EFFECT OF DIFFERENT CULTURE CONDITIONS AND INDUCERS ON PRODUCTION OF LACCASE BY A BASIDIOMYCETE FUNGAL ISOLATE *PLEUROTUS OSTREATUS* HP-1 UNDER SOLID STATE FERMENTATION

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The production of laccase by an indigenous strain of *Pleurotus ostreatus* HP-1 was studied on solid state fermentation. Culture parameters such as type and concentration of substrate, inoculum size, moisture content, pH, surfactant presence, temperature, and nitrogen source were optimized by conventional "one factor at a time" methodology. A maximum laccase yield of 3952 U g^{-1} of dry substrate optimized was obtained with wheat straw as substrate with five agar plugs as the inoculum, 60% moisture content, pH 5.0, surfactant concentration 0.015 gl^{-1}, and nitrogen source (combination of L-asparagine and NH_4NO_3 at 10 mM concentration each) at incubation temperature 28^oC. Enhancement in laccase activity was achieved with the use of various aromatic inducers and copper sulphate. Highest laccase activity of 14189 U g^{-1} of dry substrate was achieved using 0.28 mM copper sulphate under optimized conditions. Thus, the indigenous isolate seems to be a potential producer of laccase using SSF and can be exploited for further biotechnological applications. The process also promises economic utilization and value addition of agro-residues.

Key words: Solid state fermentation; Laccase; Lignocellulosic substrates; Inducer; Optimization; Pleurotus ostreatus HP-1.

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INTRODUCTION

Laccases (benzinediol: oxygen oxidoreductases; EC 1.10.3.2) are glycosylated polyphenol oxidases. They are among the important enzymes that have attracted tremendous attention in recent years due to their important applications in different industries. They are copper-containing enzymes which catalyze the oxidation of a variety of phenolic and non phenolic compounds with the concurrent reduction of O_2 to H_2O. The broad substrate specificity of laccases holds promise to use them for biotechnological purposes, such as biomechanical pulping, bleaching of pulp, degradation of dye, and transformation and detoxification of xenobiotic and other aromatic compounds (Nyanhongo et al. 2002; Patel et al. 2008). Laccase production by white-rot fungi is associated with their lignin degrading ability, although laccases play a role in some other functions such as sporulation, pigment production, and fruiting body development (Thurston 1994). The application of laccases in biotechnological processes requires the production of high amounts of enzyme at low cost, and hence the current focus of laccase research is oriented towards the search for efficient production systems. Production of

laccase from white-rot fungi has been carried out using submerged as well as solid-state fermentation (SSF). SSF is generally defined as the growth of microorganisms on solid materials in the absence or near absence of free water. SSF provides many advantages over submerged fermentation, which include higher product titers, lower wastewater output, reduced energy requirements, simpler fermentation media, etc. (Pandey et al. 2001). *Pleurotus ostreatus* is one of most extensively studied white-rot fungi for its exceptional ligninolytic properties, fast growth, and easy handling under field condition. In basidiomycete fungi, extracellular laccases are constitutively produced in small amounts, and they are affected by many typical fermentation factors such as medium composition, carbon and nitrogen ratio, pH, temperature, aeration rate, etc. (Revankar et al. 2006). The production of laccase can be stimulated by the presence of a wide variety of inducing substrates, mainly aromatic or phenolic compounds related to lignin or lignin derivatives, such as ferulic acid, 2,5-xylidine, p-anisidine, and veratryl alcohol (Barbosa et al.1996). Other aromatic compounds that acts as inducers include abietic acid; various phenols (catechol; 4-chlorophenol: 2,6-dimethoxyphenol; guaiacol); several derivatives of benzoic acid (benzoic, 2,6-dimethoxybenzoic, syringic, vanillic, veratric acids); veratrylaldehyde; the lignin precursors such as coumaric and ferulic acids; lignosulfonates; and copper sulphate (Ikehata et al. 2004).

The present work was directed towards the utilization of lignocellulosic substrates for the production of laccase from a local isolate of *Pleurotus ostreatus* HP-1 (Genbank Accession No. EU420068) in SSF. Attempts were made to optimize various cultural and nutritional parameters by the conventional "one factor at a time" approach methodology. Further investigations were carried out for enhancement of laccase activity by different inducer compounds in millimolar concentrations under optimized culture conditions.

EXPERIMENTAL

Chemicals

2,2-Azino-bis (3-ethyl benzthiozoline-6-sulphonic acid) (ABTS), Veratryl alcohol, and Guaiacol were purchased from Sigma (St. Louis, M.O., U.S.A). Tween 80, Tween 20, and phenol reagents were purchased from Merck (Mumbai, India). Copper sulphate, catechol, and ortho-dianisdine was procured from CDH, (Mumbai, India). Gallic acid and Vanillin was purchased from SD Fine Chemicals, (Mumbai, India). All other chemicals used were of analytical grade procured from Qualigens (Mumbai, India). Wheat straw, wheat bran, rice bran, sugarcane bagasse, and animalfeed corn were collected locally and used as lignocellulosic substrates.

Screening and Isolation of Fungal Strain

The rotted wood samples were collected from different forest localities in Gujarat, India. A small portion of wood sample was transferred in plates containing 2% (w/v) malt extract agar (MEA) added with chloramphenicol (0.05%), and incubated at 28 ± 2°C. After 8 days of incubation at 28 ± 2°C fungal cultures were transferred on the same medium without antibiotics until pure colonies were obtained. Fungal cultures with similar microscopic characteristic were selected for storage. The presence of

Bavendamm's reaction was observed on Sabouraud dextrose agar (SDA) incorporated with ortho-dianisidine (0.01% W/V). Culture was maintained on MEA at 28 ± 2°C and stored at 4 °C. The culture was subcultured once in a month.

Production Medium and Inoculation of Fungal Strain

All of the lignocellulosic substrates were milled and sieved before use to obtain a 50 mesh size. Each flask containing 5 g of substrate was autoclaved at 121°C for 15 min. Basal medium described by Asther et al. (1988) was used to moisten support material (5 g wheat straw in 20 ml basal medium) to attain the desired saturation of moisture level. Tween® 80 was added at the time of inoculation to stimulate the secretion of extracellular enzyme. Cultivation was carried out in 250 ml Erlenmeyer flasks under solid state conditions. Five agar plugs (8 mm diameter) from a fungal colony growing on malt extract agar plate per flask were used as inoculum. All the flasks were incubated at 28 ± 2 °C for 8 days. After 8 days 10 milliliters of acetate buffer (pH 4.5, 120 mM) were added to each flask and kept on a shaker for 2 hrs at 28 ± 2°C. The contents were transferred to muslin cloth and squeezed. Liquid extract obtained was centrifuged at 10,000 rpm at 4°C for 10 min and supernatant was analyzed for enzyme activity.

Enzyme Assay

Laccase activity (E.C. 1.10.3.2) was determined by measuring the oxidation of 2,2 -Azino-bis-3-ethyl-benzthiozoline-6-sulphonic acid (ABTS). Increase in absorbance for 3 min was measured spectrophotometrically (Elico BL-198, Hyderabad, India) at 420 nm (ε = 36000 cm^{-1} M^{-1}) (Niku-Paavola et al. 1990). The reaction mixture contained 100µl of 50 mM ABTS and 800 µl of 20 mM Na-Acetate buffer (pH 4.5) and 100 µl of appropriately diluted enzyme extract. One unit of enzyme activity was defined as the amount of enzyme that oxidized 1µM of substrate per min.

Estimation of Fungal Biomass

To determine fungal biomass, the squeezed mycelia was dried to constant weight at 60°C. Dry weights were corrected for organic and inorganic components in the medium by subtracting measurements made for squeezed uninoculated (abiotic) control flasks (Patel et al. 2008).

Data Analysis

Data in subsequent sections represent arithmetic mean values of three experimental repetitions (each one was made in duplicate).

RESULTS AND DISCUSSION

Screening and Identification of Fungal Culture

Extracellular laccase activity was found in twenty five isolates belonging to the group basidiomycetes. The isolates showed a brown coloured zone surrounding the growth on SDA plate containing ortho-dianisidine, which is a characteristic of phenol oxidase production on solid medium by basidiomycetes fungi. The culture designated as

HP-1 was found to be the best amongst the twenty five isolates. Thus, this screening allowed us to select a promising basidiomycetes strain HP-1. Further testing of the culture involved its examination of morphological characteristics. The strain grew well, covering the entire petri-plate surface during 8 days. The isolate was non-sporulating, with abundant calmp connection forming in mycelia, which is characteristic of basidiomycte. The identification of HP-1 was further corroborated with studies on its 18S rRNA, 5.8S rRNA, and partial 28S rRNA gene sequencing carried out by Bangalore Genei, India. The isolate was identified as *Pleurotus ostreatus* HP-1 (Genbank Accession no. EU420068). The boot strapped unrooted tree was structured by the neighbour-joining method from the distance data generated by multiple alignment of the nucleotide sequences (Fig. 1).

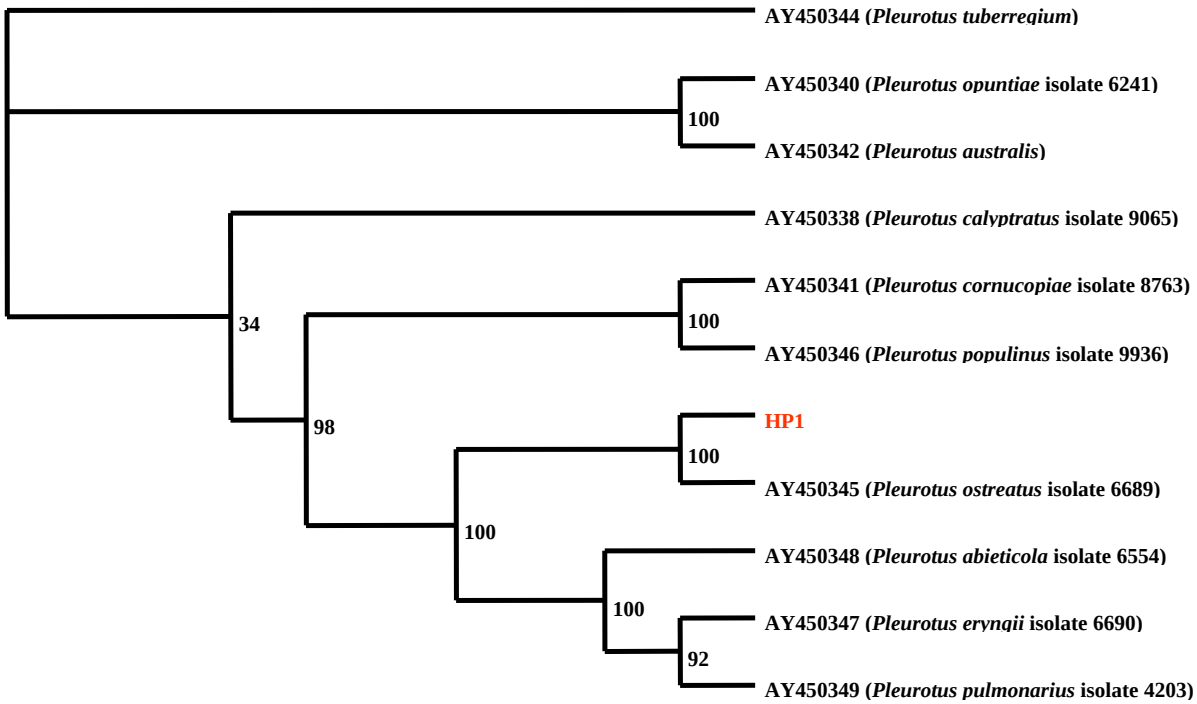

Fig. 1. Phylogenetic relationship of our isolate with *Pleurotus* genera of the family *Basidiomycete* based on 18S rRNA, 5.8S rRNA and partial 28S rRNA gene sequencing. The accession numbers for the sequences are given in parentheses. The bootstrapped unrooted tree was constructed by Neighbour-Joining method from the distance data generated by multiple alignments of nucleotide sequences. The bootstrap values for major groupings of the members included in the analysis are shown on the main branches.

Screening of Different Lignocellulosic Substrates

Selection of an appropriate substrate is a key factor in SSF, which determines the success of the process. It has been a practice to use lignocellulosic agroindustrial residues for the production of laccase. Different lignocellulosic substrates (animal feed corn, wheat bran, rice bran, wheat straw, and sugarcane bagasse) were screened for production of laccase. The culture showed maximum activity of laccase on the 8th day of incubation with all the different substrates (Fig. 2). All the five different agro-residues that were

tested supported good growth and laccase production. The first sign of growth was seen after 2 to 3 days of inoculation, and complete colonization of fungus was observed within 8 days of incubation. Among the various substrates, wheat straw was found to be the best substrate, and maximum laccase activity obtained was 2652 U g^{-1} of dry substrate. Various lignocellulosic substrates have also been used by other researchers for laccase production (Gupte et al. 2007; Vares et al. 1995). Higher laccase activities were found in *P. ostreatus* HAI 493 and *P. pulmonarius* HAI 572 under conditions of SSF of grapevine sawdust (Stajic et al. 2006). The present study has proved the utility of wheat straw, which is an inexpensive and easily available raw material as a suitable substrate for laccase production in SSF.

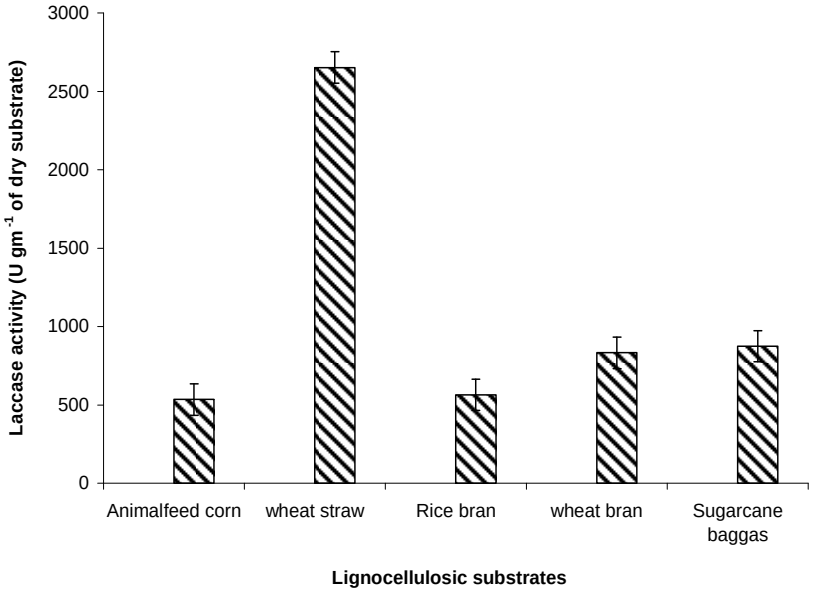

Fig. 2. Effect of different lignocellulosic substrates on laccase production on 8th day of incubation

Effect of Inoculum Size

Inoculum plays a significant role in enzyme production in SSF. A lower level of inoculum may not be sufficient to initiate the growth, whereas a higher level may cause competitive inhibition (Sabu et al. 2005). Thus, determination of optimum inoculum size becomes a crucial step in SSF. For optimization of the inoculum size for laccase production, a varying number (one to ten) of agar plugs (8 mm in diameter) were cut from actively growing fungal mycelium and inoculated in the production medium. The laccase activity increased with increase in the number of agar plugs (Fig. 3). Maximum laccase activity of 2700 U $g^{-1}$of dry substrate was obtained with five agar plugs. An increase in inoculum size enhanced the utilization of the solid substrate, thereby improving laccase activity. However, with further increase in inoculum above five agar plugs, laccase production was decreased because of fast depletion of the nutrients, resulting in a decrease in metabolic activity.

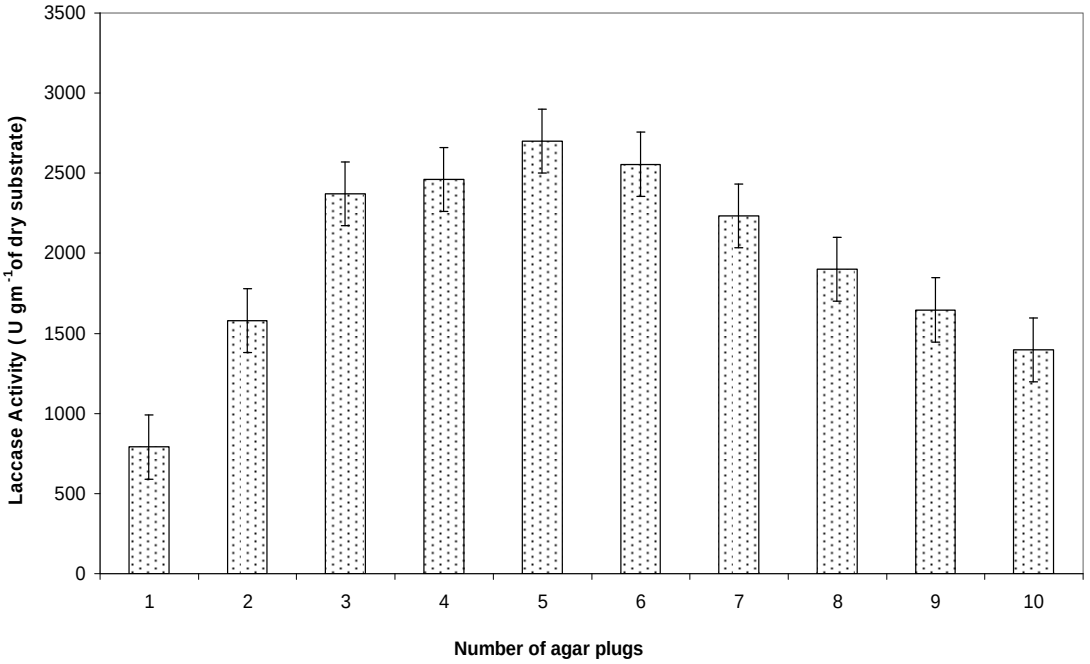

Fig. 3. Effect of inoculum size on production of laccase.

Effect of Carbon and Nitrogen Sources

The effect of conventional organic carbon and nitrogen sources on adaptation of the fungus for the production of laccase is of importance. Many previous studies have proved that both the nature and concentration of nitrogen sources are powerful nutrition factors regulating ligninolytic enzyme production by wood-rotting basidiomycetes (Galhaup et al. 2002). The purpose of supplement of glucose with lignocellulosics has two reasons. First, it promotes the growth and rapid establishment of the fungus within solid raw material. Second, the white rot fungus needs additional easily metabolizable carbon sources to degrade lignin from lignocellulosic substrates (Kaal et al. 1995). Different glucose concentrations showed different activity of laccase. A maximum laccase activity 3000 U $g^{-1}$of dry substrate was obtained with 1 % (w/v) glucose containing medium (Fig. 4). Mansur et al. (1997) showed that the use of fructose instead of glucose resulted in a 100 fold increase in the specific laccase activity of basidomycetes.

The ligninolytic enzymes have been seen to be regulated by the usable concentration of nitrogen in the medium. The low nitrogen level can stimulate the ligninolytic enzyme production, whereas a high nitrogen level represses it (Kirk and Chang 1990). Similar results were obtained with different concentrations of nitrogen in the medium. Maximum laccase production 3120 U g^{-1} of dry substrate was achieved when a combination of two different organic and inorganic nitrogen sources was used (L-asparagine and NH_4NO_3 each 10 mM) (Table. 1). Staji'c et al. (2006) showed 396 Ul^{-1} activity of laccase by *Pleurotus ostreatus* HAI 493 and 100 Ul^{-1} by *Pleurotus eryngii* with $(NH_4)_2SO_4$ as nitrogen source of 20 and 300 mM, respectively.

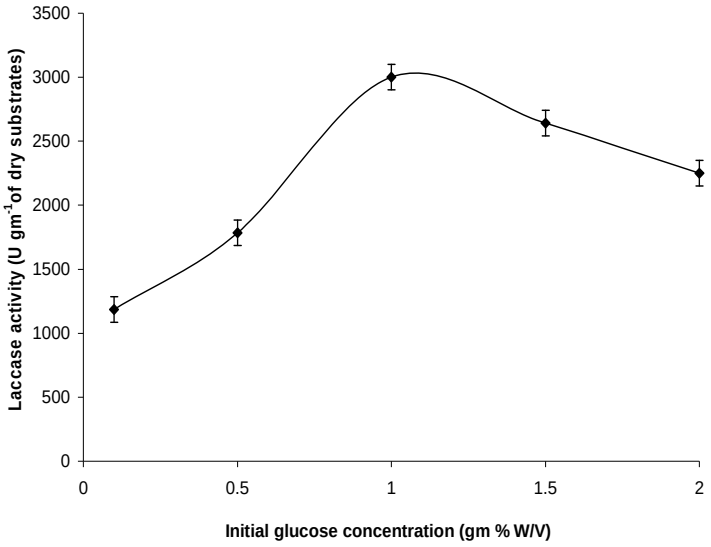

Fig. 4. Effect of different concentration of glucose on production of laccase

Table 1. Effect of Different Nitrogen Sources on Laccase Production by *Pleurotus ostreatus* HP-1

Organic Nitrogen Source (10 mM)	Activity* (U/ gm of dry substrate)
Thiamine-HCl	1903
L- Asparagine	2565
Inorganic Nitrogen Source (10 mM)	
$(NH_4)_2SO_4$	2012
NH_4NO_3	2626
Combination of Organic and Inorganic Nitrogen Sources	
2.0 mM (L-Aspargine, 1mM + NH_4NO_3, 1mM)	2142
20 mM (L-Aspargine, 10mM + NH_4NO_3, 10mM)	3120
200 mM (L-Aspargine, 100mM + NH_4NO_3, 100mM)	1629
*Laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per min at room temperature.	

Effect of Moisture Content

Optimization of initial moisture content is essential in SSF, as it affects the substrate utilization and laccase production. Laccase produced by the fungus was positively affected by increases in moisture content (Fig. 5). With increase in initial

moisture content from 40 to 60%, a considerable increase in laccase activity was observed. Maximum laccase production of 3214 U g^{-1} of dry substrate was obtained at 60% moisture. However, when the moisture content was increased beyond 60%, a substantial decrease in laccase activity was observed. During the growth of *Trametes versicolor* on wheat straw, the optimum moisture content was found to be 55 % (Yadav and Tripathi 1991). Similar results for laccase production after five days of cultivation was obtained using cultures of *P. pulmonarius* CCB-19 at 75% initial moisture content (Farani De Souza et al. 2006). The moisture levels on SSF processes vary between 30 and 85%, and for most of filamentous fungi, the optimum moisture content for growth and substrate utilisation is between 40 to 70%. However, this depends upon the organism and the substrate used for cultivation (Raimbault 1998). Increasing the moisture level is believed to reduce the porosity of the substrate, thus limiting oxygen transfer. For this reason, the use of high moisture content limited the growth within the whole substrate, resulting in surface growth.

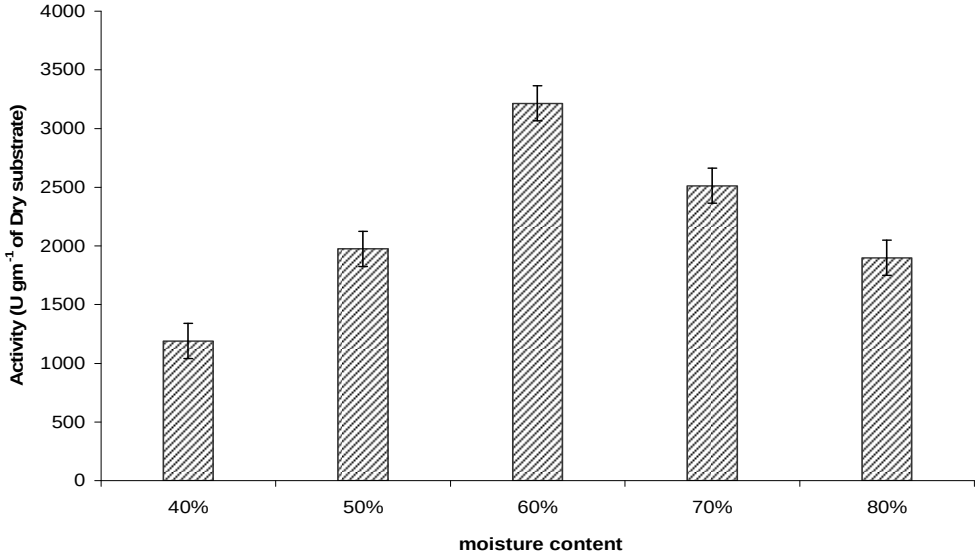

Fig. 5. Effect of different moisture content on production of laccase

Effect of Various pH

pH is one of the important parameters in fungal cultivation. Various pH (3.0 to 9.0) of the Asther's medium were adjusted with Pthalate buffer. Optimal pH for maximum laccase production (3356 U g^{-1} of dry substrate) was observed at pH 5.0. Exponential increase in laccase activity was observed from pH 3.0 to 5.0, but thereafter laccase production was decreased with increase in pH (Fig. 6). This may be attributed to the fact that change in pH may alter the three-dimensional structure of the enzymes (Shulter and Kargi et al. 2000).

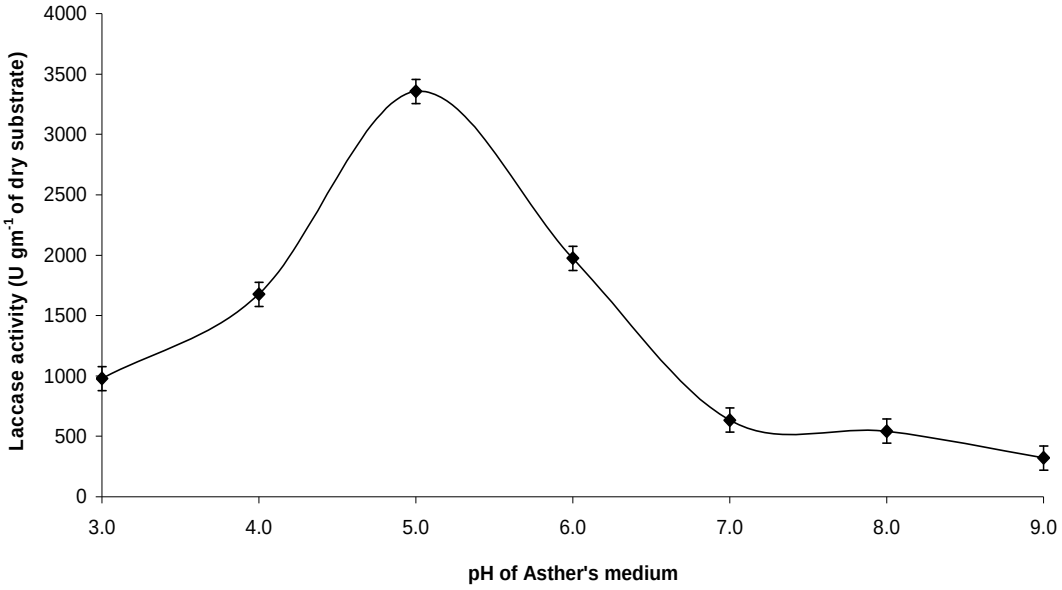

Fig. 6. Effect of different pH on production of laccase

Effect of Different Temperature

Temperature is of much significance in the SSF systems, because during fermentation there is a general increase in the temperature of the fermenting mass due to respiration (Niladevi et al. 2007). Even though the impact of temperature is more prominent in the scale-up processes, it remains an inevitable factor in all systems due to its impact on microbial growth and enzyme production. Results of the present study showed that the temperature of 28°C was optimum for laccase production (3467 U g^{-1} of dry substrate), and no considerable activity was observed at any of the other temperatures considered (i.e. 20, 40 and 50°C) (Fig. 7). The culture of *Cyathus bulleri* showed maximum laccase production at 30°C (Vasudev et al. 2005). At 50°C temperature no fungal growth was observed, due to the drying of substrate at higher temperature.

Effect of Different Surfactant

Surfactants, especially Tween®-80, can increase the bioavailability of less soluble substrates for the fungi and stimulate growth of the fungal spores (Zheng and Obbard 2001). The present study determined the effect of surfactants. Tween® 20 and Tween® 80 were added to the medium at various concentrations of 0.005 - 0.03 gl^{-1}. Tween® 80 with a concentration of 0.015 gl^{-1} stimulated higher laccase production (3952 U g^{-1} of dry substrate) as compared to the control (Fig. 8). A trend of decreasing enzyme production at increasing concentrations above 0.015 gl^{-1} Tween® 80 was observed. On the other hand, no stimulatory effect of Tween® 20 was observed for any of the concentrations used in this study. The specific mechanism by which surfactants enhance extracellular enzyme production in filamentous fungi has not been elucidated (Wang et al. 2008).

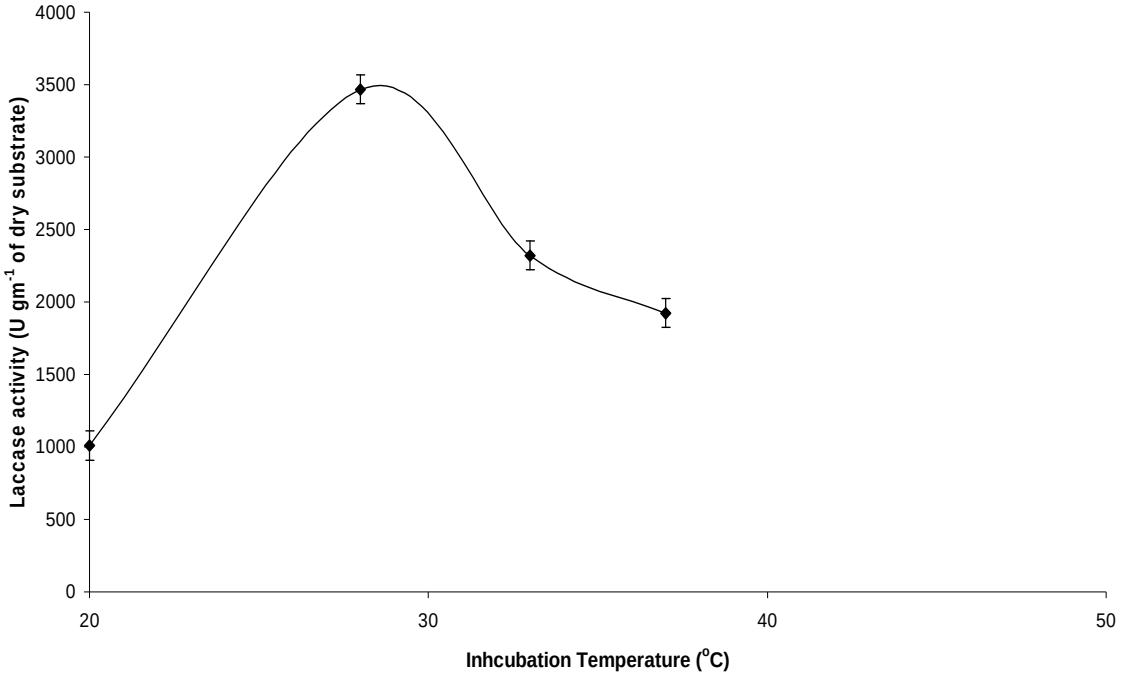

Fig. 7. Effect of different incubation temperature on production of laccase

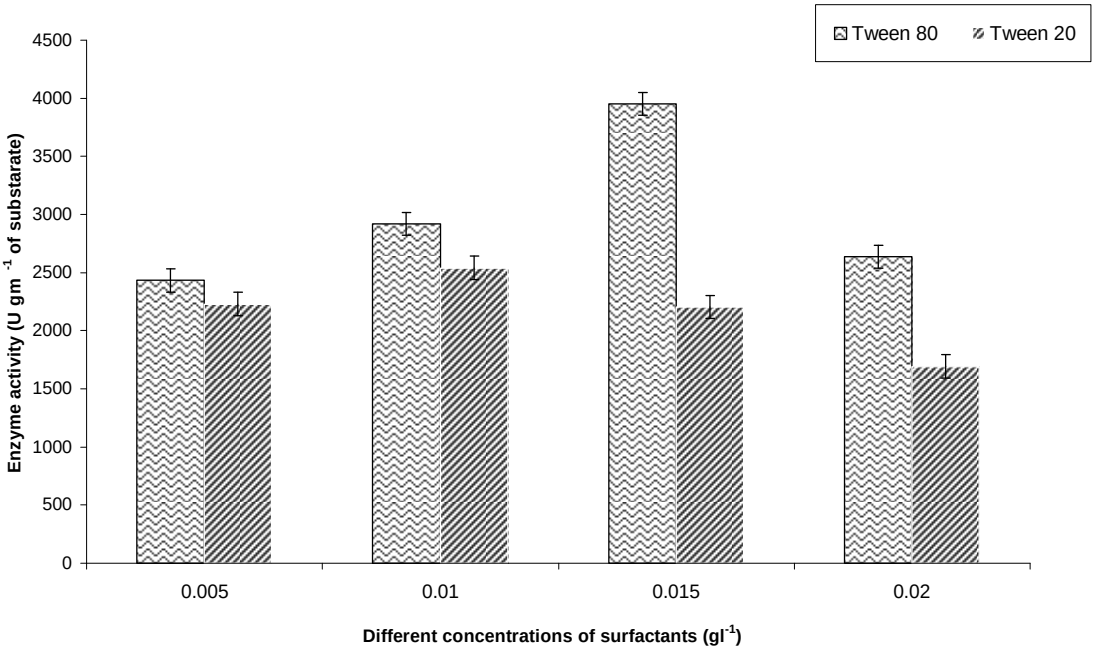

Fig. 8. Effect of different concentrations of surfactants on production of laccase

Effect of Different Inducer Compounds for Laccase Production under Optimized Culture Conditions

The production of laccase is affected by several factors, including the composition of growth medium, pH, carbon: nitrogen ratio, and temperature. The production of laccase by white-rot fungi was increased or induced when an aromatic compound (such as veratryl alcohol, vanillic acid, 2,5-xylidine, ferulic acid, syringaldazine, or guaiacol) or Cu^{+2} was added to the growth medium (Ikehata et al. 2004). Copper as micronutrient has a key role as a metal activator, induces both laccase transcription, and plays an important role in laccase production (Palmieri et al. 2000). Hence, the effect of copper on laccase production by *Pleurotus ostreatus* HP-1 was studied under optimized culture conditions. In our study all inducer compounds were filter-sterilized and added at the time of inoculation.

The increasing concentration of copper sulphate from 0.06 to 0.28 mM increased laccase production. Maximum laccase production was 14,189 U g^{-1} of dry substrate (i.e. wheat straw) on 8th day of incubation. At the same time, the control flask (without any inducer) showed laccase production of 3,952 U $g^{-1}$of dry substrate, which was about 4 times lower than that of flask supplemented with 0.28 mM copper sulphate (Fig. 9).

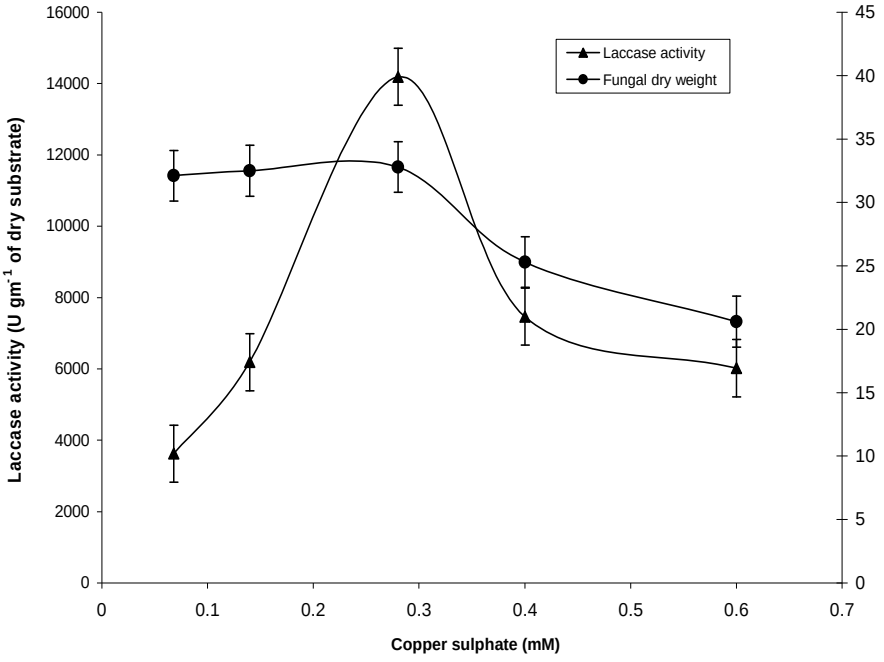

Fig. 9. Effect of different concentrations of copper sulphate on laccase production and fungal growth on 8th day of incubation.

These results clearly show the positive effect of copper sulphate as an inducer of laccase production. The optimum concentration of copper sulphate (0.28 mM) was compared for growth and enzyme production profile with the control flask (without any inducer) (Fig. 10). During various phases of incubation, twin peak activation of enzyme production was observed, which was in accordance with our earlier study (Gupte et al. 1998). At maximum laccase production no further increase in fungal biomass was observed, since production of ligninolytic enzymes occurs during their secondary metabolism of the growth phase (Zhou et al. 2007). A possible explanation for this stimulatory effect of copper on laccase biosynthesis could be a role for this enzyme activity in terms of a defense mechanism against oxidative stress, with laccase involved in the synthesis of pigments to prevent the uptake of metals (Trupkin et al. 2003). However, when the concentration of copper was increased beyond 0.28 to 0.6 mM, significant decrease in fungal growth and laccase production was observed (Fig. 10). This may be attributed to an inhibitory effect of copper at higher concentrations. Galhaup et al. (2003) and Stajic et al. (2006) reported that the addition of copper sulphate in various concentrations (1 – 10 mM) stimulates laccase production in *T. pubescens*, *P. eryngii*, *P. ostreatus* HAI 493 and *P. ostreatus* HAI 494. Our findings are in accordance with those findings. The copper concentration used was within the range of 0.002 mM to 0.6 mM that is used in typical cultivation media for the production of laccase both in wild-type and recombinant strains of different basidiomycetes fungi (Shicheng et al. 2003).

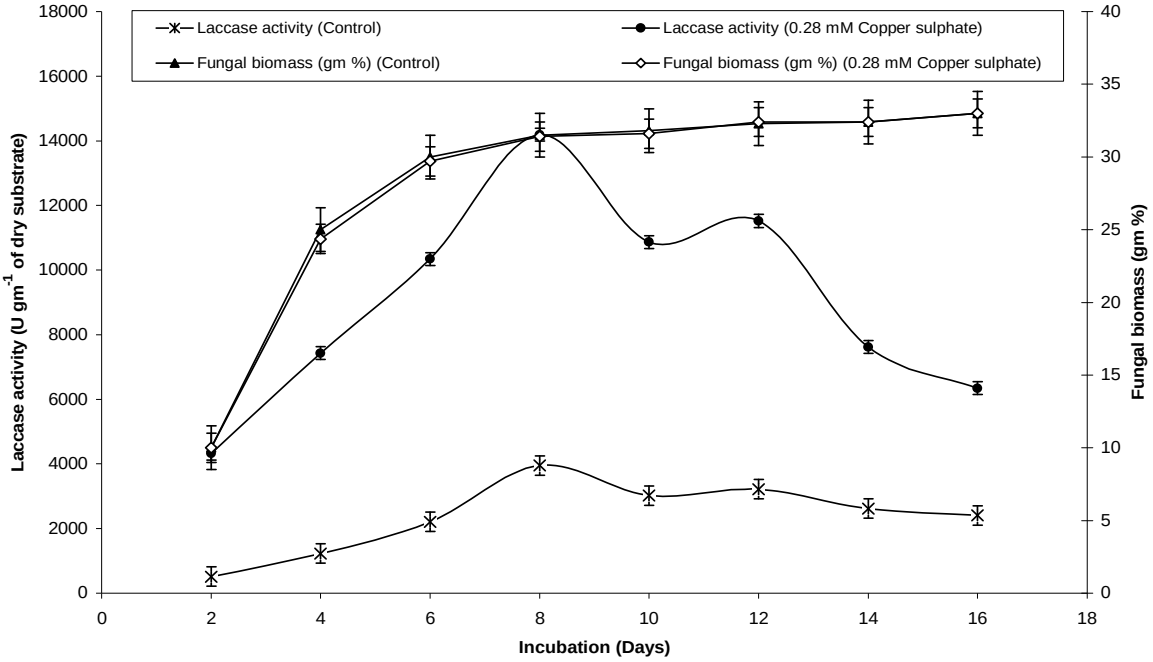

Fig. 10. Comparision of growth and enzyme production profile of control and optimum copper sulphate (0.28 mM)

The inductive effect of veratryl alcohol (3, 4-Dimethoxybenzyl alcohol) on laccase production by *Dichomitus squalens*, *Ganoderma lucidum* and *Trametes versicolor* has been reported by Arora and Gill (2001). The production of laccase 1939, 2512 and 1250 U g^{-1} of dry substrate for 4, 16, and 20 mM of veratryl alcohol concentrations respectively, were lower than that of the control flask (Fig. 11). This may be due to the toxic effect of veratryl alcohol, which affects the both growth of the fungi and production of enzyme (Barbosa et al. 1996).

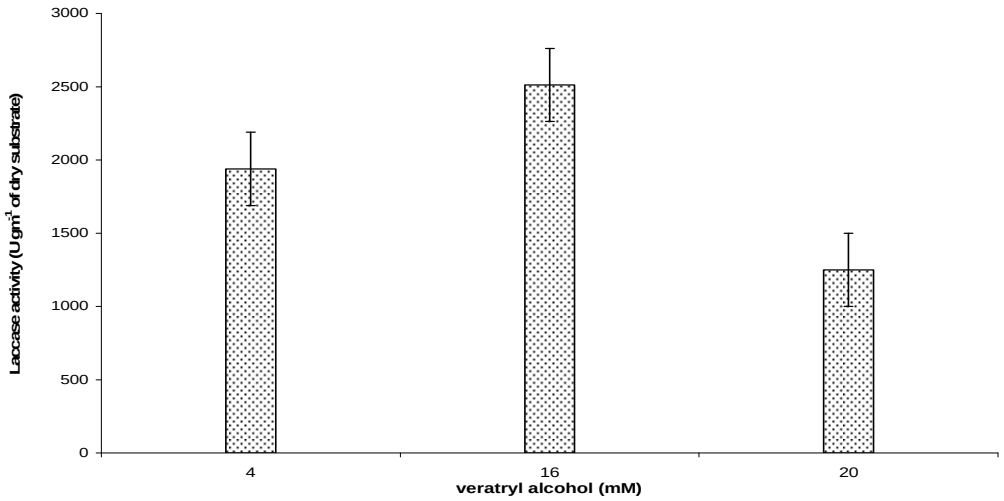

Fig. 11. Effect of different concentration of veratryl alcohol on production of laccase

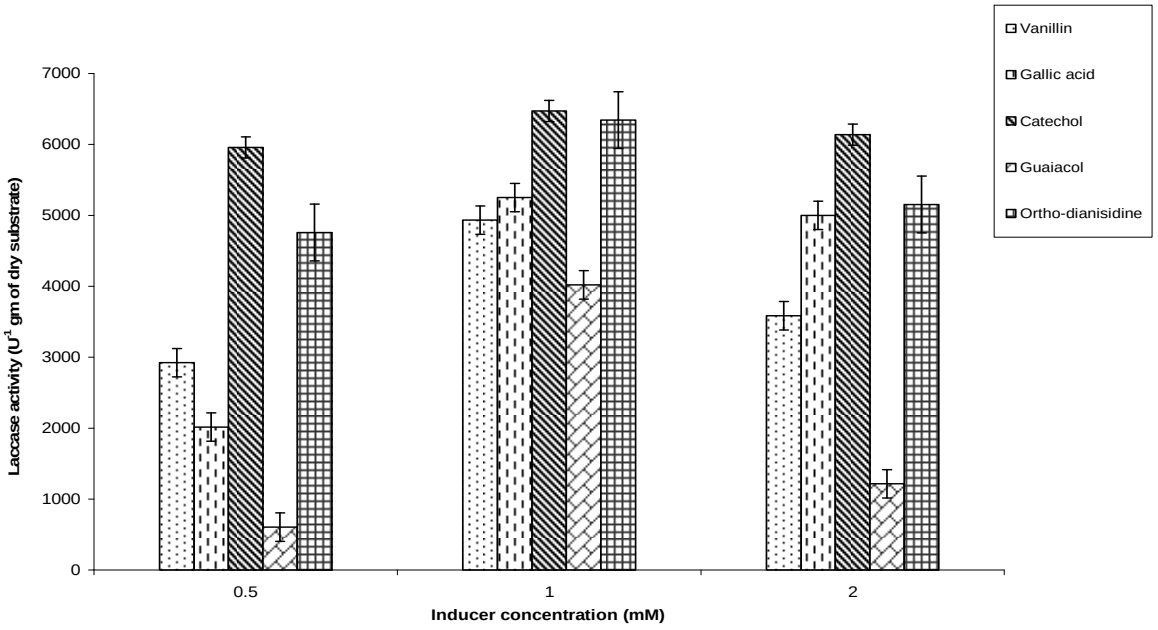

Fig. 12. Effect of different inducer concentrations on production of laccase

The guaiacol supplement showed 4021 U g^{-1} of dry substrate activity of laccase at 1 mM concentration (Fig. 12). However, there are reports on suppression of laccase production by guaiacol in *Pleurotus florida* and *Pycnoporus cinnabarinus* (Eggert et al. 1996). In case of *Phanerochaete chrysosporium* NCIM 1197, induction of laccase in presence of 300 mM of each guaiacol (1-hydroxy-2-methoxybenzene) and gallic acid (3,4,5-Trihydroxybenzoic acid) was seen independently (Gnanamani et al. 2006).

The differences between the present results and the cited work, might be due to the higher concentration of guaiacol used in this study. Out of six different compounds tested, only two, namely gallic acid and catechol, both carrying more than one hydroxyl group, showed a less positive effect on laccase production, increasing laccase activity approximately 1.3 fold for gallic acid and 1.6 fold for catechol at 1.0 mM concentration of each as compared to the control (Fig. 12). The study was further exploited by taking a combination of the two inducers 1.0 mM catechol and 0.28 mM $CuSO_4 \cdot 5H_2O$ to check the synergistic effect of the different inducers (data not shown); it was observed that laccase activity was higher in the case of 0.28 mM $CuSO_4 \cdot 5H_2O$ alone. Vanillin and ortho- dianisidine were not found to be efficient inducers of laccase by our isolate (Fig. 12).

CONCLUSIONS

The present isolate *Pleurotus ostreatus* HP-1 is found to be a very efficient producer of laccase under solid state fermentation. The present investigation has confirmed and evaluated the use of wheat straw as an inexpensive and easily available agroindustrial waste for laccase production under solid state fermentation. Optimization of the fermentation process by conventional method resulted in higher laccase production. The enzyme production ability of this organism can be enhanced by supplementing the basal media with various inducer compounds, namely copper sulphate (0.28 mM). An overall 3.5 fold increase in laccase production was attained as compared to the control. The substrate and inducer are safe, cheap, and could be suggested for prospective application for the higher production of enzyme.

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