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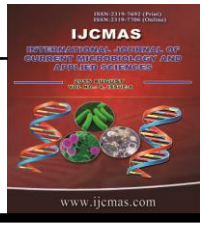

Original Research Article

Production of Laccase Enzyme by Basidomycetes *Coriolus versicolor* through Solid State Fermentation

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A B S T R A C T

Keywords

Solid state fermentation, Coriolus versicolor, Laccase enzyme, Glucose, Chickpea powder

The cultivation of *Coriolus versicolor* for laccase production and cell growth were strongly dependent on experimental conditions namely physical and chemical parameters as well as nutrient availability and inducer stimulation. In the present study, *C. versicolor* was selected for the production of laccase in solid state fermentation. The best results for laccase induction were obtained in glucose (3%) and chickpea powder (3%) respectively. The optimization studies showed that the laccase yield was maximum in rice bran (0.98 U/ml and 0.89U/ml) and in peanut shell (0.79U/ml and 0.67U/ml) at pH 6.0 and temperature 37^{o}C. As well as laccase activity was in peak (0.43 U ml) on 36th day of incubation period.

Introduction

Lignocellulose is the predominant component of woody plants and dead plant materials and the most abundant biomass on earth. White-rot fungi (Basidiomycetes) produce various extracelluar enzymes, such as laccase (Lac), manganese peroxidase (MnP) and lignin peroxidase (LiP), which are involved in the degradation of lignin and their natural lignocellulosic materials (Nagai *et al*., 2007). This ligninolytic system of white-rot fungi is also directly involved in the degradation of various xenobiotic compounds and dyes (Hofrichter, 2002).

Besides white-rot fungi, some microorganisms can also degrade and decolorize wide range of recalcitrant organic compounds (Hammel *et al*., 1985; Gopinath *et al*., 2005). Through intensive study of ligninolytic fungi, it has been determined that these organisms produce extracelluar enzymes with very low substrate specificity, enabling them to mineralize a wide range of highly recalcitrant organ pollutants that are structurally similar to lignin (Hofrichter, 2002).

C. versicolor a basidiomycete produces three ligninolytic enzymes which has an efficient degradation capacity of lignin, polycyclic aromatic hydrocarbons, a polychlorinated biphenyl mixture and a number of synthetic dyes (Tanaka *et al*. 1999; Novotný *et al*. 2004). Recently several works are being done to search specific mechanisms of enzymatic lignin oxidation by each ligninolytic enzyme particularly by laccase (Castro *et al*. 2003). After determining correct oxidative mechanisms industrial biocatalyses can be utilised on a wide type of applications. *T. versicolor* cultures or separated ligninolytic enzymes can also be used as biocatalysts on other different industrial processes namely for decolourisation of industrial dyes (Swamy and Ramsay, 1999; Amaral *et al.* 2004) and for waste-water treatments (Modi *et al.* 1998).

Large-scale production of *T. versicolor* or its ligninolytic enzymes would be very interesting for the oxidation of ligninocelulosic renewable materials. Selection of experimental conditions for fungi growth is very important for industrial applications and providing increasing ligninolytic enzymes production. Few studies have been done to find a good experimental strategy (Adejoye, and Fasidi, 2010). From a practical standpoint, proposed biotechnological applications will depend on a better understanding and control of the nutritional regulation of the ligninolytic system.

T. versicolor has been grown on rice bran and pea nut shell to see the lignolytic activity at different temperatures and pH. Glucose and chick pea effect is also analysed in order to improve laccase production or activity and effect of combination of both, inducer is evaluated for laccase industrial applications.

Material and Methods

Organisms

In this study, we used mycelia of *Coriolus versicolor* belong to mushroom species to determine their ligninolytic activities. Mycelia obtained from fruit body of mushroom by tissue culture method and were transferred on Potato Dextrose Agar. Stock culture were maintained on Tein and Kirk medium (Tein and Kirk 1988) at 4^{o}C and transferred monthly.

Screening of the fungal strain for the production of laccase.

The screening of laccase producing *C. versicolor* was carried out on plates on defined medium with 3.0 g/L peptone, 10.0 g/L glucose, 1.0 g/L KH_2PO_4, 0.001g/L Zn SO_4, 0.4g/L, 0.4g/L K_2HPO_4, 0.0005g/L $FeSO_4$, 0.05g/L $MnSO_4$, 0.5g/L Mg SO_4 and 20g/ L agar were used. The media was supplemented with 0.02% guaiacol and then sterilized by autoclaving at 121^{o}C $\pm$ 1^{o}C and 15 lb/ in^2 for 15 minutes. Before pouring media into the plates, chloramphenicol was added to avoid any bacterial contamination. *C. versicolor* was inoculated into these plates and then plates were incubated at 25 $\pm$ 1^{o}C for 7 days. 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 (Da Re *et al* 2008).

Laccase production on solid substrates.

Solid State fermentation (SSF) was used for the production of laccase on solid substrates. Mycelial plugs from 3-day-old culture of *C. versicolor* on PDA were transferred in 250ml, Erlenmeyer flask containing 5g solid substrate and 15 mL distilled water. The

solid substrates were rice bran and peanut shell. Mixture was incubated at 37°C in the dark for 36 days. Enzyme was extracted by adding 50mL of distilled water to the culture and mixed at 4°C for 1 hour. The culture was filter through cotton cloth and filtrate was used as enzyme solution (Claye *et al* 1996).

Optimization of different culture conditions for production of Laccase.

To find out optimal culture conditions for efficient production of laccase enzyme, the following factors were investigated i.e. initial pH, Incubation period, temperature, carbon and nitrogen sources.

Influence of initial pH

Optimization of initial pH was done by adjusting it at different range from 4.5 to 7 pH was maintained using 1M HCl and 1M NaOH. Each flask containing 5g substrate, was autoclaved and then inoculated with *C. versicolor* under sterilized condition. The entire flasks were incubated at 37°C for 36 days. The variation in enzyme yield was determined after 36 days of incubation (Zahida *et al* 2007).

Influence of incubation time (days)

For the best production of laccase, each flask contained 5g substrate was autoclaved and inoculated. Incubation was carried out at 37°C for 12, 18, 24, 36 and 42 days. Enzyme was harvested after 12, 18, 24, 36 and 42 days. The influence was examined on each 6 days of interval of inoculation (Jonathan and Fasidi, 2001).

Influence of temperature

The flask fermentation was carried out at different temperatures, ranging from 25°C to 37 °C for 36 days (Zahida *et al* 2007).

Influence of Carbon and Nitrogen source

For the greatest production of laccase, glucose was used as carbon source and chickpea powder was used as nitrogen source. Each flask contained 1%, 2%, 3% and 5% glucose and chickpea powder in 5g substrate. Inoculated flasks were incubated at 37°C for 36 days. Enzyme was harvested after 36 days (Zahida *et al* 2004).

Enzyme assay

The enzyme activity was determined spectrophotometrically at 37°C using sodium acetate buffer. Oxidation of 2mM of guaiacol was monitored at 450nm. Guaiacol (2 methoxy phenol) was used as substrate. Sodium acetate buffer was prepared. A 1.36g of sodium acetate was dissolved in 900mL distilled water to make 10mM solution. Then, 50 mL of acetic acid was added drop wise in 900mL sodium acetate, until 5.0pH was obtained. Then volume was made up to 1000mL, and then 0.02mL guaiacol was dissolved in 100 mL distilled water to make 2mM solution. Total volume of reaction mixture and blank was 5mL. It contained 3mL acetate buffer having 1mL of 2nM guaiacol and 1 mL culture filtrate in reaction mixture while blank contained 1mL deionized water in place of culture filtrate. The enzyme activity was assayed by monitoring the absorbance at 450 nm just after the reaction mixture was prepared, and then it was incubated at 37°C, after 10 minutes absorbance was noted again at 450nm. One unit was defined as the amount of the laccase that oxidized 1 mM of substrate per min. Assay were carried out independently in triplicate (Robles *et al* 2000).

Statistical analysis

The data was subjected to analysis of variance using ANOVA LSD (Least significant difference) computer package. The mean values were compared with the LSD (Domenic and Byron, 1999)

Results and Discussion

Influence of initial pH

The value of laccase activity observed at pH 4.5 was 0.69± 0.003 (in rice bran) and 0.46+0.003 (in peanut shell) which increased and the optimum point observed at pH 6 which was 0.97± 0.003 (in rice shell) and (0.78+0.003) in pea nut shell.

Table 1 and 2 shows that the effect of different pH on the production of laccase. With the increase in pH, laccase production increased and become maximum at pH 6 while further increased in pH adversely affected the laccase activity and fungal biomass production. Substantial production was also observed at pH 5.5 in both substrates.

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Effect of incubation time on laccase production in rice bran and peanut shell

In table 3 and 4, the effect of different incubation period (days) on the yield of laccase production was demonstrated. Laccase production was highest on 37th to 42nd day. The value of laccase activity observed at 12th day was 0.22± 0.005 which increased and the most advantageous point of production observed at 42 days of incubation which was 0.67±0.005. Similarly, in case of peanut most excited laccase production was observed at 37th to 42nd of incubation.

Influence of temperature

The value of laccase activity observed at 25^{o}C was 0.38± 0.005 (in rice bran) and 0.26+0.005 (in peanut shell) which increased and the optimum point observed at 37^{o}C which was 0.88± 0.003 (in rice shell) and (0.66+0.004) in pea nut shell.

There was significant effect of incubation temperature on enzyme activity in case of rice bran and peanut shell. Laccase production was highest at 37^{o}C, and then decreased at 45^{o}C. The effect of different temperature on the yield of laccase was represented by Table 5 and 6.

Influence of carbon and nitrogen sources

The value of laccase activity observed at 1% glucose and chick pea powder concentration was 0.87± 0.005 and 0.89 ± 0.003 (in rice bran) and 0.67± 0.005 and 0.68 ± 0.005 (in peanut shell) which increased and the optimum point observed at 3% glucose and peanut shell which was 0.83± 0.005 and1.005±0.005 (in rice shell) and (0.82+0.005) in pea nut shell.

There was significant effect of glucose and chickpea powder values on laccase production using rice bran and peanut shell. The effect of different concentrations of glucose and chick pea powder on the yield of laccase was represented by in table7 and 8. Laccase production was highest at 3% glucose and 3%chickpea powder, subsequently decreased at 4% to 5% concentrations.

White rot fungi produce various extracelluar

enzymes, such as laccase (Lac), manganese peroxidase (MnP) and lignin peroxidase (LiP), which are involved in the degradation of lignin and their natural lignocellulosic materials. Laccase is mainly responsible for the decolorization of aromatic compounds. It is able to oxidize substrates such as ABTS, guaiacol. In *C. versicolor* inoculated plate, the appearance of dark reddish brown color takes place within 24h and the complete color change was observed in fourth day. Laccase production in white rot fungi is known to be influenced by a number of factors; little work has been done to study the regulation of laccase production.

*C. versicolo*r colored indicator compounds (guaiacol) that enable the visual detection of laccase production. The use of colored indicators is generally simple handling and measurement is required, as laccase oxides guaiacol has been used as indicators for laccase production.

Results from this study showed that pH value of 6 was found to be the optimum for laccase activity. A similar pattern was observed in *Trametes trogii*. Gopinath *et al* (2005) reported that pH ranged for enzyme production of 4.5 and 6.5 (Fig- 1 and 2). It was also reported that culture pH is an index of fungi enzyme activity. The laccase activity in rice bran was up to 0.58-0.98 U/ml and in peanut shell it was recorded 0.36-0.79U/ml. 4.5 and 7 pH in our study showed lower laccase activity (0.692 and 0.58 in rice bran and 0.46-0.36 in peanut shell) and higher in pH 6 (0.98 in rice bran 0.79 in peanut shell) (Fig -1 and 2).

Table.1 & 2 Descriptive statistics of pH and laccase activity (Rice bran and peanut shell)

	Rice bran	
pH	Mean	Std. Deviation
4.5	0.6920	0.00300
5.0	0.8850	0.00300
5.5	0.9650	0.00300
6.0	0.9750	0.00300
6.5	0.7650	0.00300
7.0	0.5750	0.00300
Total	0.8095	0.15034

	Peanut shell	
	Mean	Std. Deviation
4.5	0.4620	0.00300
5.0	0.6850	0.00300
5.5	0.7680	0.00300
6.0	0.7880	0.00300
6.5	0.5950	0.00300
7.0	0.3550	0.00300
Total	0.6088	0.16240

Table.3 & 4 Descriptive statistics of Incubation time and laccase activity (rice bran & peanut shell

Incubation time (days)	Mean	Standard Deviation
12	0.2770	0.00300
18	0.4800	0.00300
24	0.6950	0.00300
36	0.8880	0.00300
42	0.8900	0.00300
Total	0.6460	0.24680

Incubation time (days)	Mean	Standard Deviation
12	0.2770	0.00300
18	0.4800	0.00300
24	0.6950	0.00300
36	0.8880	0.00300
42	0.8900	0.00300
Total	0.6460	0.24680

Table.5 & 6 Descriptive statistics of Incubation temperature & laccase activity (Rice bran & pea nut shell)

Incubation temperature (days)	Mean	Standard Deviation
25	0.3850	0.00500
30	0.4900	0.00500
35	0.7900	0.00500
37	0.8870	0.00300
45	0.6250	0.00500
Total	0.6354	0.19155

Incubation temperature (days)	Mean	Standard Deviation
25	0.2660	0.00500
30	0.3850	0.00500
35	0.4650	0.00500
37	0.4660	0.00400
45	0.5560	0.00300
Total	0.4672	0.0.14187

Table.7 & 8 Descriptive statistics of glucose and chick pea concentration & laccase activity in Rice bran & pea nut shell

Glucose Concentration (%)	Mean	Standard Deviation
1%	0.8750	0.00500
2%	0.9750	0.00500
3%	1.0000	0.00200
4%	0.7650	0.00500
5%	0.5537	0.00451
Total	0.8337	0.16852

Chickpea powder concentration (%)	Mean	Standard Deviation
1%	0.6850	0.00500
2%	0.7850	0.00500
3%	0.8250	0.00500
4%	0.6650	0.00500
5%	0.5650	0.00500
Total	0.7050	0.09541

Effect of pH on laccase production using rice bran and pea nut shell

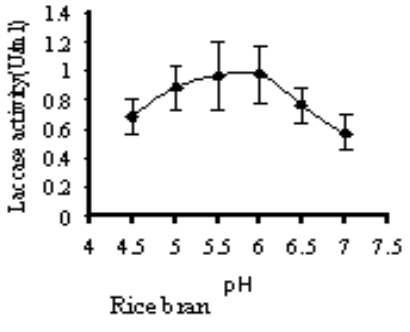

Fig.1

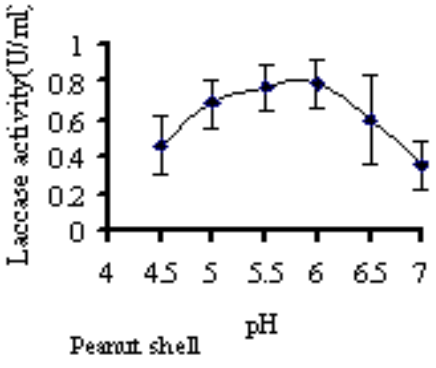

Fig.2

Effect of incubation time on laccase production in rice bran and pea nut shell

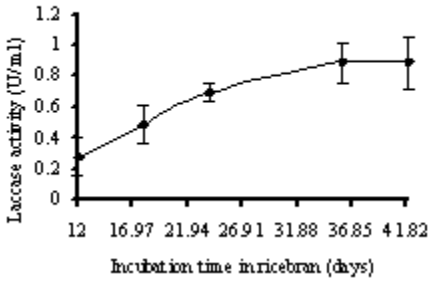

Fig-3

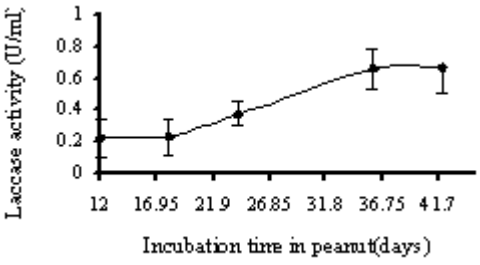

Fig-4

Effect of incubation temperature on laccase production in rice bran and pea nut shell

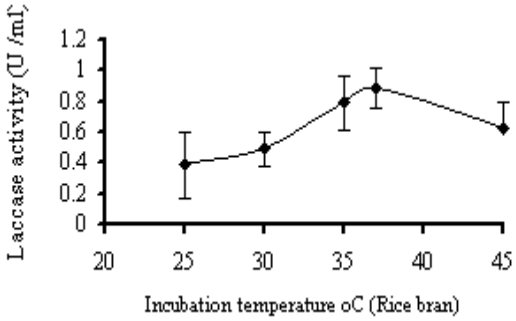

Fig-5

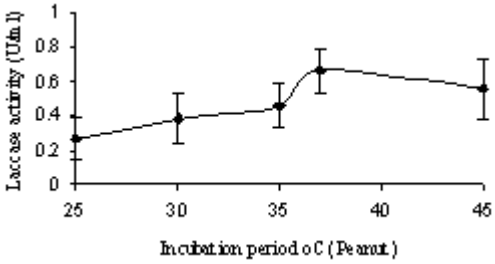

Fig-6

Effect of different concentration of glucose and chick pea powder on laccase production in rice bran and pea nut shell

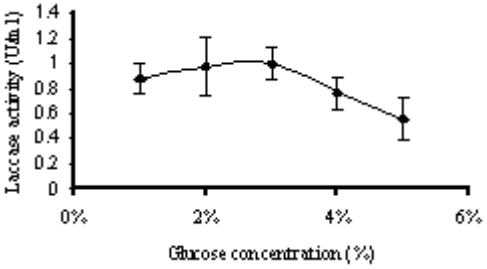

Fig- 7

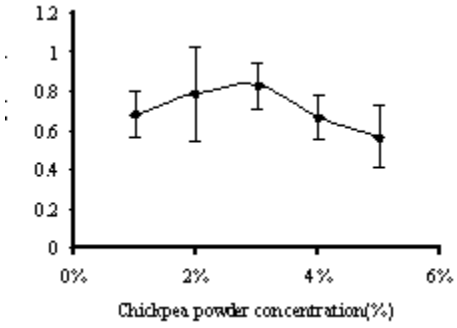

Fig-8

Laccase activities in solid medium of *C. versicolor* are also depend on incubation period. The highest laccase activity after 36 days of cultivation was obtained on rice bran (1.00 U/g substrates) and in peanut shell (0.82U/g substrate). Laccase production in solid substrate, rice bran was higher than peanut shell (Fig 3 and 4). These results are agreement with the findings of Munoz *et al* (1997) who worked on *C. versicolor* for the

production of laccase using rice bran. Machuca and Ferraz (2001) suggested that *C. versicolor* produced the highest laccase from solid state culture at 42 days of incubation of rice bran.

Cultivation on rice bran and peanut shell clearly exhibited the highest laccase production and, if the time course of enzyme production is considered, it is evident that laccase activity still increased after 36 days cultivation and this have to be further studied (Kasinath *et al* 2004) (Fig3 and 4).

It was also noticed that laccase production is maximum at temperature 30-35^{o}C, with optimum temperature 37^{o}C. Laccase activity of this fungus was not favoured by low or high temperature. Temperature higher than 37^{o}C reduced the activity of ligninolytic enzymes (Fig-5 and 6). Similar observations are made by Saparrat (2000). He study that laccase activity in solid medium of *C. versicolor* are also dependent on temperature.

It was observed that carbon requirement affected enzyme activity of *C. versicolor*. Glucose supported highest enzyme activity (0.4850-0.8350 U/ml) after 36 days of incubation (Fig-7).The different concentrations of glucose (ac carbon source) that were tested for laccase production, (1, 2, 3, 4, and 5%) 3% glucose (0.8350 U/ml) stimulated the highest laccase production (Fig-7). Stajic *et al* (2006) obtained similar results with *Trametes versicolor* and obtained highest yield of laccase on barley bran at 4%. It was also evident that 2 and 4% concentrations of supplement slightly favorable for enzyme activity. As well as 5% treatment depicted minimum diminution.

Laccase activity was also observed in solid substrates in the study containing different concentration of nitrogen source. Among various concentration of chickpea (as nitrogen source) used, 3% chickpea powder stimulated higher production of laccase (Fig 8). Similar observations are made by Moldes, *et al* (2004) who found that laccase was also produced earlier when fungus was cultivated in a substrate with a high nitrogen concentration.

In view of the results obtained, it can be concluded that: Laccase production by *Coriolus versicolor* has been shown to depend markedly on the composition of the culture medium, nitrogen content and parameters such as pH of the production medium, temperature and nutrition parameters. The laccase activity in rice bran and peanut shell was high at pH 4.5 and low at pH 6. Similarly, high yield of laccase was produced in case of 4% of barley bran.

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References

Adejoye, O.D.; Fasidi, I.O. (2010). Effect of cultural conditions on biomass and laccase production in submerged medium by *Schizophyllum commune* (Fries), a Nigerian edible mushroom. EJEAFChe., 9(3): 600-609.

Amaral, P.F.F.; Fernandes, L.A.F.D.; Tavares, A.P.M.; Xavier, A.M.R.; Cammarota, M.C.; Coutinho, J.A.P. and Coelho, M.A.Z. (2004). Decolorization of dyes from textile wastewater by *Trametes versicolor*. Environmental Technology. 25 (11): 1313-1320.

Castro, Ana, I.R.P.; Evtuguin, D.V. and

Xavier, Ana M.R.B. (2003). Degradation of biphenyl lignin model compounds by laccase of *Trametes versicolor* in the presence of 1-hydroxybenzotriazole and heteropolyanion [SiW11VO40]5-. Journal of Molecular Catalysis B: Enzymatic, 22(1-2): 13-20

Claye, S.S. A.; Idouraine, and Weber, C.W. (1996). Extraction of insoluble fiber from five sources. Food Chemistry, 5:305-310.

Da Re, V.; Papinutti, L.;Villalba, L.; Forchiassin, F.;Levin, L. (2008). Preliminary studies on the biobleaching of loblolly pine Kraft pulp with *Trametes trogii* crude extracts. Enzyme Microb. Technol., 43: 164–168.

Domenic, V.C and Byron, (1999). Methodological and bio statistical foundations of clinical neuropsychology and medical and health disciplines. 2nd Ed; 78-89.

Gopinath SCB; Anbu P; Hilda A (2005). Extracellular enzymatic activity profiles in fungi isolated from oil-rich environments. Myco science 46: 119-126.

Hammel, KE.; Tien, M.; Kalyanaraman, B.; Kirk, T.K. (1985). Mechanism of oxidative Ca-Cb cleavage of a lignin model dimer by *Phanerochaete chrysosporium* ligninase. J. Biol. Chem. 260: 8348-8353.

Hofrichter, M. (2002). Review: lignin conversion by manganese peroxidase (MnP). Enzyme Microb. Technol. 30**:** 454-466.

Jonathan, S.G and Fasidi, I.O. (2001). Effect of Carbon Nitrogen and Mineral …..Sources on growth of *Psathyerella autroumbonata* (Pegler), a Nigeria Edible Mushroom. Food Chem., 72: 479-483.

Kasinath, A.; Lang, E. and Sasek, V. (2004). Ligninolytic fungi in bioremediation: extracelluar enzyme production and degradation rate. Soil Biology and Biochemistry.36 (10):1545-1551.

Kirk, T.K and Tein, M. (1988). Lignin peroxidase of *Phanerochaete chrysosporium*. Method Enzymol, 161 B: 238-248.

Machuca, A and Ferraz, A. (2001). Hydrolytic and oxidative enzymes produced by white and brown rot fungi during *Eucalyptus grandis* decay in solid medium. Enzyme and microbial technology, 29: 386-391.

Modi, D.R.; Chandra, H. and Garg, S.K. (1998). Decolorization of bagasse-based paper mill effluent by the white-rot fungus *Trametes versicolor*. Bioresource Technology. 66(1): 79-81.

Moldes, D.M.; Lorenzo and Sanroman, M.A (2004). Different proportions of laccase isoenzymes produced by submerged cultures of *Trametes versicolor* frown on lignocellulosic waste. Biotehnol. Lett, 26: 327-330.

Munoz, C.F; Guillen, A.T and Martinez, M.J. (1997). Induction and characterization of laccase in the lignolytic fungus *Pleurotus eryngii*. Current. Microbiology. 34:1-5.

Nagai, M.; Sakamoto, Y.; Nakade, K.; Sato, T (2007). Isolation and characterization of the gene encoding a manganese peroxidase from *Lentinula edodes*. Mycoscience 48: 125-130.

Novotny, C.; Svobodova, K.; ErbanovA, P.; Cajthaml, T.; Songulashvili, G; Elisashvili, V; Wasser, S.P.; Nevo, E.; Hadar, Y.(2007). Basidiomycetes laccase and manganese peroxidase activity in submerged fermentation of food

industry wastes. Enzyme. Microb. Technol. 41: 57-61.

Robles, A.; Lucas, GA.; Cienfuegos and Galvez, A. (2000). Phenol-oxidase (laccase) activity in strains of the hyphomycete Chalara paradoxaisolated from olive mill wastewater disposal ponds. *Enzyme Microb*. Technol, 26: 484-490.

Saparrat, M.C.N. (2004). Optimizing production of extracellular laccase from Grammothele subargentea CLPS no. 436 strain. World J Micro. Biotehnol; 20:583-6

Stajic, M.; Persky, L.; Friesem, D.; Hadar,Y.; Wasser, S.P.; Nevo, E.; Vokojevic, j.(2006). Effect of different carbon and nitrogen sources on laccase and peroxidases production by sele cted *Pleurotus* species. Enzyme microb. Technol. 38:65-73.

Swamy, J. and Ramsay, J.A (1999). The evaluation of white rot fungi in the decolouration of textile dyes. Enzyme Microbial Technology, 24(3-4):130- 137.

Tanaka, H.; Itakura, S. and Enoki, A. (1999). Hydroxyl radical generation by an extracellular low molecular-weight substance and phenol oxidase activity during wood degradation by the white-rot basidiomycete *Trametes versicolor*. J. Biotechnology 75(1): 57-70.

Zahida, N.; Tasnim, K.; Shahjahan, B.; Quratul ain, S.; Nadeem, M. and Rukshana, B. (2007). Bistage control of pH, temperature and agitation speed for improving mycelial growth of edible and medicinal mushrooms in shake flask culture. Pak. J.Biochem. Mol. Bio, 40 (1)**:** 15-22.

Zahida, N.; Rukshana, B.; Tasnim, K. and Shahjahan, B. (2004). Influence of carbon and nitrogen source on the biodegradation of agricultural wastes with *Ganoderma lucidum***.** Pak. J Biochem. Mol. Bio, 40 (1)**:** 15-22.