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Lignolytic and lignocellulosic enzymes of *Ganoderma lucidum* in liquid medium

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ABSTRACT

Lignin is probably one of the most recalcitrant compounds synthesized by plants. This compound is degraded by few microorganisms. White-rot fungi have been extensively studied due to its powerful ligninolytic enzymes. Ganoderma lucidum is one such organism that can degrade lignin and hence it was isolated and screened qualitatively by guaiacol plate assay. Spectrophotometric enzyme assays were also carried out to examine the production of laccase, manganese peroxidase and lignin peroxidase quantitatively. Manganese peroxidase activity of G. lucidum increased from 0.05 units/ml to 0.1 units/ml during incubation. Also high quantities of exo and endoglucanases (C_x & C_1) were produced by 20th day culture. Therefore, the results of the present study allow us to conclude that wild G. lucidum is a good candidate for scale-up ligninolytic enzyme production.

Keywords: Lignolytic enzymes, Guaiacol assay, Laccase, Lignin peroxidase, Manganese peroxidase

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INTRODUCTION

Lignin, the second most abundant renewable organic polymer on earth, is a major component of wood. Because of the importance of wood and other lignocellulosics as a renewable resource for the production of paper products, feeds, chemicals, and fuels, there has been an increasing research emphasis on the fungal degradation of lignin [1, 2]. White rot fungi are believed to be the most effective lignin-degrading microbes in nature. A majority of the previous studies have focused on the lignin-degrading enzymes of *Phanerochaete chrysosporium* and *Trametes versicolor* [3, 4]. Recently, however, there has been a growing interest in studying the lignin-modifying enzymes of a wider array of white rot fungi, not only from the standpoint of comparative biology but also with the expectation of finding better lignin-degrading systems for use in various biotechnological applications [5-8].

White rot fungi produce various extra cellular 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. These enzymes can oxidize phenolic compounds creating phenoxy radicals, while nonphenolic compounds are oxidized via cation radicals [1, 2, 6, 9]. This lignolytic system of white- rot fungi is also directly involved in the degradation of various xenobiotic compounds and dyes [10].

Some of the strains of white rot fungi were subjected to lignin degradation by [11]. All strains that produced more EPS showed good lignin degradation activity. Some of these strains were studied by [12] for laccase and peroxidase production and all of them showed enzyme activity.

Laccase probably participates in lignin degradation by oxidizing phenolic lignin units or even non-phenolic units in the presence of some laccase substrates [13, 14]. However, similar enzymes could play various physiological roles (e.g. melanin biosynthesis, morphogenesis, detoxification, etc.) in other fungal groups.

MnP production by *Ganoderma australe, D. squalens, P. ostreatus* and *T. hirsut*a had already been described [15-19]. The production of lignin-modifying enzymes (LME) varies in the *Ganoderma* genus and depends on substrate type and culture conditions [8]. Laccases were detected as the unique oxidative enzyme in submerged liquid cultures of *G. lucidum* [5].

A work by [20] with four *Ganoderma* spp. strains indicated that all strains produced laccase, while LiP and MnP were detected in only two of them. The apparent discrepancy among several reports emphasizes that LME production varies in a genus and that it depends on the substrate type and the culture conditions.

Xylanases belong to the group of hemicellulolytic enzymes which are required for the hydrolysis of β-1, 4-xylans present in lignocellulosic materials. Several microorganisms have been reported as xylanolytic, and most of the bacteria, fungi and yeasts producing xylanases secrete the enzyme extracellularly. Exoglucanase and endoglucanase are the two cellulose degrading enzymes. Cellulases are a complex enzyme system, comprising endo-1,4- β-D-glucanase (EC-3.2.1.4), exo-1,4-β-glucanase (exocellobio hydrolase, EC-3.2.1.91) and β-D-glucosidase (β-D-glucoside glucan hydrolase, EC-3.2.1.21). These enzymes together with other related enzymes, *viz.*, hemicellulases and pectinases are among the most important group of enzymes that are employed in the processing lignocellulosic materials for the production of feed, fuel and chemical feed stocks.

Ganoderma lucidum is a saprophytic fungus that tends to grow more prolifically in warm climates on decaying hardwood logs and stumps. Under commercial cultivation conditions, *Ganoderma lucidum* is normally grown on artificial saw dust logs. Previous studies of *G. lucidum* have mainly concentrated on the medicinal properties of this fungus [21] and, except for two brief preliminary reports [22, 23] little is known about the ligninolytic system of this organism. In this report, we describe the production of ligninolytic, xylanolytic and cellulolytic enzymes by *G. lucidum*.

MATERIALS AND METHODS

Isolation and maintenance of macrofungi

The strain of *G. lucidum* was collected from the Bharathidasan University campus, Tiruchirappalli District, Tamil Nadu from decaying wood and identified as *G. lucidum* by using conventional description method. Culture was prepared on malt extract agar (MEA) by tissue culture from the basidiocarp. Slants were incubated for 5 to 7 days and were observed. The mycelium collected from the growing edge was transferred into new malt agar slant and incubated further for 5 to 7 days. This was repeated 2 to 3 times to get pure isolates and was stored at 4°C. Approximately 2 mm^2 of mycelial mat was removed from slants and was allowed to grow on malt agar slants for 7 days.

i) Lignolytic activity

Ganoderma lucidum was screened qualitatively for its ability to produce ligninases. Malt agar plates supplemented with 0.02% guaiacol were used for screening of the fungi.

2 mm^2 of the each fresh fungal mycelium pre-cultured on malt agar plates were inoculated on to the center of these plates and incubated for 3 days. The fungus containing the enzyme activity developed circular zones of reddish brown colour due to the oxidation of guaiacol and noted as ligninase positive. The cultures which did not produce zones were further incubated up to seven days and observed. If there is no activity then discarded. The fungi was inoculated into malt extract broth and incubated for 10 and 20 days at room temperature. After incubation, cultures were harvested, the mycelial mat was separated and the culture filtrate was used for quantification of cellulolytic (c_x and c_1) and lignolytic (Lip, Mnp and Lac) enzymes.

Lignolytic Enzymes
Laccase was measured according to the method of [2]. Sodium acetate buffer (50 mM, pH 4.5), 2 mM guaiacol, 0.5 ml enzyme source and distilled water were taken in a total volume of 2.5 ml. The activity was measured at 440 nm and increase in absorbance was noted for 2 minutes. The activity was expressed as the amount of tetraguaiacol formed per min per ml of enzyme extract.

Lignin Peroxidase was assayed in an assay mixture consisting of 250 µl sodium tartarate (pH 2.5), 2 mM veratryl alcohol, 0.4mM H_2O_2 and 50-275 µl of enzyme in a total volume of 0.5 ml. The reaction was initiated by addition of H_2O_2 and oxidation of veratryl alcohol to veratralatehyde was determined by an increase in absorbance at 310 nm [24].

Manganese peroxidase was assayed as follows : 0.5 ml sodium tartarate buffer (100 mM) (pH 5.0) was taken and added with 0.5 ml $MnSO_4$ (100 mM) followed by 0.5 ml guaiacol (100 mM) and 0.1 ml of enzyme culture filtrate and 1 ml of distilled water. The contents were shaken thoroughly and 0.5 ml of H_2O_2 (50 mM) was added and immediately the absorbance was observed at 465 nm for every 20 seconds and enzyme activity was expressed in units/ml/min[25].

Cellulolytic Enzymes
Cellulases (C_x) or endo-glucanase activity was assayed by viscometric method suggested by [26]. Ostwald Fenske viscometers made up of Pyrex glass were used. The reaction mixture consisting of 15 ml 0.8% carboxy methyl cellulose (CMC), 5.0 ml of enzyme and 1.0 ml of citrate buffer pH 5.6 was poured in to the viscometer up to the mark and time taken for flow was noted. Loss of viscosity was measured after every 10 minutes and expressed in terms of relative enzyme activity (REA). REA was calculated by using the formula $1000/t_{50}$ where t_{50} is the time taken for 50% loss of viscosity.

The percentage of viscosity change was calculated as

$$\text{Loss of viscosity (\%)} = \frac{t_i - t_a}{t_i - t_a} \times 100$$

t_i = initial flow time of reaction mixture with control (inactivated enzyme)
t_a = initial flow time of reaction mixture with enzyme
t_o = initial flow time of water with enzyme

Cellulases C_1 – or Exo-glucanase activity was measured by DNS method [27]. Reaction mixture consisting of 3.5 ml of 0.8% CMC, 1.0 ml of citrate buffer, pH 5.6 and 0.5 ml of enzyme was incubated at room temperature for 6 hours. From this mixture 0.2 ml was taken and added with3 ml of 3, 5 - dinitrosalysilic acid (DNS) reagent and placed in a boiling water bath for 15 minutes. Two ml of 20% sodium potassium tartarate was added while the tubes were hot. Then the tubes were cooled immediately to room temperature under running tap water. The absorbance was measured at 575 nm and activity was expressed in terms of mg/ml of reducing sugars liberated in 6 hours.

ii) **Xylanolytic Activity**
The screening of the fungi for their extra cellular xylanolytic (hemi cellulolytic) ability was evaluated on Malt Extract Agar (MEA) containing 0.1% (w/v) birch wood xylan. The pH of the medium was adjusted with 1N NaOH and 1N HCl. 75 mg of streptomycin was added prior to sterilization to avoid bacterial contamination. After autoclaving media was cooled and poured to sterile petriplates aseptically. On solidification, the plates were inoculated in the centre with 1 cm^2 mycelium disc of fungal culture under study and incubated at 28 ± 1^0C for a week. Three replicates were maintained for each set of observations. Positive xylanolytic isolates were selected based on the clear zones of hydrolysis after flooding the plates with 0.1% aqueous Congo red for 15 minutes followed by repeated washing with 1M NaCl [28].

RESULTS AND DISCUSSION

White coloured mycelium of *G. lucidum* appeared in MEA plates and grows by spreading over the medium two days after incubation. Green halo was not formed during the incubation period in the guaiacol plate indicating less or no lignolytic activity. The diameter of the halo and the color intensity indicating a positive extracellular oxidoreductase secretion from mycelium was used to screen the level of ligninolytic enzyme production. Some white rot fungi produced all lignin modifying enzymes [29-31] whereas most white rot fungi lack one or more lignolytic enzymes indicating that not all enzymes are essential for lignin degradation. Similar to present observations [14] and [32] analyzed the biochemical characterization of lignolytic enzymes from different macrofungi with special reference to white rots. Zone of hydrolysis on Malt Extract Agar (MEA) containing 0.1% (w/v) birch wood xylan was 6.6 cm (Table 1), indicates the presence of xylanolytic enzymes in *G. lucidum*.

Table 1. Screening of Lignolytic and Xylanolytic Activity on Solid Media

Enzymic Activity	Diameter of Zone of hydrolysis
Lignolytic Activity	-
Xylanolytic Activity	6.6 cm

Different enzyme activities are depicted in the Table 2. Laccase activity was more in 10th day culture and decreased after further incubation. The low values for laccase activity reported in this study may have resulted because of the fungi grown at unsuitable conditions, or the presence of inhibitors in the growth media.

Table 2. Screening of Lignolytic and Cellulolytic Enzyme Activity in Liquid Media after 10 and 20 days of Incubation

Enzyme		10th Day	20th Day
Laccase		5 units/ml	0.045 units/ml
Lignin peroxidase		0	0
Manganese Peroxidase		0.05 units/ml	0.1 units/ml
Cellulolytic Enzyme	C_x	29.790	53.07
	C_1	0.233 mg/ml	0.388 mg/ml

Lignin peroxidase content was reported to be nil after 10 and 20 days of incubation. Manganese peroxidase activity increased from 0.05 units/ml after 10 days of incubation to 0.1 unit/ml after 20 days of incubation. The above result was supported by the findings of [33]. Manganese peroxidase is the most common lignin-modifying peroxidase produced by almost all wood-colonizing basidiomycetes causing white-rot and various soil-colonizing litter-decomposing fungi [34]. *G. lucidum* also produced high quantities of exo and endoglucanases (C_x & C_1) with high production exhibited by 20th day culture. The present investigation findings are similar as reported by several workers with different basidiomycetes [35, 36]. These extracellular lignolytic enzymes have potential biotechnological applications in degradation of polyaromatic hydrocarbons (PAHs) [37], heavy metal removal [38], decolorization of industrial dye [39] and solid west management [33].

CONCLUSION

To conclude, the present study clearly demonstrated that *Ganoderma lucidum* is a good candidate for scale-up production of ligninolytic and lignocellulosic enzymes.

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