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Isolation, Culture Optimization and Physico-chemical Characterization of Laccase Enzyme from *Pleurotus fossulatus*

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Abstract: *Pleurotus fossulatus* (Cooke) Sace is member of oyster mushroom can produced extracellular laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) in submerged fermentation. To analyze the optimum production for laccase *P. fossulatus* was cultured both in stationary and shaking condition in different media. Partial purification of laccase was done after 0-80% ammonium sulphate precipitation, followed by DEAE (Diethylaminoethyl) Sephadex (A-50) anion exchange chromatography. Potato-sucrose peptone (PSP) medium and Potato-dextrose (PD) medium showed highest laccase production in shaking and stationary conditions, respectively. Though the time required for optimum laccase production in stationary condition was much more than the shaking condition but the amount of laccase was about 2.75t greater in former condition. The laccase produced in stationary condition was more stable than the enzyme produced in shaking condition. The partially purified enzyme showed highest affinity towards o-dianisidine than guaiacol and ABTS (2,2'- Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) as evidenced by their K_m. The physico-chemical properties of the laccase suggested the significance of this enzyme in industrial applications.

Key words: Laccase, *Pleurotus fossulatus*, Submerged fermentation

INTRODUCTION

Oyster mushroom (*Pleurotus* spp.) occupies second position after the button mushroom (*Agaricus bisporus*), in quantitive terms produced around the world (Sanchez, 2010). More than twenty species of *Pleurotus* are reported from different parts of the world within which a few are commercially cultivated. The agro-climatic condition of India is very much conducive to the production of different species of this edible group of fungi. The members of oyster mushroom not only produce the protein rich nutrients and a number of pharmaceuticals but also some biotechnologically important enzymes within which laccase is most important.

Pleurotus fossulatus (Cooke) Sace., is a member of oyster mushroom which appears in plenty during April-May on dead, decaying basal parts and roots of *Ferula* sp. in some parts of India, Pakistan and Afghanistan (Kaul, 1999). This mushroom is also efficiently cultivated on rice straw (Das *et al*., 2010).

Laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) is a copper containing enzyme though found in plants, bacteria and some lower group of animals (mainly insects) but the primary source of this enzyme is fungi (Das *et al*., 2011a). The enzyme is very much abundant in almost all white rot fungi including *Pleurotus* spp. (Youn *et al*., 1995; Desai and Nityanand, 2011). The number of isoenzymes of laccase not only varies from species to species but also in same species depending upon cultural conditions (Giardina *et al*., 1996). Das *et al*. (2011b) recently showed that one of this laccase isozymes is ubiquitous to all *Pleurotus* species and might be utilized as marker character for strain differentiation, assessment and improvement of fruiting efficiency of oyster mushroom. Different biological role of the enzyme is reported in various organisms viz. lignin degradation (Thurston, 1994), rapid cell growth (Das *et al*., 1997), formation of primordia (De Vries *et al*., 1986), detoxification of pollutants (Eggert *et al*., 1997), Pathogenesis (Galhaup *et al*., 2002) etc. The enzyme has very much applications in paper and pulp, textile, food, pharmaceuticals industry and also utilized for various biotechnological purposes (Couto and Toca-Herrera, 2006; Gilaki, 2010). Ray *et al*. (2012) recently conjugated laccase with immunoglobulin which might be utilized very efficiently in immunoblots and immunoassay experiments than other commonly used methods.

In the present investigation we have isolated laccase from the newly cultivated species *Pleurotus fossulatus* (Das *et al*., 2010; Das *et al*., 2011a), optimized the production of laccase and studied the various physico-chemical properties of semipurified enzyme.

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MATERIALS AND METHODS

Mushroom strain: *Pleurotus fossulats* Cooke (MTCC 1800) was collected from Microbial Type Culture Collections, Institute of Microbial Technology, Chandigarh, India and was maintained on potato-dextrose agar (pH 7.0) containing 20% potato extract with 2% dextrose and 2% agar as reported earlier (Das *et al*., 1997).

Inoculum source and Liquid submerged fermentation (SMF): An inoculum was taken from the periphery of colonies growing on PDA for 7 days. The production of laccase was studied in liquid medium containing potato extract(20%) with 2% carbon source (Dextrose/Fructose /Sucrose) and or one 0.5% nitrogen source (Peptone/Yeast extract/Malt extract) normally at pH-7.0 unless otherwise mentioned in shaking/stationary condition at 25±2°C. The fungus was cultured for 30 days in stationary condition and aliquots of culture filtrate was collected in 5 day interval from starting day. The volume of the culture medium was adjusted to its original volume with fresh medium after each collection. For shaking cultures (100 cycles min^{-1}) aliquots were taken for one day interval from 2nd to seventh day and the volume of the culture medium was adjusted to its original volume with fresh medium after each collection. Extracellular culture filtrates were assayed for enzyme/protein activity after mycelium was removed by filtration.

Laccase activity assay: Laccase activity was performed spectrophotometrically as described by Das *et al*. (1997) with guaiacol , o-dianisidine or ABTS (Bose *et al*., 2007) as the substrate. The reaction mixture generally contained 0.1 mL 1 M acetate buffer pH 7.0, 0.2 mL Culture filtrate 0.1 mL 3 mM 0-dianisidine/0.5 mL 20 mM guaiacol (in 20% acetone) or 0.1 mL 1M ABTS. The volume was adjusted to 1 mL. The absorbances were taken in 460 nm for o-dianisidine (ε = 11300 M^{-1} cm^{-1}), 470 nm for guaiacol (ε = 6740 M^{-1} cm^{-1}) and 420 nm for ABTS ($\varepsilon = 3.6\times10^4$ M^{-1} cm^{-1}). Enzyme activity was expressed in International Unit.

Protein estimation: Protein concentration was determined according to the method of Lowry using bovine serum albumin as standard.

Activity staining: Activity staining of the partially purified enzyme after native Polyacrylamide Gel Electrophoresis (PAGE) was done with solutions of o-diamisidine, in acetate buffer, pH 5.0 as reported earlier (Das *et al*., 2011a).

Partial purification of laccase: The laccase activity present in the culture filtrate was partially purified after 0-80% ammonium sulphate precipitation, followed by DEAE Sephadex (A-50) anion exchange chromatography eluted with 0.6 M NaCl (Das *et al*., 1997).

Physico-chemical studies of laccase enzyme: The physico-chemical studies of laccase enzyme were done using guaiacol as substrate unless otherwise mentioned.

Determination of pH optima: The pH optima of laccase was determined in the pH range of 3.0 to 9.0 with an intervals of 1.0 min. Twenty units of laccase was incubated with substrate at 30°C in different pH conditions. The buffer systems used were: 0.1 M Na-citrate buffer (pH 3.0-4.0), 0.1 M Na-acetate buffer (pH 4.0-5.0) , 0.1 M Na-phosphate buffer (pH 6.0-8.0) and 0.1 M Tris buffer (pH 8.0-9.0).

Determination of pH stability: pH stability was estimated by incubating 20 U of enzyme at different pH values (3.0-9.0). The incubation was carried out at 30°C up to 5 h. The buffer systems used were the same as mentioned during determination of pH optima. The highest enzyme activity obtained was taken to be 100% and other values were expressed as percentage relative to the highest value.

Determination of optimum temperature: For determination of optimum temperature, assay mixture were incubated at different temperature in the range of 20-70°C in a temperature regulated spectrophotometer (Systronics). Prior to enzyme-substrate reaction, substrate and buffer were also incubated separately so that at the time of reactions all the components could reach the specific temperature. Twenty units enzyme was used to start the reaction.

Determination of temperature stability: Temperature stability of laccase was studied by incubating 20 U of enzyme at different temperatures in the range of 40-70°C for upto 5 h. The samples were taken out at different time intervals and assayed at 30°C with substrate.

Study of some effector molecules: Effects of some metal ions and effector molecules were determined by co-incubating the respective compounds at 100 mM concentration with the substrate and enzyme (20 U) solution followed by measurement of laccase activity.

Study of substrate specificity: Substrate specificities of culture filtrate enzymes were tested by using different substrates (0.1 mM) of laccase in 0.1 M acetate buffer,

pH 5.0 and 20 U of enzymes, the enzyme activities were measured either spectrophotometrically. The absorbance changes were measured at the respective wavelengths (as indicated in text) at 30°C .

Solvent stability: Stability of laccase in organic solvents was determined by incubating 20 U enzyme/mL in 1:1 (v/v) buffer /organic solvents upto 2 h . For enzyme assay in biphasic condition of buffer/chloroform aliquots were drawn from the upper aqueous phase.

Dye decolorization studies: The dyes tested for the in vitro studies were phenol red, congo red Eosin yellow and malachite green. The reaction mixture for dye decolorization consisted of equal volume of an aqueous solution of dye and 20 U crude laccase in acetate buffer (pH-5.0). The residual dye conc. was measured spectrophotometrically at 475 nm for phenol red, 497 nm for congo red, 516 nm for Eosin yellow and 618 nm for malachite green. A control set containing the same amount of heat killed laccase was performed simultaneously (Stoilova *et al*., 2010).

Statistical analysis: Two-way Analysis of Variance (ANOVA) was done using GraphPad Prism ver. 5.01 (2007) software.

RESULTS

Production of laccase in liquid medium in Shaking condition

Carbon sources: Laccase production was studied in *P. fossulatus* using three different carbon sources dextrose, sucrose and fructose. It was found that dextrose is the best substrate followed by fructose. Sucrose showed the least effect on laccase production. The optimum laccase production was found in fourth day in shaking condition using all the sugars (Fig. 1).

Nitrogen sources: Different nitrogen sources e.g. malt extract, peptone and yeast extract were given along with different carbon sources as media for *P. fossulatus*. Table 1 and 2 showed that laccase activity and protein content varied significantly high with each other as well as in different media. The highest laccase activity (70.67 U mL^{-1}) was observed in PDM medium followed by PSP medium and lowest was recorded from PFY (2.52 U mL^{-1}). Again, PFP and PDY media showed the maximum (104.44 mg mL^{-1}) and the minimum (4.0 mg mL^{-1}) protein content, respectively. Nevertheless, the calculated specific activity values gave a completely different picture, where PSP medium showed the highest (5.39 U mg^{-1} protein) and PFY showed the lowest (0.31 U mg^{-1} protein) value.

Table 1: Production of laccase in *P. fossulatus* in different media in shaking condition

Media	Laccase activity (U mL^{-1})	Protein (mg mL^{-1})	Specific activity (U mg^{-1} protein)
PDP	56.35±2.33	53.53±2.31	1.05
PDY	11.62±1.62	4.00±0.63	2.91
PDM	70.67±2.87	31.43±1.50	2.25
PFP	67.57±1.92	104.44±3.32	0.65
PFY	2.52±0.38	8.01±1.10	0.31
PFM	53.34±1.71	96.49±3.28	0.55
PSP	68.88±2.03	12.78±0.70	5.39
PSY	26.59±1.49	8.04±1.20	3.31
PSM	11.54±0.83	6.00±1.02	1.92

The enzyme activity (Mean±SD) was measured using guaiacol as substrate. Fourth day's culture filtrate was used in all experiments

Table 2: Two-way ANOVA table of activity (laccase and protein) and Media

Source of variation	Df	SS	MS	F
Interaction	8	20400.0	2550	717.1***
Activity	1	547.1	547.1	153.8***
Media	8	73950.0	9244	2599***
Residual	72	256.1	3.556	

***: Highly significant at $p<0.001$

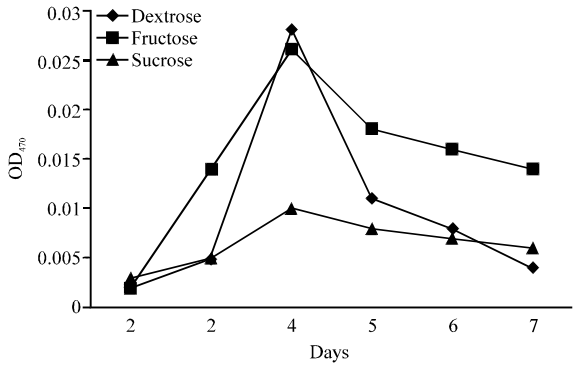

Fig. 1: Effect of different carbon sources on laccase production in *P. fossulatus* in shaking condition at 25±2°C. The enzyme activity was measured using 0.1 mL culture filtrate using 10 mM guaiacol as substrate in 0.1M acetate buffer (pH 5.0)

Effect of pH: The optimum laccase production was found in pH 6.0 in PSP and PSY media (Fig. 2). In higher pH the laccase production sharply decreased in both media.

Comparison of laccase production in Different media in stationary conditions: The laccase production was studied in PD and PSP media in stationary condition. Though PSP medium showed highest laccase production in shaking condition but in stationary condition laccase production was less in PSP medium than PD medium. The optimum day for laccase production in stationary condition was 15 day (Fig. 3). PD medium showed about

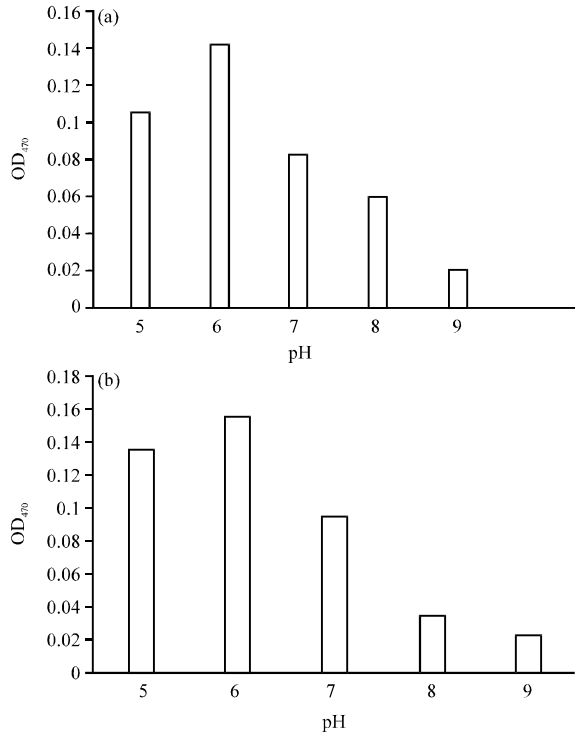

Fig. 2: Effect of pH on laccase production in *P. fossulatus* in shaking condition at 25±2°C a) In PSP Medium (pH 5.0-9.0) b) In PSY Medium (pH 5.0-9.0). The enzyme activity was measured using 10 mM guaiacol as substrate in 0.1 M acetate buffer (pH- 5.0). 0.1 mL fourth day's culture filtrate was used as source of enzyme

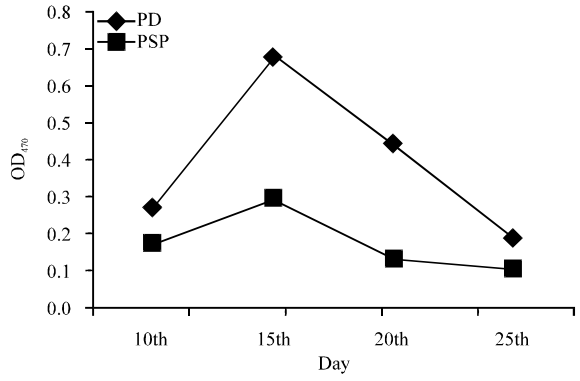

Fig. 3: Optimum day for laccase production in PD and PSP medium in stationary condition in *P. fossulatus* at 25±2°C. OD value of 1 mL culture filtrate was shown in Fig. 3. The enzyme activity was measured using 10 mM guaiacol as substrate in 0.1M acetate buffer (pH 5.0)

2.75 times more laccase production than PSP medium. The optimum laccase production was found in 15 days in both

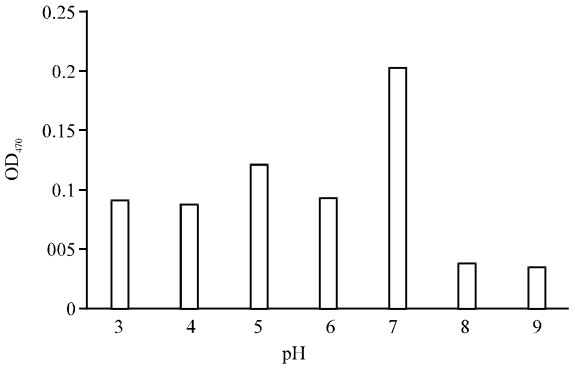

Fig. 4: pH optima of laccase of *P. fossulatus*. 20U of laccase was incubated with substrate at 30°C in different pH conditions. Enzyme activity was determined using 10 mM guaiacol as substrate

cases. After optimum laccase production, a sharp decrease in laccase activity was found in PD medium. The 15 day's PD culture filtrate after partial purification was taken as the extracellular enzyme source for further studies unless otherwise mentioned.

Physico-chemical properties of laccase from partially purified culture filtrate from PD medium in stationary condition

Stability: Though optimum laccase production required much time (about 15 days) in stationary submerged fermenting condition than the shaking condition (4 days) but it was found that the laccase produced through stationary condition is much stable than the later one (data not shown).

Substrate specificity: A number of phenolic and non-phenolic aromatic compounds were reported as laccase substrates. In the present experimental conditions three substrates viz., guaiacol, ABTS and o-dianisidine were tested for laccase activities. It was found that *P. fossulatus* culture filtrate showed maximum laccase activity against *o*-dianisidine followed by ABTS and guaiacol.

Kinetic properties: Three different substrates viz. guaiacol, ABTS and o-dianisidine were tested for laccase activities. The K_m of laccase against guaiacol (0.083 mM) was about two times than *o*-dianisidine (0.041 mM) whereas for ABTS, it was much more (0.454 mM) (Table 3). The result prove the specificity of this enzyme towards o-dianisidine.

pH optima: The optimum pH of the laccase reaction is 7.0 with guaiacol as substrate (Fig. 4).

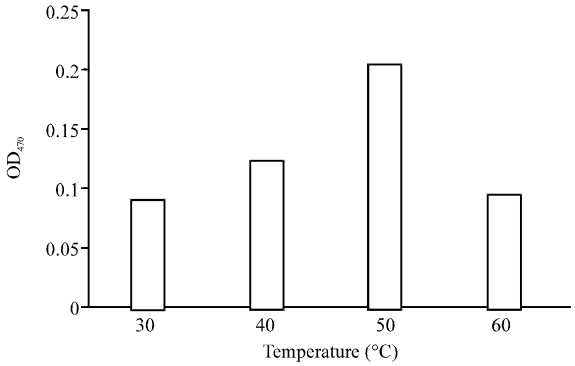

Fig. 5: Temperature optima of laccase. 20U of laccase was incubated with substrate at different temperature. Enzyme activity was determined using 10 mM guaiacol as substrate in 0.1 M acetate buffer (pH 5.0)

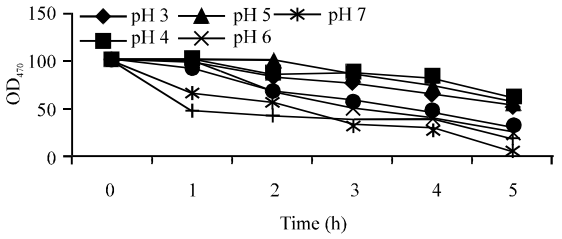

Fig. 6: pH stability of laccase of *P. fossulatus*. pH stability was estimated by incubating 20 U of enzyme at different pH values (3.0-9.0). The incubation was carried out at 30°C up to 5 h. Enzyme activity was determined using 10 mM guaiacol as substrate

Table 3: V_{max} and K_m of partially purified laccase of *P. fossulatus* against three different substrates

Substrate	V_{max} (U min^{-1})	K_m (mM)
o-dianisidine	17.67	0.041
Guaiacol	18.37	0.083
ABTS	31.54	0.454

Temp. optima: Temperature optima of laccase was 50°C (Fig.5).

pH stability: Laccase was full stable for 1 h in pH 3.0-6.0. It is three hour stable in pH 6.0 but less stable in alkaline pH (Fig. 6).

Temp. stability: Laccase was 100% stable for at least 5 h in 30 and 40°C. More than 50% stable for 5 h in 50°C. More than 50% of laccase activity was found in 60°C for 2 h (Fig. 7).

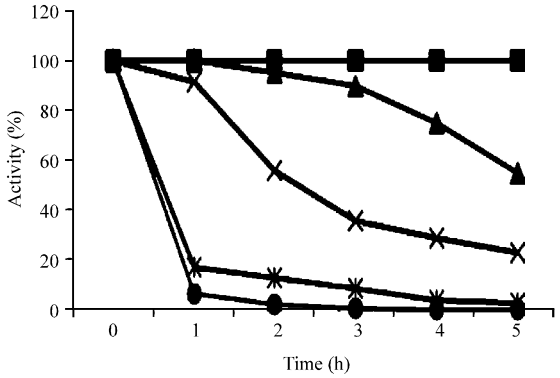

Fig. 7: Temperature stability (30-80°C) of laccase of *P. fossulatus*. 20 U of enzyme was incubated in respective temperature for 5 h. Enzyme activity was determined using 10 mM guaiacol as substrate in 0.1 M acetate buffer (pH 5.0)

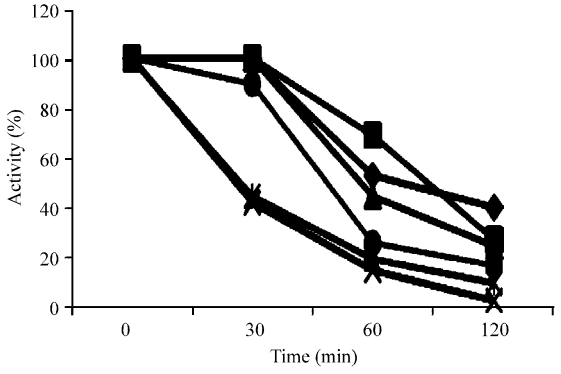

Fig. 8: Stability of laccase of *P. fossulatus* on different solvents. Stability of laccase in organic solvents was determined by incubating 20 U enzyme/mL in 1:1 (v/v) buffer /organic solvents upto 2 h. Enzyme activity was determined using 10 mM guaiacol as substrate in 0.1 M acetate buffer (pH 5.0)

Solvent stability: Solvent stability of laccase was measured in presence of DMSO, methanol, ethanol, phenol, benzene and chloroform. Laccase was full stable in Benzene, DMSO, Acetone for 30 min, more than 50% stable in benzene and DMSO for 1 h and least stable in chloroform (Fig. 8). After two hour incubation only 3% activity was retained in chloroform.

Inhibitor studies: Laccase was assayed in presence of a number of compounds like EDTA, $MgSO_4$, $ZnSO_4$, $FeSO_4$, $CuSO_4$, NaCl, $MnCl_2$, SDS, Azide and H_2O_2 (Fig. 9). The highest inhibition was found against azide (99.3%) followed by $FeSO_4$. More than 50-55% inhibition was

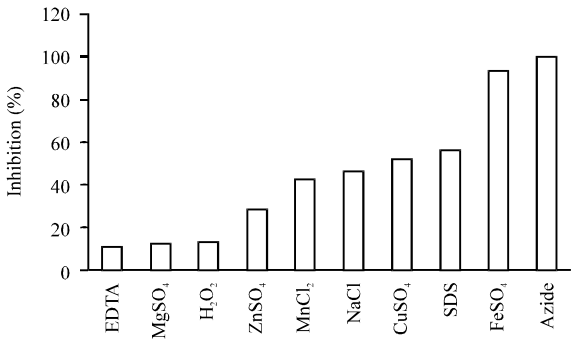

Fig. 9: Effect of different inhibitors on laccase activity of *P. fossulatus*. Twenty units of enzyme was incubated with 100 mM of effector molecules. Enzyme activity was determined using 10 mM guaiacol as substrate in 0.1 M acetate buffer (pH 5.0)

Fig. 10: Laccase isoenzymatic pattern of *P. fossulatus*. 20 U partially purified enzyme (20 U) was loaded in native polyacrylamide gel electrophoresis (PAGE) The zymogram was developed after two hour incubation with 0.3 mM o-diansidine in 0.1M acetate buffer (pH 5.0)

found against $CuSO_4$ and SDS whereas the other compound showed less than 50% activity. EDTA showed the lowest inhibition (about 10%).

Dye decolorization: In presence of 20 U laccase 50% colour of phenol red was decolorized in 26 h whereas 15% malachite green was decolorized in 10 days. Congo red and Eosin yellow were not decolorized in present experimental conditions.

Zymogram: The zymogram showed a smeared laccase band in presence of o-dianisidine as substrate (Fig. 10).

DISCUSSION

Laccase production was studied in *P. fossulatus* in both stationary and shaking condition in 25+2°C. It was found that laccase production was better in stationary condition than in shaking condition but the time required for optimum laccase production is much more (15 day) in stationary condition than in shaking condition (4 day) (Fig. 1 and 3). Variation of laccase production was reported due to shaking and stationary submerged conditions by different reporters. Dong *et al.* (2005) reported the marked diffences of laccase production in *Trametes gallica* in shaking and stationary submerged conditions. Highest laccase activity was reported in *P. eryngii* in shaking submerged fermentations (Stajic *et al.*, 2006). In our previous study we have showed the optimum laccase production of *P. fossulatus* in 26th day due to the lower incubation temperature (Das *et al.*, 2010).

Many researcher have found that laccase activities were dependent not only on the shaking/stationary cultural conditions but the composition of media i.e., carbon source, nitrogen source, pH, temperature, inducing substrates etc., De Souza *et al.* (2004). Laccase can be produced in very simple potato extract medium containing a carbon source.

Das *et al.* (1997) previously showed laccase production in potato extract in *P. florida* and also showed the supplementation of different carbon and nitrogen source (s) can influence the laccase activity in *P. florida* though in *T. gallica*, potato extract can lowered the laccase activity (Dong *et al.*, 2005). In the present investigation within three carbon sources it was found that in shaking condition, dextrose is the better carbon source than fructose and sucrose (Fig. 1). When three different nitrogen sources (peptone, yeast extract and malt extract) were individually added with different carbon sources in presence of potato extract it was found that Potato Sucrose Peptone (PSP) showed the best result than even potato dextrose peptone and others (Table 1, 2). Though sucrose has least effect as individual carbon source but there might any synergistic effect of sucrose and peptone, so that the combined results are more than the other combinations (Table 1, 2) and the result is significant. PSP showed more than 2.5 times greater activity than the PD media (Fig. 3). Again though peptone showed an inducing effect in sucrose containing medium but it has more or less little effect on dextrose or fructose containing medium (Table 1, 2). In each and

every media the optimum laccase production has been seen in fourth day in shaking conditions. The pH of culture media was also a factor for laccase production. The optimum pH for laccase production was 6.0 in PSP and PSY media (Fig. 2).

Though optimum laccase production required much time (15 days) in stationary submerged fermenting conditions than the shaking conditions (4 days) but it was found that the laccase produced through stationary conditions are much stable in storage than the later one (Data not shown). Possibly due to slow but steady production in stationary condition the enzyme is gradually accustomed with the external environment. In stationary condition PD medium showed better laccase production even than PSP media (Fig. 3). So, the laccase obtained from extracellular culture filtrate of PD media under stationary conditions has been taken for further studies. There are a number of laccase substrates and different enzyme has its own substrate specificity. Like other *Pleurotus* spp, the culture filtrate of *P. fossulatus* can oxidize o-dianisidine, ABTS and guaiacol with different efficiencies. In the present condition o-dianisidine was found suitable than guaiacol and ABTS. The L1 enzyme of *P. florida* showed more specificity towards o-dianisidine than guaiacol (Chakraborty *et al*., 2000). Inefficiency of o-dianisidine as laccase substrate over guaiacol and ABTS was further established by their Km and Vmax/Km (Table 3). It was now well established that laccase can oxidize a wide range of substrates like phenols, polyphenols, benzenethiols, aromatic amines and different non-phenolic substrates (Gianfreda *et al*., 1999) and different laccase enzymes have affinity towards different substrates.

The enzyme has a pH optima of 7.0 (Fig. 4) and temperature optima of 50°C (Fig. 5). Like most of the reported laccase the enzyme showed stability against a wide range of temperature and pH (Fig. 6, 7). The laccase is most stable in pH 5.0 and stable at least for 5 h in 40°C. More than 50% stability of laccase was found in 50°C after 5 h of incubation. Though laccase is considered as a cu-containing enzyme and the production is induced in some cases in presence of lower conc. of $CuSO_4$ as in *Volvariella* (Chen *et al*., 2003) but here $CuSO_4$ act as an inhibitor, possibly due to its high conc. (100 mM) in reaction mixture. Like most of the laccase the enzyme is inhibited by azide but the concentration required for azide inhibition is much more than other reports (Fig. 9). Das *et al*. (2000; 2001) reported the 100% inhibition of L1 and L2 laccase of *P. florida* in 1mM and 0.1 mM, azide conc.. respectively but in present condition in 100 mM of azide still there are a little (<1%) laccase activity. Gianfreda *et al*. (1999) commented that azide can bind to the type 2 and type 3 copper which resulted the interruption of the internal electron transfer and inhibition of laccase activity. The stability of the present enzyme might be due to the partial purified condition where the enzyme might be conjugated with other factor etc. Like other *Pleurotus* laccase it is inhibited by $FeSO_4$ but not with EDTA.

When analyzed in 1:1 (V/V) organic solvent- buffer solution, faster inactivation of laccase was found in chloroform and ethanol. . However, 100% of enzyme activity was retained in DMSO, benzene and acetone after 30 min (Fig. 8). Das *et al*., (2000) showed that L1 laccase of *P. florida* was 100% stable in chloroform for more than two hours. Burton *et al*. (1993) reported chloroform as a suitable medium for polyphenol oxidase activities. The high stability of laccase in some solvents confirms their possible involvement in bioremediation of xenobiotics. Like other laccases azide showed maximum inhibition followed by other inhibitor like $FeSO_4$, SDS etc.

The capability of different fungi to decolorize dyes of industrial importance has been extensively studied (Couto and Toca-Herrera, 2006; Khammuang and Sarnthima, 2007). In this work four dyes are tested for their decolorization in presence of laccase and without any mediator substances. Though phenol red showed about 50% decolorization in 26 h, malachite green showed only 15% decolorization after 10 days. Eosin yellow and congo red did not show any decolorization in present experimental conditions. Stoilova *et al*. (2010) showed more than 90% decolorization of congo red in 13 days in *Trametes versicolor*. The reasons possibly that they used more concentrated enzyme (1000/2000 U mL^{-1}) whereas in the present condition the enzyme conc. was much less (20 U). The other worker used mediator substances like ABTS which increase the rate of decolorization also. Again different fungal species produced different laccase enzymes which be at variance in their efficiencies.

The number of laccase isoenzyme varies not only from species to species but also some times within the same species depending upon the cultural conditions. Das *et al*. (2011b) showed variation in laccase isoenzyme pattern in different *Pleurotus* species. Two laccase isoenzymes were present in *P. florida*, *P. ostreatus* and *P. flabellatus*, three in *P. pulmonarius* and four in *P. sajorcaju*. Previously, Das *et al*. (2011a) four isoenzymes of laccase of *P. fossulatus* . In the present work after ammonium sulphate saturation and DEAE Sephadex (A-50) anion exchange chromatography followed by NaCl (0.6) M elution the number of laccase band decreased. The semipurified enzyme showed a smeared activity band possibly due to higher glycosylated portion (Fig. 10).

CONCLUSION

Like other *Pleurotus* species, *P. fossulatus* produced laccase enzymes extracellularly both in stationary as well as shaking condition. The optimum time required for laccase production is very less (4 days) in shaking condition than the stationary condition (15 day). The highest enzyme activities were observed in Potato-sucrose Peptone (PSP) medium (pH-7.0) in shaking culture and PD medium in stationary condition. The enzyme activity in latter condition was about 2.75t greater than the previous condition. The enzymes produced in stationary condition are much more stable than its counterpart in shaking condition. After partial purification the laccase showed more affinity towards o-dianisidine than guaiacol and ABTS. Possibly the enzyme is more stable than other reported laccases as evident from their requirement of high conc. of inhibiting substances like azide etc. It is apparent from the present studies that the laccase isolated from *P. fossulatus* might be industrially significant as they have a wide range of temperature and pH stabilities, stability in solvent (benzene, DMSO and acetone) and can decolorize the dyes (phenol red and malachite green) which were generally used in textile industries. The more detailed study of this laccase may open other avenues which can add not only the art of knowledge but also the other biotechnological applications of this enzyme in addition to the physiological role (s) in the producer organism, the edible mushroom *P. fossulatus*.

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