found to be reduced. Our results were similar to the findings of Guo *et.al.,*[22] who has reported that initial pH for the optimization was 5.5. These findings are in agreement with previous reports as most fungal enzymes, especially laccases, have maximum activity when the initial pH of the nutrient medium ranges from 4 to 6[23-25].

Similarly, the optimum temperature for the maximal enzyme activity was 30°C. Further increase in the temperature resulted in the reduction of the enzyme activity. This is probably due to the fact that increasing the temperature could have inhibited the fungal growth and hence, low/decreased enzyme activities. The same trend has also been demonstrated by Zadrazil *et al.,*[26] when *Pleurotus* specie and *Dichomitus squalens* were cultivated at temperatures higher than 30°C. Similar results have been reported by Nakamura *et al*.,[27] whereby, maximum lignolytic activity from cultures of *B. adusta* were attained at 30°C, but above 37°C there was no activity observed.

The inoculum size is an important factor in enzyme production. The enzyme production was optimum when the medium was inoculated with 3.0 ml of inoculum. Increase in the inoculum size resulted in the reduction of the enzyme activity. This could be due to the fact that clumping of cells which could have reduced sugar and oxygen uptake rate and also enzyme release[28]. Our results were supported by Patrick *et.al.,*[29] who reported production of laccase from *Pleurotus sajorcaju.*

The carbon source in the medium plays an important role in ligninolytic enzyme production[30]. In our study, the maximal enzyme activity was obtained when Xylose was used as the carbon source at 3% concentration where as Ticklo *et.al.,*[31] obtained maximal activity of laccase when fructose was used as the carbon source.

Laccase production is triggered by nitrogen depletion[32] but some nitrogen strains do not affect the enzyme activity[33]. Some studies show that the elevated laccase activity was achieved by using low carbon-to-nitrogen ratio[34] while others show that it was achieved at high carbon-to-nitrogen ratio[35]. Among the different nitrogen sources we used, KNO_3 showed the maximum production of the enzyme followed by NH_4NO_3. But Sivakumar *et.al.,*[36] reported that Yeast extract supported the maximum laccase production where as ammonium nitrate favored high laccase production in many white rot fungi, namely, Basidiomycete PM123, *Lentinula edodes*24.

The purification of crude enzyme through DEAE cellulose column chromatography gave 5.11 folds increase in purity with 53% recovery of laccase from *Hypsizygus ulmarius.* The molecular weight of the laccase is predicted between the ranges of 50-97 kDa. The molecular mass of the purified

laccase was determined using SDS–PAGE. It was calculated to be 63 kDa by comparison of the single laccase protein band with the protein molecular weight standards. The native PAGE of the purified laccase resulted in the detection of a single band, indicating that the laccase was a monomeric enzyme. Our results were similar to the findings of Irshad *et al.,*[37] who reported that the molecular weight of laccase from *Schizophyllum commune.*

The purified laccase showed optimum activity at pH 6.0 identical to the optimum pH observed for *Coprinus friegii* laccase for ABTS substrate[38]. Several fungal laccases, which have been reported, previously, show maximum activity at pH close to 5.0[39, 40]. Likewise, the purified laccase exhibited its optimum activity at 40 °C which is identical to the findings of Murugesan *et.al.,*[41]. The loss of enzyme activity above 40 °C may be due to the release of copper ions from the enzyme laccase[5].

Investigation into the effect of metal ions revealed that Cu^{2+}, Mn^{2+} promoted the laccase activities, whereas Na^{2+}, Co^{2+} inhibited the laccase activities which were in agreement with other investigations[42]. Fe^{2+} Completely inhibited the laccase activity, showing the same effect as other findings[43].

CONCLUSION

In view of the results obtained, it can be concluded that the enzyme laccase was able to oxidize the phenolic substrate ABTS. The optimization of various cultural and nutritional parameters for the production of laccase by *H. ulmarius* showed that the enzyme production by this isolate is governed by parameters such as pH of the production medium and other nutrition parameters. The enzyme was purified by ammonium sulphate precipitation and characterized for its optimum pH and temperature. In future we are interested to study the effect of the enzyme against the cancer cell lines and also to test the ability of this enzyme for various industrial applications.

ACKNOWLEDGEMENT

We, the authors are thankful to our Chancellor, Advisor, Vice Chancellor and Registrar of Karpagam University for providing facilities and encouragement for this study.

REFERENCES

1. Wang C, Zhao M, Lu L, Wei X and Li T, Characterization of spore laccase from *Bacillus subtilis* WD23 and its use in dye decolorization, Afr J Biotechnol, 10 (11): 2186-92, (2011).
2. Sunil SM, Renuka PS, Pruthvi K, Swetha M, Malini S and Veena SM, Isolation, Purification, and Characterization of Fungal Laccase from *Pleurotus sp*, Enzyme Res, Article ID 248735: doi:10.4061/2011/248735: 1-7, (2011).
3. Mayer AM and Staples RC. Laccase: new functions for an old enzyme, Phytochemistry, 60: 551-65, (2002).
4. Claus H, Laccases and their occurrence in prokaryotes, Arch Microbiol, 179 (3): 145-50, (2003).
5. Palonen H, Saloheimo M, Viikari L and Kruus K, Purification, characterization and sequence analysis of a laccase from the Ascomycete *Mauginiella sp*, Enzyme Microb Tech, 33 (6): 854–62, (2003).
6. Thurston CF, The structure and function of fungal laccases, Microbiology, 140: 19-26, (1994).
7. Baldrian P, Fungal laccases - occurrence and properties, FEMS Microbiol Rev, 20: 1-28, (2005).
8. Shraddha, Shekher R, Sehgal S, Kamthania M and Kumar A, Laccase: Microbial Sources, Production, Purification, and Potential Biotechnological Applications, Enzyme Res, Article ID 217861: doi:10.4061/2011/217861: 1-11, (2011).

9. Baldrian P, Fungal laccases-occurrence and properties, FEMS Microbiol Rev, 30 (2): 215–42, (2005).
10. Vianello F, Ragusa S, Cambria MT and Rigo A, A high sensitivity amperometric biosensor using laccase as biorecognition element, Biosens Bioelectron, 21: 2155-60, (2011).
11. Wang H and Ng TB, Eryngin, a novel antifungal peptide from fruiting bodies of the edible mushroom *Pleurotus eryngii*, Peptides, 25(1):1–5, (2004).
12. Kiiskinen LL, Ratto M and Kruus K, Screening for novel laccase producing microbes, J Appl Microbiol, 97: 640-646, (2004).
13. Minussi RC, Pastore GM and Duran N, Potential applications of laccase in the food industry, Trends Food Sci Tech, 13: 205–216, (2002).
14. Couto RS and Herrera JLT, Industrial and biotechnological applications of laccases: A review, Biotech Adv, 24: 500–513, (2006).
15. Camarero S, Lbarra D, Marinez AT, Romero J, Gutierrez A and Del Rio JC, Paper pulp delignification using laccase and natural mediators, Enzyme Microb Technol, 40: 1264–1271, (2007).
16. Myasoedova NM, Chernykh AM, Psurtseva NV, Belova NV and Golovleva LA, New efficient producers of fungal laccases, Appl Biochem Microbiol, 44: 73–77, (2008).
17. Meera KS, Sudha G, Rajathi K and Manjusha GV, Antidiabetic effect of aqueous extract of Hypsizygus ulmarius on Streptozotocin- Nicotiinamide induced diabetic rats, Asian J Pharm Biol Res, 1(2): 151-57, (2011).
18. Slomczynski D, Nakas JP and Tanenbaum SW, Production and characterization of Laccase from *Botrytic cinerea* 61-34, Appl. Environ. Microbiol, 61: 907-912, (1995).
19. Quaratino D, Federicic F, Petruccioli M, Fenice M and D'Annibale A, Production, purification and partial characterization of a novel laccase from the white-rot fungus *Panus tigrinus* CBS 577.79, Antonie van Leeuwenhoek, 91(1): 57-69, (2007).
20. Liu Z, Zhang D, Hua Z, Li J, Du G and Chen J, Improvement of laccase production and its properties by low-energy ion implantation, Bioprocess Biosyst Eng, 33:639–646, (2010).
21. Lowry OH, Rosebrough NJ, Farrand AL and Randall RJ, Protein measurement with the folin phenol reagent, J Biol Chem, 193: 265–275, (1951).
22. Guo LQ, Lin SX, Zheng XB, Huang ZR and Lin JF, Production, purification and characterization of a thermostable laccase from a tropical white-rot fungus, World J Microbiol Biotechnol, 27: 731–735, (2011).
23. Galhaup C, Wagner H, Hinterstoisser B and Haltrich D, Increased production of laccase by the wood-degrading basidiomycete *Trametes pubescens*, Enzyme Microb Technol, 30: 529-536, (2002).
24. Jang MY, Ryu WR and Cho MH, Laccase production from repeated batch cultures using free mycelia of *Trametes* sp, Enzyme Microb. Technol, 30: 741-746, (2002).
25. Chen SC, Ma D, Ge W and Buswell JA, Induction of laccase activity in the edible straw mushroom *Volvariella volvacea*, FEMS Microbiol. Lett, 218: 143-148, (2003).
26. Zadrazil F, Gonser A and Lang E, Influence of incubation temperature on the secretion of extra cellular ligninolytic enzymes of *Pleurotus sp*. and *Dichomitus squalens* into soil, Proceedings of the conference on Enzymes in the environment. Activity, Ecol. Applications Granada, Spain. 526, (1999).
27. Nakamura Y, Mtui GYS, Sawada T and Kuwahara M, Lignin degrading enzyme production from *Bjerkandera adusta* immobilized on polyurethane foam, J. Biosci. Bioeng, 88: 41-47, (1999).
28. Kalisz HM, Microbial proteinases, Adv Biochem Eng Biotechnol, 36: 1-65, (1998).
29. Patrick F, Mtui G, Mshandete AM and Kivaisi A, Optimization of laccase and manganese peroxidase production in submerged culture of *Pleurotus sajor caju,* African J Biotechnol, 10(50): 10166-10177, (2011).

30. Desai SS and Nityananda C, Microbial Laccases and their applications: A review, Asian J Biotechnol, 2: 98-124, (2011).
31. D'Souza-Ticlo D, Verma AK, Mathew M and Raghukumar C, Effect of nutrient nitrogen on laccase production, its isoenzyme pattern and effluent decolorization by the fungus NIOCC #2a, isolated from mangrove wood, Indian. J.Mar.Sci, 35: 364-372, (2006).
32. Keyser P, Kirk TK and Zeikus JG, Ligninolytic enzyme system of *Phanerochaete chrysosporium*: synthesized in the absence of lignin in response to nitrogen starvation, J Bacteriol, 135 (3): 790–797, (1978).
33. Leatham GF and Kirk TK, Regulation of ligninolytic activity by nutrient nitrogen in white-rot basidiomycetes, FEMS Microbiol Lett, 16 (1): 65–67, (1983).
34. Monteiro MC and De Carvalho MEA, Pulp bleaching using laccase from *Trametes versacolor* under high temperature and alkaline conditions, Appl Biochem Biotechnol, 70–72: 983, (1998).
35. Buswell JA, Cai Y and Chang ST, Effect of nutrient nitrogen and manganese on manganese peroxidase and laccase production by Lentinula (Lentinus) edodes, FEMS Microbiol Lett, 128 (1): 81–88, (1995).
36. Sivakumar R, Rajendran R, Balakumar C and Tamilvendan M, Isolation, Screening and Optimization of Production Medium for Thermostable Laccase Production from *Ganoderma* sp, Int J Eng Sci Technol, 2(12): 7133-7141, (2010).
37. Irshad M, Asgher M, Sheikh MA and Nawaz H, Purification and characterization of laccase produced by *Schyzophylum commune* IBL-06 in solid state culture of banana stalks, BioResource, 6 (3): 2861-2873, (2011).
38. Heinzkill M, Bech L, Halker T, Schneider P and Anke T, Characterization of laccases and peroxidases from wood-rotting fungi (Family *Coprinaceae*), Appl Environ Microbiol, 64: 1601- 1606, (1998).
39. Sethuraman A, Akin DE and Eriksson KEL, Production of lignolytic enzymes and synthetic lignin mineralization by the bird's nest fungus *Cyathus stercoreus*, Appl Microbiol Biotechnol, 52: 689–697, (1999).
40. Dedeyan B, Klonowska A, Tagger S, Tron T, Iacazio G and Gil G, Biochemical and molecular characterization of a laccase from *Marasmius quercophilus*, Appl Environ Microbiol, 66: 925–929, (2000).
41. Murugesan K, Arulmani M, Nam IH, Kim YM, Chang YS and Kalaichelvan PT, Purification and characterization of laccase produced by a white rot fungus Pleurotus sajor-caju under submerged culture condition and its potential in decolorization of azo dyes, Appl Microbiol Biotechnol, 72: 939–946, (2006).
42. Liu Z, Zhang D, Hua Z, Li J, Du G and Chen J, A newly Paecilomyces sp. WSH-L07 for laccase production: Isolation, identification and production enhancement by complex inducement, J. Indust. Microbiol. Biotech, 36: 1315-1321, (2006).
43. Murugesan K, Kim YM, Jeon JR and Chang YS, Effect of metal ions on reactive dye decolorization by laccase from *Ganoderma lucidum*, J Hazard Mat, 168:523–529, (2009).