Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

European Journal of Experimental Biology, 2014, 4(4):196-201

ISSN: 2248 –9215
CODEN (USA): EJEBAU

Production of Laccase by *Alternaria alternata* and *Lasiodiplodia theobromae*

Ashwani Tapwal[1], Shiju Varghese[2], Uttam Kumar[3]* and Juhi Arora[4]

[1]*Forest Research Institute, Dehradun, Uttarakhand, India*
[2]*Doon (P.G.) College of Agriculture Science and Technology, Dehradun, Uttarakhand, India*
[3]*Alpine Institute of Management and Technology, Dehradun, Uttarakhand, India*
[4]*Banasthali University, Banasthali, Rajasthan, India*

ABSTRACT

Laccase is a copper-containing polyphenol oxidase that acts on a wide range of substrates. This enzyme is found in many plant species and is widely distributed in fungi including wood-rotting fungi where it is often associated with lignin peroxidase, manganese dependent peroxidase, or both. Because of its importance in bioremediation, fungal cultures were screened for laccase positive production by plate test method using the indicator compound guaiacol. The biotechnological application of laccase has been expanded by the introduction of laccase- mediator systems, which are able to oxidize non-phenolic compounds that are otherwise not attacked and are thus able to degrade lignin in kraft pulps. Both cultures were found to be laccase-positive with Alternaria alternata and Lasiodiplodia theobromae being the best potential cultures. Laccase activity was determined using ABTS as substrate. Higher level of laccase activity was observed by Alternaria alternata (3.221U/ml) than that of Lasiodiplodia theobromae (2.318 U/ml). Laccase was purified by Ammonium sulfate salt saturation, dialysis and gel filtration chromatography. The purified laccase was a monomer showed a molecular mass of 45±1kDa as estimated by SDS-PAGE and a 4.02-fold purification with a 2% yield.

Keywords: Laccase, ABTS, *Alternaria alternata*, *Lasiodiplodia theobromae*, Purification, Characterization

---

INTRODUCTION

Laccases (phenol oxidases; E.C. 1.10.3.2.), also known as multicopper blue oxidases, belong to the oxidoreductase group of enzymes. Fungi,belongingtophylum like ascomycetes,basidiomycetes anddeuteromycetesare known to produce laccases of ecological as wellas biotechnological importance,suchasbiodegradation and bioremediation [**1, 2**].During cultivation of such fungi for their optimum utilization at industrial scale, laccase production needed to be enhanced by modifying nutritional and physiological conditions.Besides nutritional supplements, inducers like organic solvents and metal ions also play importantroleinproductionoflaccases [**3, 4**]. Laccases oxidize mediator compoundslike phenol, aniline, 4-hydroxybenoic acid and 2, 2'-azino-bis-(3-ethyl benzothiazoline-6-sulphinic acid) (ABTS) which in turn degrade dyes and a wide variety of organic and inorganic substrates [**5**].20-50% glycosylation are involved in the polymerization reaction in laccases produced by plants. In comparison, the fungal laccases have 5-25% glycosylation and are involved in depolymerization reactions [**6**].The biotechnological application of laccase has been expanded by the introduction of laccase- mediator systems, which are able to oxidize non-phenolic

compounds that are otherwise not attacked and are thus able to degrade lignin in kraft pulps. Aromatic and phenolic compounds have been widely used to elicit enhanced laccase production by different organisms [**7, 8**].The nature of compound that induces laccase activity differs greatly with the species. Laccases can degrade several dye structures and transform toxic compounds into safer metabolites and may be useful to manage environmental pollution [**9**]. Laccases are also useful for the decomposition of azo dyes by oxidative methods [**10**]. The ability of laccase producing microorganisms or purified laccases to eliminate a wide range of pollutants is currently one of the most interesting subjects for researchers in environmental biotechnology [**6**]. Laccases can be used in detection of catecholamine neurotransmitters like dopamine and norepinephrine, [**11].** Some fungal laccases are also responsible for degradation offungal toxins like aflatoxin B1 [**12**] demonstrating their utilization in food industry.

In the present study,an attempt has been made to screen and select one of the cultures of *Alternaria alternata and Lasiodiplodia theobromae* obtained from National Type Culture Collection (NTCC) Forest Research Institute, Dehradun which has high laccase production capabilityin pre-optimized conditions. The extracellular fungal laccase in the culture medium was subjected to purification and characterization.

MATERIALS AND METHODS

Chemicals

All chemicals were of the AR grade, Guaiacol and ABTS were obtained from Himedia. Dialysis kit and Sephadex G-100, Protein Molecular weight marks (Bangalore Genei, Pvt, Ltd).

Fungal Strains and Inoculum Preparation

Pure culture of *Alternaria alternata and Lasiodiplodia theobromae* were obtained NTCC, Forest Research Institute-Dehradun, India. Both species were grown in Potato Dextrose Broth at pH 5 and incubated at 35°C for 6 days.

Plate ScreeningAssay

Selected fungal species were cultivated on media containing (3.0 peptone; 10.0 glucose; 0.6 KH_2PO_4; 0.001 $ZnSO_4$; 0.4 K_2HPO_4; 0.0005 $FeSO_4$; 0.05 $MnSO_4$; 0.5 $MgSO_4$; 20.0 Agar (pH-6) supplemented with 0.02% guaiacol. Laccase catalyzes the oxidative polymerization of guaiacol to form reddish brown zones in the medium [**13**].

Laccase Assay

Laccase activity was determined bytheoxidationofABTSmethod [**14**].Thenon-phenolicdye ABTS is oxidized by laccase to the more stable and preferred state of the cation radical. The concentration of the cation radical responsible for the intense blue-green color can be correlated to enzyme activity and is read at 420nm [**15**]. The assaymixturecontained0.5mMABTS,0.1M sodium acetate (pH 4.5), and a suitable amount of enzyme. Oxidation of ABTS was monitored by determining the increase in A420 (ε420, 3.6×10^4 $M^{-1} \cdot cm^{-1}$). The reaction mixture contained0.5mMsubstrate(ABTS),2.8mLof0.1Msodium acetate buffer of pH 4.5, and 100µL of culture supernatant and incubated for 5min. Absorbance was read at 420nm in a spectrophotometer against a suitable blank. One unit was defined as the amount of the laccase that oxidized 1µmolofABTSsubstratepermin.Proteinconcentrationwas determined by the dye-binding method of Bradford using BSA as standard [**16**].

Purification, fractional precipitation and dialysis of Enzyme

The Crude extract of selected fungi was centrifuged at 3000xg for 15 min at 4°C. The crude enzyme concentrate was placed in ice bath and crystals of ammonium sulphate were added to attain 60% saturation at 0°C. It was kept overnight at 4°C and centrifuged again at 3000xg for 30 min at 4°C. The pellet was discarded and more ammonium sulphate crystals were added to supernatant to attain 80% saturation at 0°C. It was kept overnight at 4°C and centrifuged again. This time supernatant was discarded and pellet was collected. The pellet was dissolved in a minimum quality of buffer and dialyzed against 50 mM phosphate buffer several times to remove ammonium sulphate. The laccase activity and total protein content of the dialyzed fraction were determined.

Gel filtration Chromatography

The dialyzed fraction was loaded on a Sephadex G-100 gel filtration column that was leveled with 50mM malonate buffer (pH 4.5). Small amount of sample was applied on every run, 100mM phosphate buffer (pH 6.0) having 0.15M NaCl used as elution buffer. After every single purification step, the total protein content and enzyme activity were determined to calculate specific activity and purification factor.

Molecular weight determination

To verify the purity and subunit molecular mass, the enzyme was run on sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE) following the method described by Laemmli, 1970 [**17**]. The purified enzyme was dissolved in a minimum amount of distilled water and subjected toSDS-PAGE on 10% separating and 5% stacking polyacrylamide gels at 25°C using low and medium molecular weight protein markers. The gels loaded with enzyme samples and molecular markers (Bangalore Genei) were run at 50 V till the dye front reached the end of separating gel. The protein bands were visualized by staining with Coomassie brilliant blue G-250 stain prepared according to Merril, 1990 **[18].**

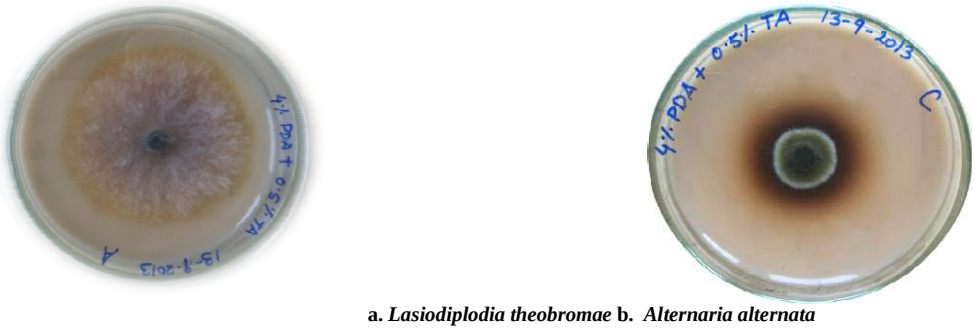
Characterization of Laccase

Optimum temperature and pH were determined by performing enzymatic assay at different temperature (25-75°C) and pH level (3-9), respectively. The pH level was adjusted using the following buffers: 0.1M citrate buffer (pH 3–5), 0.1M phosphate buffer (pH 6–8), and 0.1M carbonate buffer (pH 9). The stability of the purified laccase at various temperatures was investigated bypreincubating the purifiedlaccaseatdifferent temperatures between 4 and 70 °C for 1h, followed by determinationoftheresidualactivity.TheeffectofpHonthe laccase stability was determined by incubating the purified enzymeat4°CindifferentpHlevelsfor24handdetermining the residual activity.

RESULTS AND DISCUSSION

Confirmation of Laccase activity by selected fungi

In plate assays, conducted at optimum growth conditions, both *A. alternata andL. theobromae* oxidized guaiacol and developed reddish brown color around the colony indicating the production of laccase (Figure 1a, b). The fungus also produce laccase through oxidization of other three substrates likely ABTS, syringaldazine and DMP. Guaiacol, however, was found to exhibit sensitivity towards laccase activity.



a. *Lasiodiplodia theobromae* b. *Alternaria alternata*

Figure 1: Oxidative polymerization of guaiacol to form reddish brown zones in the medium

Laccase Activity

A. alternata and L. theobromae were cultivated on potato dextrose broth under optimum conditions (pH 5; temperature 35°C; 0.5mM ABTS). Sample was drawn from the culture for every 48 h to assay the production of laccase.Significant laccase activitywasrecorded for both fungi. The efficiency for production of ligninolytic enzymes was found to be inversely proportional to the temperature. In qualitative estimation conducted for the production of laccase varied with culture conditions. Maximum laccase production (3.221 U/ml) was recorded at 35C on 8th day of incubation by *A. alternata*. Laccase production was recorded minimum (2.318 U/ml) at 35C on 8th day of incubation by *L.theobromae*Figure 2.

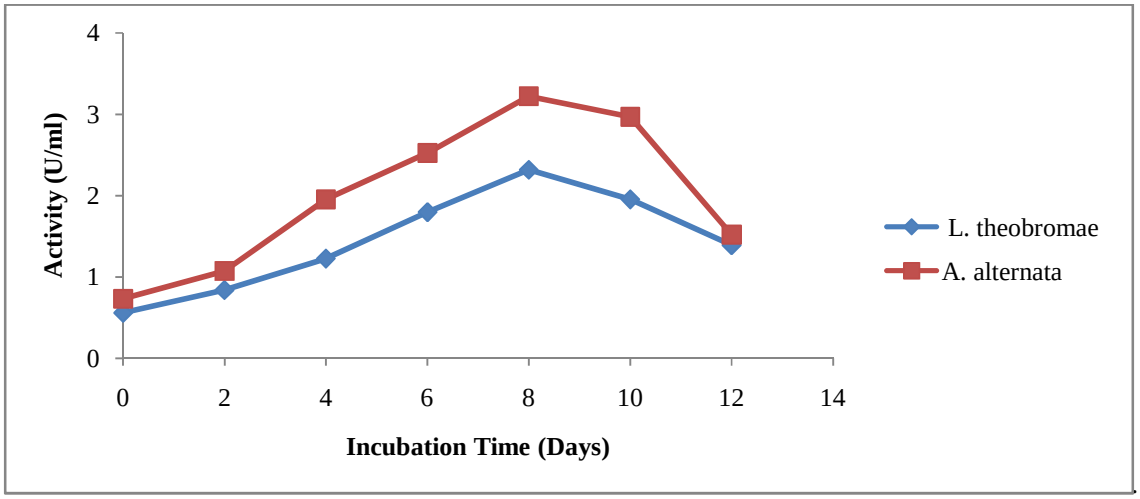


Figure 2: Graph displaying the activity of enzyme produced after particular days of incubation and at 35°C [activity (U/ml) v/s incubation time (days)]

Purification of Laccase

The total laccase activity of crude extract (500 ml) of selected strain was 206000 U with specific activity of 42 U/mg. During each purification step, specific activity and fold purification increased (Table 1). Laccase was treated with ammonium sulfate saturation of 80%. After dialysis and gel filtration the enzyme was purified to 4.02 fold with specific activity 169 U/mg.

Table 1: Summary of Purification of Laccase from *Alternaria alternata*

Purification Steps	Total Volume (mL)	Total Enzyme activity (U)	Total Protein content (mg)	Specific Activity (U/mg)	Purification (fold)	Yield (%)
Culture	500	206000	4850	42	1	100
$(NH_4)_2SO_4$	100	91550	975	93	2.2	44
Dialysis	50	43379	419	103	2.45	21
Sephadex G100	10	5439	32	169	4.02	2

SDS-PAGE

The purified laccase resolved on SDS-PAGE was a homogenous monomeric protein as indicated by a single band corresponding to (45±1kDa) relative to the standard molecular weight markers (Figure 3). A single band on SDS-PAGE showed that the enzyme is a single polypeptide protein.Molecular weights of most fungal laccase proteins fall between 43,000 and 110,000 Daltons [**19, 20**]. A majority of laccases from basidiomycete fungi were reported to have molecular weights in the range of 55,000 to 72,000 Daltons [**19, 21**].

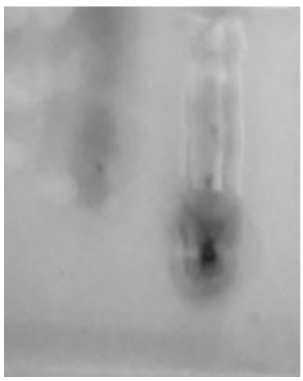


Figure 3:SDS-PAGE of purified laccase from *Alternaria alternata*

Effect of pH and temperature on purified laccase

The purified laccase was active in broad pH range of 3–5with optimum activity at pH 4.5 (Figure 4). The purified laccase has a broad temperature sensitive 35–70°C and the optimum temperature for the laccase was observed at 65°C (Figure 5). Temperature kinetics of the laccase suggests that the enzyme activity increases sharply from 60 to 65°C followed by a decline after 70°C. The laccase was stable in at 60°C for 8hrs. The enzyme at 75°C was stable up to 30min, and after 90min it retained 38% of the activity. The purified enzyme at room temperature was stable for 20 days and stable for 60 days when stored at4°C.

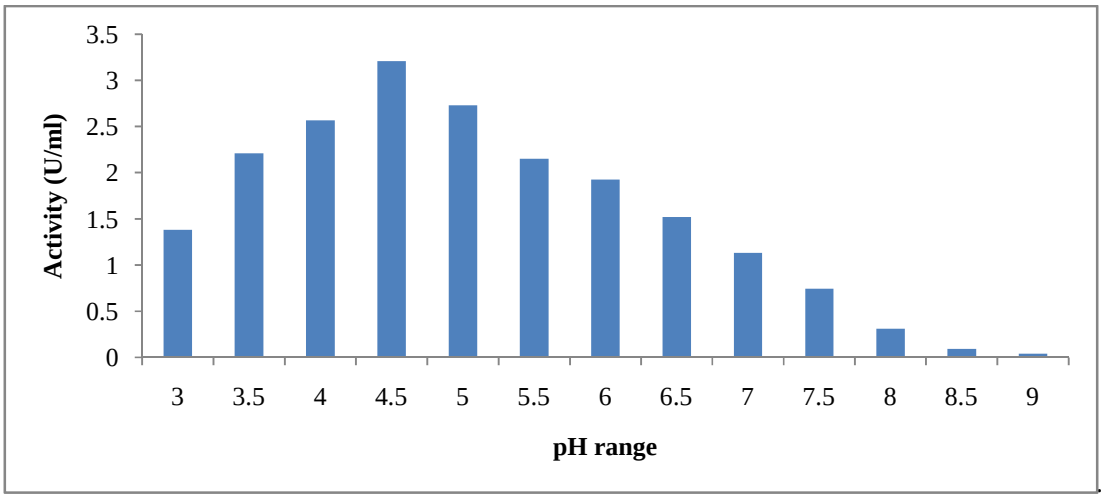


Figure 4:Optimum activity at different pH range with ABTS as substrate

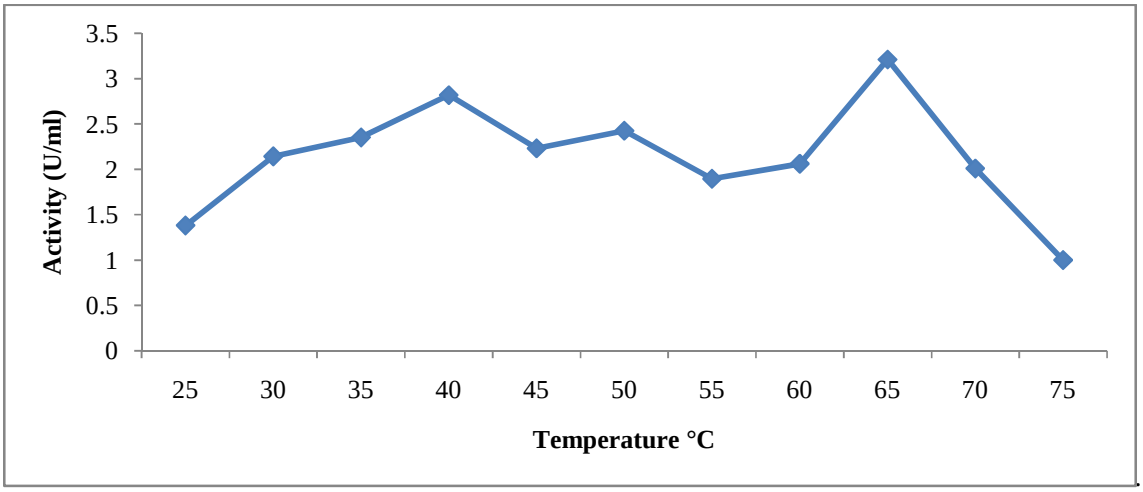


Figure 5:Optimum activity at different temperatures with ABTS as substrate

CONCLUSION

It can be concluded that increased production of laccase at optimal conditions, as recorded in the present study, is likely to be advantageous from ecological as well as biotechnological prospects. This study showed that high laccase producers can be discovered from environmental samples by very simple plate test screening methods. Guaiacol is a sensitive substrate for screening the laccase producing organisms. Laccase was purified from theculture filtrate of *A. alternata* during purification and subsequently characterized against pH and temperature. The molecular mass of the enzyme was determined to be 45±1kDa with a 4.02-fold and 2 % yield. The production level of laccase by *Lasiodiplodia theobromae* was quite low (2.318 U/ml), whereas by *Alternaria alternata* resulted adequate levels of laccase yields. The findings of the present study, based on the selection of laccase producing fungal strain,

followedbytheoptimizationfornutritionalandphysiological conditions, are likely to be useful in further upgrade of the process.

REFERENCES

[1]Desai SS, Nityanand C. *Asian Journal of Biotechnology*.**2011**; 3(2):98–124
[2]Shraddha, Shekher R, Sehgal S, Kamthania M, Kumar A. *Enzyme Research*. **2011**; 2011:11 pages.217861
[3]Piscitelli A, Giardina P, Lettera V, Pezzella C, Sannia G, Faraco V. *Current Genomics*. **2011**; 12(2):104–112.
[4]Brijwani K, Rigdon A, Vadlani PV. *Enzyme Research*, **2010**; vol.2010, Article ID 149748, 10pages.
[5]Asgher, M., Bhatti, H. N., Ashraf, M., and Legge, R. L. *Biodegradation*. **2008**; 19, 771-783.
[6]Baldrain P, *FEMS Microbiology Reviews*, **2006**; vol. 30, no. 2, pp. 215–242.
[7]Desouza CGM, Tychanowicz GK, De Souza DF, Peralta RM. *J. Basic Microbiol*. **2004**; 44: 129-136.
[8]Revankar MS, Lele SS. *Proc. Biochem*. **2006;** 41: 581-588.
[9]Gianfreda L, Xu F, Bollag JM. *Bioremed. J.* **1999;** 3:1-25.
[10] Michael MT, Georg MG, Astrid R. *Appl. Environ. Microbiol*. 2005; 71: 2600–2607.
[11] Couto SR and HerreraJLT. *Biotechnology Advances*, **2006**; vol. 24, no. 5, pp. 500–513.
[12] Alberts JF, GelderblomWCA, Botha A, and van Zyl WH. *International Journal of Food Microbiology*, **2009**; vol. 135, no. 1, pp. 47–52.
[13] Coll PM, Abalos JMF, Villanueva JR, Santamaria R, Perez P. *Appl. Environ. Microbiol*. **1993**; 59: 2607-2613.
[14] Bourbonnais R, Leech D, and Paice MG. *Biochimica et Biophysica Acta*, **1998**; vol. 1379, no. 3, pp. 381–390.
[15] Majcherczyk A, Johannes C, and H¨uttermann A. *Enzyme and Microbial Technology*, **1998**; vol.22, no. 5, pp. 335–341.
[16] Bradford MM. *Analytical Biochemistry*, **1976**; vol. 72, no. 1-2, pp. 248–254.
[17] Laemmli UK. *Nature*, **1970**; vol. 227, no. 5259, pp. 680–685.
[18] Merril, C.R. *Methods Enzymol*. **1990**; 182, 477-488.
[19] Thurston CF. *Microbiology*, **1994**; vol. 140, no. 1, pp. 19–26.
[20] Xiao YZ, Tu XM, Wang J et al. *Applied Microbiology and Biotechnology*, **2003**; vol.60, no.6, pp.700– 707.
[21] Petroski RJ, Peczynska-Czoch W, and Rosazza JP. *Applied and Environmental Microbiology*, **1980**; vol. 40, no. 6, pp. 1003–1006.