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Full Length Research Paper

Screening and assessment of laccase producing fungi isolated from different environmental samples

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Laccase is a copper-containing polyphenol oxidase that acts on a wide range of substrates. This enzyme is found in many plant species and is widely distributed in fungi including wood-rotting fungi where it is often associated with lignin peroxidase, manganese dependent peroxidase, or both. Because of its importance in bioremediation, fungal cultures were screened for laccase positive production by plate test method using the indicator compound guaiacol. Out of 12 cultures tested, six cultures were found to be laccase-positive with *Stereum ostrea* and *Phanerochaete chrysosporium* being the best potential cultures. Laccase production on 5 different liquid media was compared using these two white rot fungi.

Key words: Laccase, guaiacol, isolation, *Stereum ostrea, Phanerochaete chrysosporium*.

INTRODUCTION

Laccases (benzenediol:oxygen oxidoreductases; EC 1.10.3.2) are multicopper enzymes belonging to the group of blue oxidases which exist widely in nature and are defined in the Enzyme Commission (EC) nomenclature as oxidoreductases which oxidize diphenols and allied substances and use molecular oxygen as an electron acceptor (Thurston, 1994; Kiiskinen et al., 2002; Kiiskinen and Saloheimo*,* 2004). They are predominantly found in higher plants and fungi (Thurston, 1994; Mayer and Staples, 2002). Their substrate flexibility makes laccases highly interesting for diverse applications, including textile dye bleaching, pulp bleaching and bioremediation, where enzymatic catalysis could serve as a more environmentally benign alternative than the currently used chemical processes (Gianfreda et al., 1999; Xu, 1999). In contrast to most enzymes, which are generally very substrate specific, laccases act on a surprisingly broad range of substrates, including diphenols, polyphenols, different substituted phenols, diamines, aromatic amines, benzenethiols and even some inorganic compounds such as iodine (Xy, 1996). When oxidized by a laccase, the reducing substrate loses a single electron and usually forms a free radical (Kersten et al., 1990; Thurston, 1994). The unstable radical may undergo further laccase-catalysed oxidation or non-enzymatic reactions including hydration, disproportionation and polymerization (Thurston, 1994). Figure 1 shows the schematic laccase-catalyzed oxidation of a *p*-diphenol. In laccase-mediated reactions, diphenolic compounds undergo a four-electron oxidation. During this reaction, Cu(II) is reduced to Cu(I). In the next step in the reaction, Cu(I) reduced molecular oxygen (O_2) to produce two molecules of water. During this reaction Cu(I) is oxidized back to Cu (II) thus completing the reaction cycle.

Laccases from fungi have been implicated in lignin degradation, in differentiation and in protection from toxic phenolic monomers of polyphenols. Laccases 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 (Aadulla et al., 2000) transform toxic compounds into safer metabolites and may be useful to control environmental pollution (Gianfreda et al., 1999). Laccases are also useful for the decomposition of azo dyes by oxidative methods (Michael et al., 2005). In view of its importance in large scale application, the present endeavor is to search for highly efficient laccase producing fungi with secretion of abundant amounts of laccase and to reduce the cost of production. Hence, the

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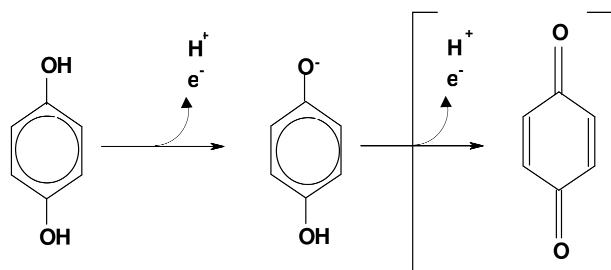
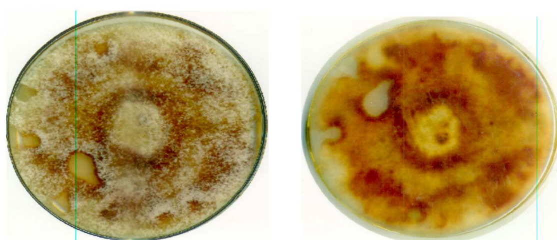


Figure 1. Laccase-catalyzed oxidation of a diphenol.

Phanerochaete chrysosporium



Stereum ostrea

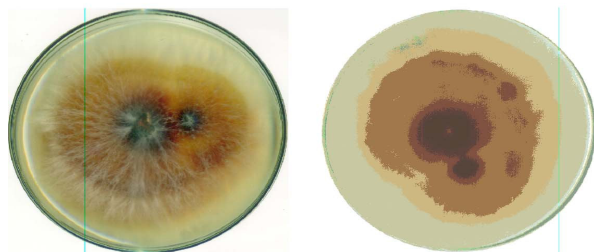


Figure 2. Oxidative polymerization of guaiacol to form reddish brown zones in the medium.

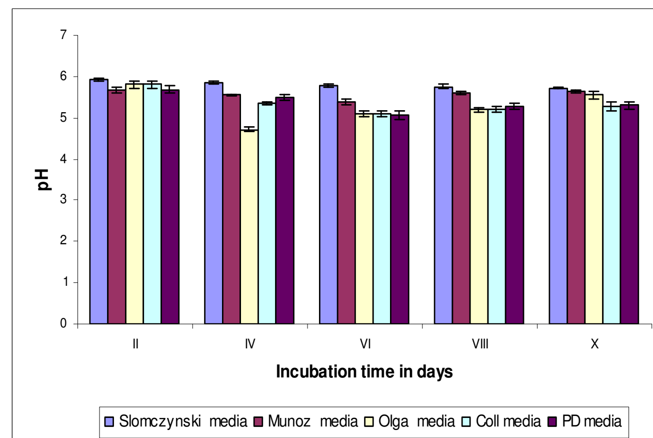


Figure 3. Changes in the pH of broths of *Stereum ostrea* cultures on different media Values are means for three replicates.

present work reports the production of laccases by a local isolates of *Stereum ostrea* and *Phanerochaete chrysosporium* by submerged fermentation.

MATERIALS AND METHODS

Fungi and laccase assay

Selection for laccase producing organisms was done on plates containing 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-6) supplemented with 0.02% guaiacol. Twelve different species of fungi i.e., *Phanerochaete chrysosporium, Theliophora terristrus, Stereum ostrea, Lenzitis betulina, Cunninghamella echinulata, Chaetomium globosum, Aspergillus flavus, Paecilomyces varioti, Penicillium rubrum* were inoculated into these plates and the plates were incubated at 30°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 (Coll et al., 1993).

Cultivation of fungal cultures

Two fungal cultures were cultivated on five media for production of laccase. Five media used in the study were those of Olga et al. (1998), medium of Slomczynski et al. (1995), medium of Munoz et al. (1997), medium of Coll et al. (1993) and potato dextrose medium. The medium of Olga et al. (1998) contained (in 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 $MgSO_4$; medium of Slomczynski et al. (1995) contained (in g/l): 40.00 glucose, 7.00 glycerol, 0.50 L-histidine, 0.10 $CuSO_4$, 1.80 $NaNO_3$, 01.80 NaCl, 0.50 KCl, 0.50 $CaCl_2.H_2O$, 0.05 $FeSO_4.7H_2O$, 1.00 KH_2PO_4, 0.50 $MgSO_4.7H_2O$; medium of Munoz et al. (1997) included ingredients (g/l) 10.00 glucose, 2.00 ammonium tartrate, 1.00 KH_2PO_4, 01.00 Yeast extract, 0.50 $MgSO_4.7H_2O$, 0.50 KCl, 1 ml mineral solution (Composition of mineral solution (in g/l) 0.10 $Na_2B_4O_7.10H_2O$, 0.07 $ZnSO_47H_2O$, 0.05 $FeSO_4.7H_2O$, 0.01 $CuSO_4.5H_2O$, 0.01 $MnSO_4.4H_2O$, 0.01 $(NH_4)_6MO_7O_{24}.4H_2O$); Medium of Coll et al. (1993) contained (in g/l) 10.00 glucose, 1.00 aspargine, 0.50 yeast extract, 0.50 K_2HPO_4, 1.00 $MgSO_4.7H_2O$, 0.01 $FeSO_4.7H_2O$; and Potato Dextrose (PD) medium which contained (g/l) 20.00 dextrose, 200.00 potato extract. All these media were adjusted to pH 6.00.

The fungal cultures *S. ostrea* and *P. chrysosporium* were maintained on medium containing (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 6). Fungal spore suspension was prepared by adding 2 ml of sterile distilled water to these freshly (7 days) grown slants of the above cultures. These suspensions were used to inoculate in 50 ml of different media into 250 ml Erlenmeyer flasks s used in the present study. The flasks were incubated on a gyratory shaker with shaking at 200 rpm at 30°C. At desired intervals, 250 ml Erlenmeyer flasks with growing culture of *S. ostrea* and *P. chrysosporium* were withdrawn during the course of different experiments. Each sample was monitored for fungal growth, pH, protein and laccase activities.

The cultures in the flasks were aseptically filtered through pre-weighed Whatman No 1 filter paper to separate mycelial mat and culture filtrate. The filter paper along with mycelial mat was dried at 70°C in an oven until constant weight and this weight was recorded. Difference between the weight of the filter paper bearing mycelial mat and weight of only filter paper represented biomass of fungal mat. Fungal growth was expressed in terms of mg/flask.

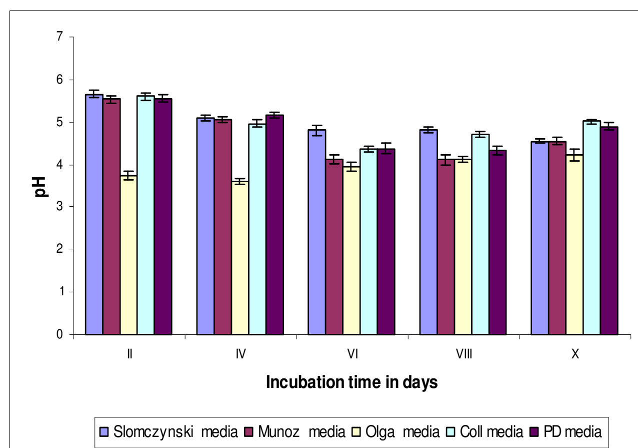


Figure 4. Changes in the pH of broths of *Phanerochaete chrysosporium* cultures on different media Values are means for three replicates

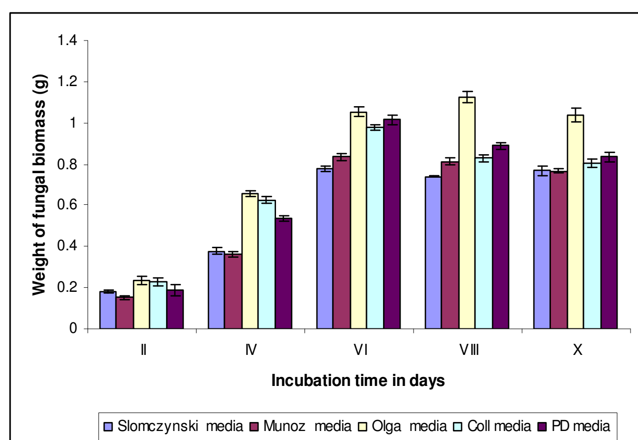


Figure 5. Growth of *Stereum ostrea* on different media Values are means for three replicates.

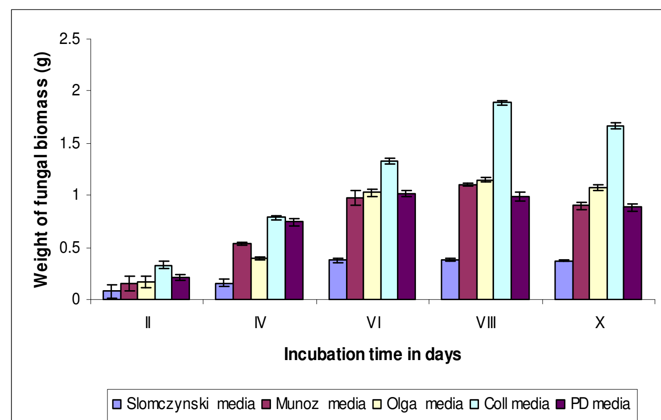


Figure 6. Growth of *Phanerochaete chrysosporium* on different media Values are means for three replicates

An aliquot of these *S. ostrea* and *P. chrysosporium* culture filtrates with appropriate dilution was used for estimation of soluble protein content according to the Lowry et al. (1951). Bovine serum albumin was used as protein standard.

Enzyme activity

Enzyme activity was assayed at 30°C by using 10 mM guaiacol in 100 mM acetate buffer containing 10% (v/v) acetone. The changes in absorbance of the reaction mixtures containing guaiacol was monitored at 470 nm (ε = 6,740 M^{-1} cm^{-1}) for 5 min of incubation. The enzyme activities were calculated using an extinction coefficient of 6,740 M^{-1} cm^{-1} and expressed as Katals (1 mol of substrate conversion/s) (Das et al., 1997).

Table 1. Screening of Laccase-producing fungi by plate tests using the indicator compound Guaiacol.

Name of the fungi	Result
Phanerochaete chrysosporium (Basidiomycete)	+
Theliophora terristrus (Basidiomycete)	+
Stereum ostrea (Basidiomycete)	+
Lenzitis betulina (Basidiomycete)	+
Cunninghamella echinulata (Zygomycetes)	-
Chaetomium globosum (Ascomycetes)	+
Aspergillus flavus (Deuteromycete)	-
Aspergillus fumigatus (Deuteromycete)	-
Aspergillus niger (Deuteromycete)	-
Aspergillus sydowi (Deuteromycete)	-
Paecilomyces varioti (Deuteromycete)	-
Penicillium rubrum (Deuteromycete)	+

RESULTS AND DISCUSSION

In order to find laccase producing fungi from the fungi isolated from various environmental samples, a simple screening method was followed using solid media containing indicator compound guaiacol. A total of 12 species were screened and among these, 6 were laccase positive (Table 1). From these laccase positive fungi *P. chrysosporium* and *S. ostrea* were selected for further studies as the oxidative polymerization of guaiacol to form reddish brown zones in the medium was high in these two organisms compared to other fungi (Figure 2).

During the course of growth of both *S. ostrea* and *P. chrysosporium*, pH changes occurred in the culture broth (Figures 3 and 4). Very low pH was observed in culture of *P. chrysosporium* grown in Olga et al. (1998) medium with 3.61 on 4th day incubation as against 4.72 in culture broth of *S. ostrea*. Likewise changes in pH were also more in the culture broth of *P. chrysosporium* on other media.

Maximum biomass of *S. ostrea* was recovered at the end of 8th day incubation from Coll et al. (1993) medium with 1.877 grams whereas in case of *P. chrysosporium*, high biomass was observed in Olga et al. (1998) medium with 1.124 on 8th day incubation. Growth of *P. chrysosporium* was slower than *S. ostrea* in all the tested media (Figures 5 and 6). Very low growth was observed in the media of Slomczynski et al. (1995). Though growth and secretion of extracellular protein reached peak on the

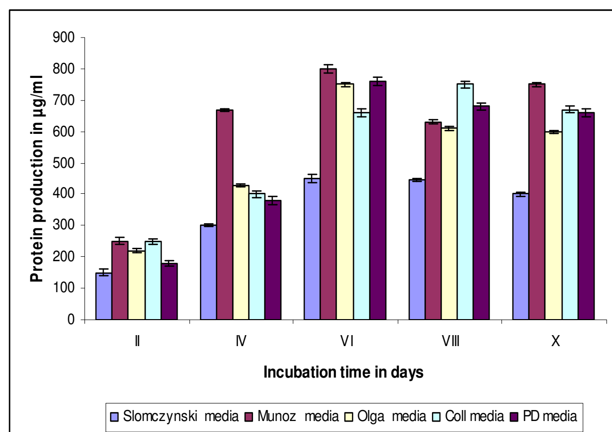


Figure 7. Secretion of extracellular protein in the filtrate of *Stereum ostrea* Values are means for three replicates.

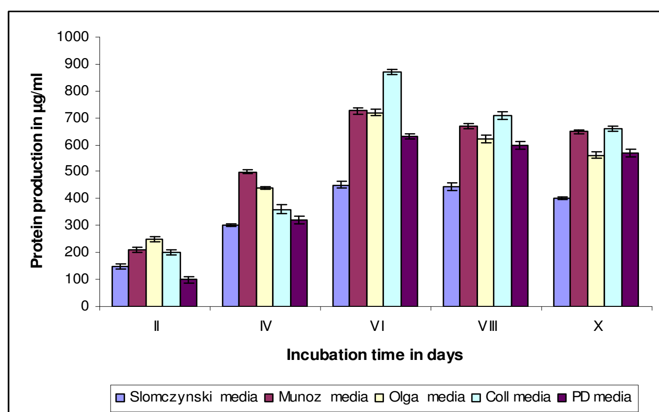


Figure 8. Secretion of extracellular protein in the filtrate of *Phanerochaete Chrysosporium*.Values are means for three replicates.

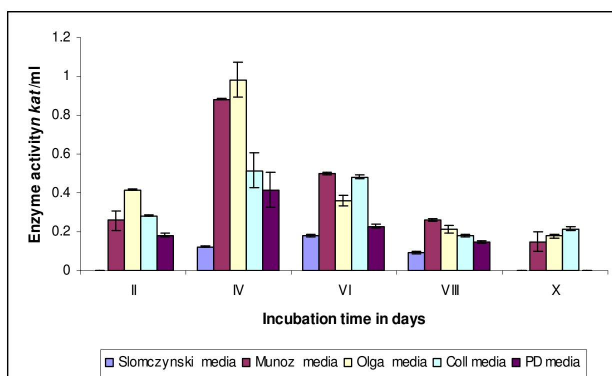


Figure 9. Laccase activity in the filtrate of *Stereum ostrea.* Values are means for three replicates.

sixth day of incubation, but maximum activity of laccase occurred on 4th day of incubation.

At the end of 6th day of incubation, maximum of about 800 µg/ml of extracellular protein was secreted into the culture medium of *S. ostrea* grown in Munoz et al. (1997) medium as against highest value of 870 µg/ml of extracellular protein in the filtrate of *P. chrysosporium* in Coll et al. (1993) medium (Figures 7 and 8).

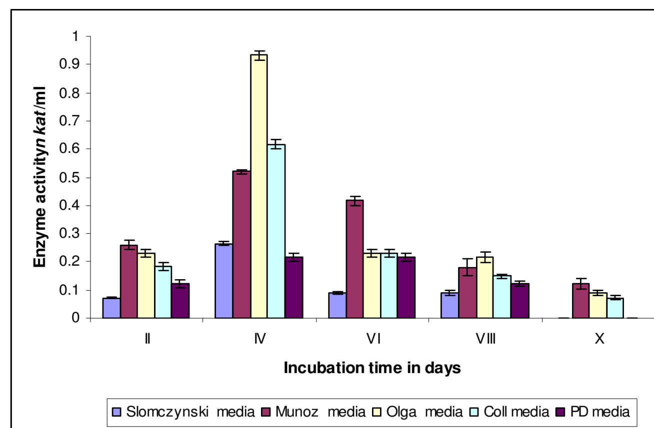


Figure 10. Laccase activity in the filtrate of *Phanerochaete chrysosporium.* Values are means for three replicates

Production of laccase by both cultures reached maximum on 4th day of incubation in culture broths of all five media used in this study and declined these onwards (Figures 9 and 10). Among the five media tested in this study Olga et al. (1998) medium yielded highest titres of laccase by both cultures. *S. ostrea* was as good as the reference culture, *P. chrysosporium*, in producing laccase.

Maximum laccase activity occurred in the filtrates of both *S. ostrea* and *P. chrysosporium* grown in Olga et al. (1998) medium with 0.983 *n* kat/ml and 0.933 *n* kat/ml at 4th day (Figures 9 and 10). In this study, nitrogen containing compound i.e., peptone might have been the crucial factor for efficient laccase production. Induction of laccase production by high medium nitrogen content has been detected in the Basidiomycete I-62 (CECT 20197) (Mansur et al., 1998), in *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 (Niku-Paavola et al., 1990; Rogalski et al., 1991; Schlosser et al., 1997). Thus further work is in progress to test various combinations of different nitrogen and carbon sources as well as compounds that have been reported to be inducers of laccase production to obtain large amount of laccase activity in both *S. ostrea* and *P. chrysosporium*.

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