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RESEARCH ARTICLE

Optimization and purification of laccase from *Pleurotus* sp. and its dye decolorization ability

Periyasamy Ashokkumar
PG and Res. Dept. of Microbiology, Sengunthar Arts and Science College, Tiiruchengode-637205, Namakkal
ashokkumarps@yahoo.co.in; +919444897602

Abstract

A *Pleurotus* sp. showed positive for laccase, lignin peroxidase and manganese peroxidase production. The growth of the test strain and laccase production was high comparable to other lignin-degrading enzymes on 10^{th} day of incubation. pH optimum for laccase production was 5.5, where the enzyme retained more than 50% production in 4.0-6.0 at 50°C. Glucose and peptone were the most suitable carbon and nitrogen source for laccase production. Slomczynski *et al.* (1995) medium composition enhanced laccase production. Laccase was purified to 31 folds from culture filtrate with the yield of 51.51% using ammonium sulphate precipitation, ultrafiltration, Hitrap Q FF cartridge and Superdex 75. *Pleurotus* sp. secreted a single monomeric laccase showing a high specific activity of 7347 U/mg. The enzyme had a molecular mass of 58.32 kDa revealed by SDS-PAGE. Crude culture filtrate was effective in decolorization of various textile dyes.

Keywords: *Pleurotus* sp., laccase, glucose, peptone, ultrafiltration, Superdex 75.

Introduction

Lignin is a recalcitrant heteropolymer of phenylpropanoid units present in woody plant tissues that confers them rigidity and resistance to biological attack (Higuchi, 1990). Lignocellulose is the predominant component of woody plants and dead plant materials and the most abundant biomass on earth. Enzymes involved in lignin degradation are laccase, lignin peroxidase, manganese peroxidase, versatile peroxidase and H_2O_2 forming enzymes such as glyoxal oxidase and aryl alcohol oxidase which are produced by lignin-degrading white-rot and litter-decomposing fungi in different combinations (Hatakka, 2001).

Laccase is an enzyme secreted by most of the lignin degrading basidiomycetes (Kahraman and Gurdal, 2002) and it has been reported as an essential enzyme for lignin degradation in fungi without peroxidases (Eggert *et al*., 1997). This enzyme catalyzes the oxidation of a wide number of phenolic compounds and aromatic amines but its substrate range have been extended to non-phenolic compounds in the presence of low molecular mass compounds acting as mediators (Eggert *et al*., 1996). White rot fungi are believed to be the most effective lignin degrading microbes in nature. They produce different kinds of extracellular oxidoreductases including laccases (Leontievsky *et al*., 1997), peroxidases (Conesa *et al*., 2002) and oxidases producing H_2O_2 (Guillen *et al*., 1992). Among the extracellular enzymes produced by filamentous fungi, the ligninolytic system is of great significance in environmental remediation process.

Environmental remediation is a process by which the biological systems are used to degrade or neutralize pollutants, such as polycyclic aromatic hydrocarbons (PAHs), a major class of hazardous contaminants that pose a potential health risk because of their detrimental biological effects (Arun *et al*., 2008). In addition, these enzymes have potential applications in a large number of fields, including chemical, fuel, food, agricultural, paper, textile and cosmetic industrial sectors (Sette *et al*., 2008). The major industrial application of laccase are used for many industrial purposes such as paper processing, prevention of wine discoloration and detoxification of environmental pollutants, oxidation of dye, production of chemicals from lignin. Laccases can degrade several dye structures (Buddolla *et al*., 2008), transform toxic compounds into safer metabolites and may be useful to control environmental pollution (Gianfreda *et al*., 1999). The potential application of ligninolytic enzymes in biotechnology has stimulated the investigation of their production with the purpose of selecting promising enzyme producers and increasing their yield (Kalmis *et al*., 2008). The major objective of this study was to investigate the effects of various nutritional and physiological conditions for laccase production by *Pleurotus* sp. and its application in decolorization of various textile dyes.

Materials and methods

Sample collection: The white rot fungi, *Pleurotus* sp. was obtained from the culture collection of CAS in Botany, University of Madras and was maintained through

periodic transfer onto potato dextrose agar (PDA) plates, at 25ºC.

Screening for ligninolytic enzyme

Pleurotus sp. was screened for laccase production on plates containing the following composition (g/L): 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 5.0) supplemented with 0.02% guaiacol. *Pleurotus* sp. culture was inoculated and incubated at 30°C for 7 d. Laccase activity was visualized on plates containing 0.02% guaiacol since laccase catalyzes the oxidative polymerization of guaiacol to form reddish brown zones in the medium (Coll *et al*., 1993).

Laccase assay

Extracellular laccase activity of cell free filtrate was assayed spectrophotometrically on 6^{th},7^{th}, 8^{th}, 9^{th}, 10^{th} and 11^{th} day in mM of ABTS as substrates in 100 mM sodium acetate buffer (pH 5.5), 0.1 ml of the culture filtrate (assay for 1 mL of reaction mixture) and absorbance was measured at 436 nm (Mansur *et al*., 1997). 0.1 mL of distilled water, 0.9 mL of sodium acetate buffer was kept as blank.

Protein content

Protein content was determined by Bradford method (1976), using bovine serum albumin as standard.

Effect of different media on laccase production

Pleurotus sp. culture was cultivated on four different media for production of laccase. Four media used in the study were those of Olga *et al*. (1998), medium of Slomczynski *et al*. (1995), medium of Munoz *et al*. (1997) and medium of Coll *et al*. (1993). All these media were adjusted to pH 5.0. The effects of culture conditions on laccase production was assayed by growing *Pleurotus* sp. in broth (g/L): 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 5.5) supplemented with 0.02% guaiacol.

Effect of pH and temperature on laccase production

The effect of pH was studied by adjusting the pH of the laccase production medium to different pHs ranging 4-8 inoculated with the test strain. Similarly to study the effect of temperature, the test isolate was inoculated in the production medium at different temperatures ranging from 20–60^{o}C.

Effect of carbon and nitrogen source

Different carbon sources namely glucose, mannose, cellobiose and maltose were tested for laccase production by the test strain. Organic and inorganic nitrogen sources like ammonium nitrate, peptone, and urea were amended to the culture medium with the test strain for laccase production. The flasks were incubated in dark condition at 28°C for 10 d.

Partial purification of laccase

The culture filtrate from the optimized production medium was centrifuged (10,000 × g) and the supernatant was was used as crude enzyme for purification. All the purification steps were carried out at 4^{o}C unless otherwise stated. The crude enzyme was subjected to ammonium sulfate precipitation (80% saturation W/V) and the resulting precipitate was centrifuged, dissolved in a minimum volume of 100 mM tartrate buffer (pH 5.0) and dialyzed against 1000 volumes of the same buffer. The dialyzed enzyme was used for further purification.

Purification of laccase

Laccase from *Pleurotus* sp. was purified from basal medium. The 10 d old culture medium was separated from mycelia by filtration on Whatman paper, concentrated and dialyzed against 100 mM tartrate buffer (pH 5.5) by ultrafiltration (3 kDa cut-off membrane). Samples of 50 mL of this crude enzyme preparation were applied to a Hitrap Q FF cartridge (Amersham Biosciences) equilibrated with the same buffer at a flow rate of 1.5 mL min^{-1}. The retained proteins were eluted using NaCl gradient (0 to 1 M). Fractions with laccase activity were pooled, concentrated (3 kDa cut-off membrane), and samples of 2.4 mL were applied to a Superdex 75 (Pharmacia HR 10/30) column equilibrated with 10 mM sodium tartrate buffer (pH 5.5) containing 150 mM NaCl, at a flow rate of 0.5 mL min^{-1}. Fractions (3 mL) were collected and assayed for protein and enzyme activity as described above. Molecular weight of the purified enzyme was determined by SDS-PAGE (Laemmli, 1970) using Sigma high molecular-weight electrophoretic standards. The separated protein was detected by silver staining method described by Blum *et al.* (1987).

Application of crude laccase on dye decolorization

Decolorization of RBBR (Remazol Brilliant Blue R) was done by plate assay method. RBBR (100 mg/L) was incorporated in the agar medium that contained (g/L) peptone 5 g, glucose 20 g and agar 20 g. The fungal strain about 9 mm agar disc was inoculated on the plates and incubated at 30°C for 2 weeks and observed for clear zones around the colonies (Kizhekkedathu *et al*., 2005).

Decolorization *of textile dyes*

Hundred mL of basal medium containing (g/L), peptone 5 g, glucose 20 g and 4 different textile dyes (congo red, fast green, Orange G and rhodamine B) of concentration (100 mg/L) was prepared and sterilized. The fungal strain about 9 mm agar disc was inoculated on the basal medium and incubated at 30°C for 2 weeks. After 5 d incubation, aliquots of culture filtrate (0.5 mL) were drawn aseptically from each tube periodically, diluted with equal volume of acetate buffer (pH 5.0), centrifuged and the supernatant was used for measurement of absorbance at appropriate λ_{max} of the dye (622 nm for fast green, 475 nm for orange G, Congo red for 486 nm

and 547 nm for rhodamine B) using a UV-VIS spectrometer (Soma *et al*., 2004).

Results and discussion

Ligninolytic enzymes are one of the important groups of enzymes involved in bioremediation, which are produced by various white rot fungi in higher extent. In this study, a *Pleurotus* sp. obtained from the Centre for Advanced Studies in Botany, University of Madras, Chennai was screened for laccase, lignin peroxidase, manganese peroxidase production in the initial screening process. The test strain showed positive for all the above enzymes screened whereas, showed high laccase activity with very low activities of manganese-dependent peroxidise, lignin peroxidise and cellulase activity. Since the test strain had the ability to secrete laccase in higher amount, further studies were carried out focusing on optimization of laccase production.

The study of growth of the organism is essential for production of enzyme, because most of the extracellular enzymes are produced during log phase of the organism. Growth study was done with slight modification and the samples collected from day 6 were checked for enzyme activity. In the growth study, we found that minimum biomass production occurred from day 6 to 8 indicated that the test strain had growth in the lag phase. Enzyme production increased gradually on 9th day onwards and the maximum production was recorded on 10^{th} day (Fig.1). This study highly supported that *Pleurotus* sp. maintained log phase from day 8 to 12. This variation of log phase timing is based on the nutrient availability present in the medium and culture condition of the organism and also environmental parameters influencing the fungal growth and enzyme production (Sahay *et al*., 2008).

The optimal pH for laccase production was determined to be around pH 5.5 (Fig. 2), but the enzyme retained more than 50% production in the pH range 4.0-8.0. Chefetz *et al*. (1998) reported similar results in *C. Thermophilium* laccase which exhibited maximum activity at pH 6 to 8 though fungal laccases are generally active at low pH values (pH 3 to 5) (Nishizawa *et al*., 1995). This result showed that the present laccase was slightly alkalophilic in nature.

The optimum temperature for laccase production by the test strain was found to be 50°C (Fig. 3). To optimize the temperature for the better growth and enzyme production, 50°C was observed to be optimal for laccase production. The results are in accordance with the findings of Shanmugam *et al.* (2008) who reported thermostable laccase from *Pleurotus eous* and *Ganoderma* sp. with maximum activity at 60°C (Sivakumar *et al.,* 2010). Among different carbon sources evaluated for laccase production, glucose was found to be best carbon source followed by maltose (Fig. 4). The results fall in line with the findings of El Zayat (2008) who reported maximum laccase production with glucose and maltose supplemented medium. Galhaup *et al.* (2002) also reported glucose and cellobiose efficiently and rapidly utilized by *Trametes pubescens* with high laccase activity. Nitrogen sources are secondary energy sources for the organisms which play an important role in the growth of the organism and the enzyme production. Organic nitrogen sources namely peptone and yeast extract favoured laccase production (Fig.4). Shanmugam *et al.* (2008) reported peptone as the best nitrogen source for laccase production when compared with yeast extract and tryptone. Among different complex nitrogen sources used, urea stimulated higher biomass yield and laccase production while inorganic nitrogen sources stimulated fungi biomass yield and laccase production (Fig. 4) (Adejoye *et al.,* 2010).

High production of laccase was obtained with medium proposed by Slomczynski *et al*. (1995) followed by Munoz *et al.* and Coll *et al.* medium (Fig. 5). In this study, the medium containing copper at lower concentration induced laccase production. Mansur *et al*. (1998) reported that laccase production is induced by high medium nitrogen content detected in Basidiomycete I-62 (CECT 20197), *Pleurotus sajor-caju* (Soden and Dobson, 2001) and in *Trametes trogii* (Colao *et al*., 2003). Laccase production by fungi has previously been shown to depend markedly on the composition of the cultivation medium; for example carbon source, nitrogen content and phenolic inducer compounds have been reported to have significant effects on laccase production (Schlosser *et al*., 1997).

Protein with laccase activity was purified to homogeneity from the basal medium. Table 1 summarizes the results obtained from 10 d old culture. During the first chromatographic step, laccase activity was separated from ammonium sulphate precipitated protein. Laccase enzyme was further purified by Hitrap Q FF cartridge and Superdex 75. At the end of the purification process, laccase had been purified about 31 folds and the molecular mass was found to be 58.32 kDa determined by SDS-PAGE (Fig. 6). The molecular mass of laccases from white-rot basidiomycetes ranged from 55-65 kDa (Vares *et al*., 1992).

The RBBR plate inoculated with *Pleurotus* sp. showed decolorization of RBBR dye, suggesting the ability of ligninolytic enzymes produced by the test strain. Similar reports were made with RBBR and Poly R-478 by lignin-degrading fungi (Barbosa *et al*. 1996), and the production of ligninolytic enzymes is observed as a colourless halo zone around microbial growth (data not shown). With guaiacol, a positive reaction is indicated by the formation of a reddish-brown halo (Nishida *et al*., 1988), while with tannic and gallic acid, the positive reaction is a dark-brown coloured zone (Harkin and Obst, 1973). Decolorization of several textile dyes was achieved by incubating the culture supernatant containing laccase enzyme from *Pleurotus* sp. was investigated.

Fig. 1. Effect of incubation period on laccase production by *Pleurotus* sp.

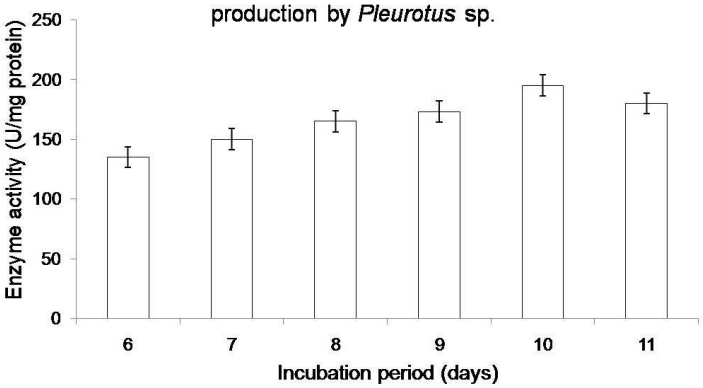


Fig. 5. Effect of media on laccase production by *Pleurotus* sp.

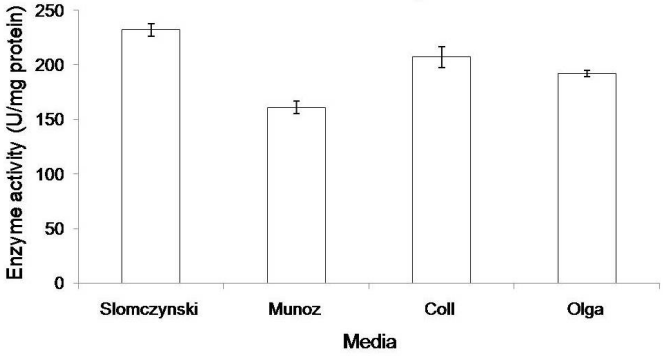
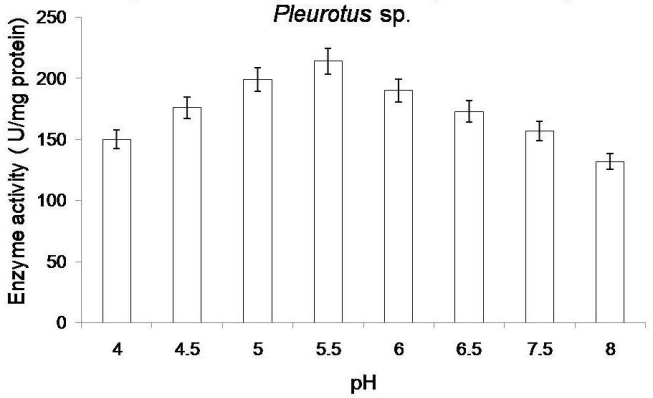


Fig. 2. Effect of pH on laccase production by *Pleurotus* sp.



Congo red showed 93% decolorization, followed by fast green (87%), Orange G (81%) and rhodamine B (71%) (Fig. 7). Similar observations were made in *Pleurotus ostreatus* which recorded various percentage of decolorization of industrial dyes (Soma *et al*., 2004). This study was highly supported by Eggert *et al*. (1996) who showed the decolorization ability of laccase in *Pycnoporus cinnabarinus*, *Phlebia tremmellosa* (Robinson *et al*., 2001) and *Pleurotus sojarcaju* (Chagas *et al*., 2001). Decolorization of textile dye industrial effluents by white-rot fungus producing laccase as the major lignin-degrading enzyme as seen in this study was reported in white-rot fungus, *Clitocybula dusenii* by Wesenberg *et al*. (2002).

Fig. 3. Effect of temperature on laccase production by *Pleurotus* sp.

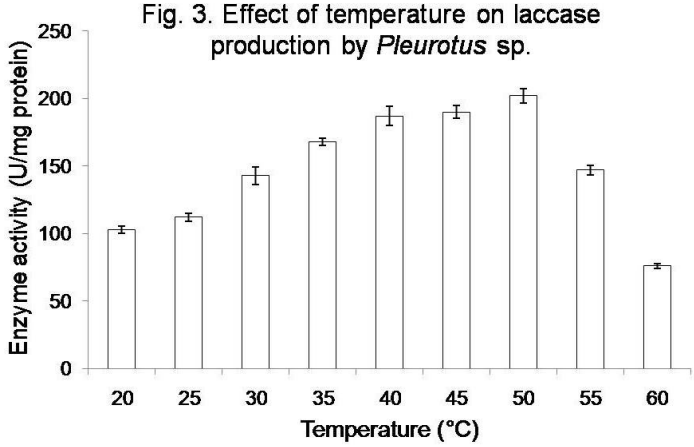


Fig. 4. Effect of carbon and nitrogen source on laccase production by *Pleurotus* sp.

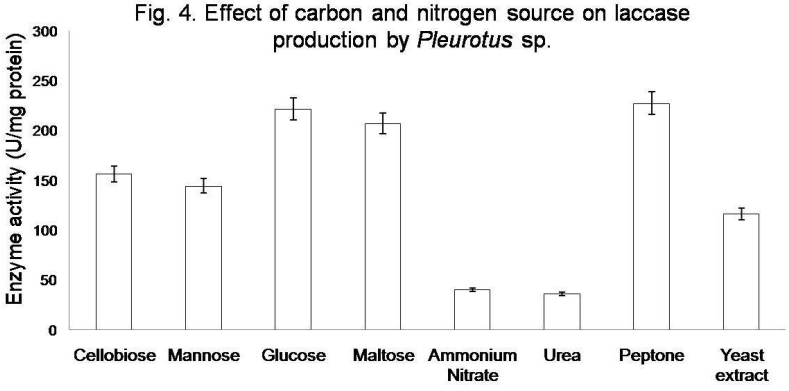
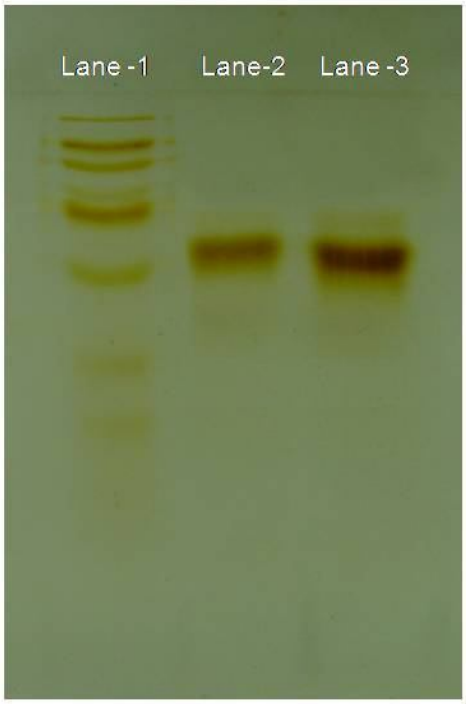


Fig. 6. SDS-PAGE of purified laccase.

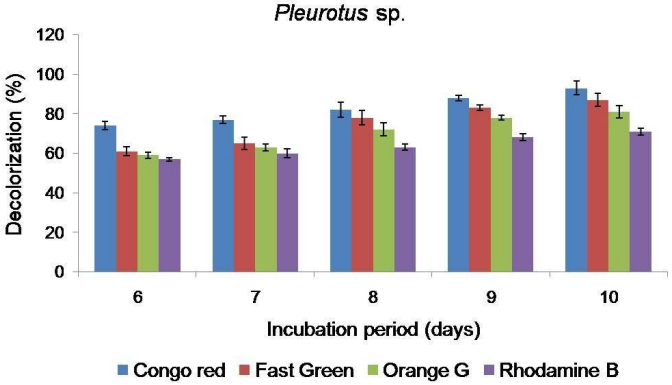


Lane -1: High molecular weight markers
Lane -2: 5 µg of purified protein
Lane-3: 10 µg of purified protein

Table 1. Purification summary of laccase from *Pleurotus* sp.

Source	Protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)	Purification fold
Culture filtrate	662	156894	237	100	1
Ammonium sulphate precipitation	243	123161.79	506.83	78.5	2.13
Hitrap Q FF	25	102451.78	4098	65.3	17.29
Superdex 75	11	80817	7347	51.51	31

Fig. 7. Decolorization of textile dyes by laccase from *Pleurotus* sp.



Conclusion

Pleurotus sp. showed its ability to secrete laccase comparable to other lignin-degrading enzymes on 10th day of incubation. pH optimum for laccase production was 5.5, where the enzyme retained more than 50% production in 4.0-6.0 at 50°C. Glucose and peptone were the most suitable carbon and nitrogen source for laccase production. Slomczynski *et al.* (1995) medium composition enhanced laccase production. Laccase was purified to 31 folds from culture filtrate with the yield of 51.51% using ammonium sulphate precipitation, ultrafiltration, Hitrap Q FF cartridge and Superdex 75. *Pleurotus* sp. secreted a single monomeric laccase showing a high specific activity of 7347 U/mg. The enzyme had a molecular mass of 58.32 kDa revealed by SDS-PAGE. Crude culture filtrate was effective in decolorization of various textile dyes. To conclude, the enzyme from the test strain may be used in dye decolorization of textile effluents after careful investigations in future.

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