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Ligninolytic Activity of a Promising Fungal Isolate from Tropical Areas of Haryana

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Abstract

Lignin is extremely recalcitrant to degradation and due to its linking with both, cellulose and hemicellulose, it creates a barrier for chemicals and enzymes to penetrate the interior lignocellulosic structure. Lignin is degraded by an enzyme complex; containing three enzymes laccase, manganese peroxidase and lignin peroxidase which are collectively known as ligninases. These enzymes are produced by several microorganisms, majorly by fungi but surprisingly most of them have high production cost. During the present investigation, sixteen distinct fungi were isolated from soil samples collected from tropical areas of Haryana and screened for their ligninolytic activities using 0.04% aniline blue dye in malt extract agar medium and the zone of dye clearance was observed which ranged from 1.03 to 1.20. Ligninase activity for five selected isolates based on their zone of clearance was measured. Out of five fungal isolates, one isolate; HST 15 showed highest lignin peroxidase activity (21 U/ml). Its laccase activity was 6.0 U/ml and manganese peroxidase activity was 1.60 U/ml. Microscopically, on the basis of their hyphae, sporangiophore and spores, it was identified to be an ascomycete.

Keywords: Ligninolytic, Ligninase, Laccase, Lignin Peroxidase, Manganese Peroxidase.

Introduction

Lignin, next to cellulose and hemicelluloses, is the third most abundant compound in plant biomass. Its molecular weight is high and various biologically stable linkages are present therefore it is resistant to microbial degradation. It provides mechanical strength and rigidity to vascular plants. When vascular plants die or drop litter, lignified organic carbon is incorporated into the top layer of the soil. This recalcitrant material has to be broken down and recycled by microorganisms to maintain the earth's carbon cycle.

Different conventional methods used for lignin degradation including pulping or enzymatic hydrolysis are being discouraged due to energy and environmental concerns. Therefore microbial lignin degradation is drawing attention as an alternative to pulping or enzymatic hydrolysis of lignocellulosic materials. However, degrading lignocellulosic biomass by current available microorganism is still far to meet the industrial demands (Shary *et al*., 2007; Afrida *et al*., 2009). Thereby, isolation of new microbial strains for degradation of lignin is essential.

Among various microorganisms, fungi are more efficient candidates for lignin degradation, due to their ability to derive energy via decomposition of organic matter and not through photosynthesis. Generally, molds secrete extracellular hydrolytic enzymes mainly from the hyphal tips. These enzymes degrade complex biopolymers such as starch, cellulose and lignin into simpler substances, which can be absorbed by the hyphae. It is reported that fungi belonging to different genera, such as *Aspergillus fumigatus, A. japonicus, A. niger, A. terreus* and *Penicillium simplicissimum*, were capable of degrading both aromatic and carbohydrate components of water-soluble lignocarbohydrate complexes (LCC) or Kraft lignin (Milstein *et al*., 1984; Zeng *et al*., 2006).

Lignin degrading fungi are either white rot fungi or brown rot fungi. White rot fungi (basidiomycetes) are reported to be the best lignin degrading fungi. (Abdel- Raheem and Shearer, 2002; Urairuj *et al*., 2003). The most well known white rot fungi, which have been studied extensively to establish for the mechanism of lignin degradation are *Phanerochaete chrysosporium* and *Tramestes versicolor* (Urairuj *et al*., 2003).

Lignin biodegradation performed by these fungi is a multistep

process mediated by enzymes of the ligninolytic complex that involve lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac) (Nagai *et al*., 2007). Laccases are blue multicopper oxidases, which catalyze the monoelectronic oxidation of a broad spectrum of substrates, for example, *ortho*- and *para*-diphenols, polyphenols, aminophenols, and aromatic or aliphatic amines, coupled with a full, four electrons reduction of O_2 to H_2O. Hence, they are capable of degrading lignin and are present abundantly in many white-rot fungi. In vitro degradation of lignin was first reported for LiP (Tien and Kirk, 1983; Hammel *et al.,* 1993). Lignin peroxidase appears to be a key enzyme in the oxidation of nonphenolic phenylpropanoid units, which lead to polymer fragmentation (Hammel *et al*., 1985). Manganese peroxidase, one of the important enzymes of fungi, oxidizes Mn^{2+} to Mn^{3+}, which in turn may attack phenolic structures in lignin as long as it is stabilized by suitable metal chelators secreted by fungi (Zhang *et al*., 2006).

In the present study, we analyzed these three ligninolytic enzymes secreted by different fungi isolated from soil samples collected from different ecological niches. Different biochemical assays were used to study their activity. Finally, one isolate with apparently good enzyme activity was identified morphologically.

Aim of the Study

The main aim of this study was to isolate lignin degrading fungi and to screen their efficacy to degrade lignin. Findings show promising results and can be used for further studies.

Materials and Methods

Collection of Soil Samples

Soil samples were collected from different sites like organic waste dumping site, leaf and litter waste site, mushroom waste, paper and pulp waste biogas slurry and compost from northern India region, Haryana, India. Samples were taken from the top 15 cm layer The soil was then homogenized and stored at $4^{O}C$ for bioaugmentation experiments.

Isolation and Screening of Fungal Isolates

Lignin degrading fungal strains were isolated from the collected soil samples using dilution plate technique in malt extract agar medium under stationary conditions at $28\pm2^{o}C$ in the dark. The Malt Extract medium (MEA) contained: malt extract 30 g/L; peptone 5 g/L and agar 20 g/L. For screening MEA medium was supplemented with 0.04% aniline blue. All fungal cultures were cultivated at $28\pm2^{o}C$ for a period of 7 days on the MEA. Ligninolytic enzyme producers decolorized the dye on the plates and a zone of clearance was formed. Zone efficiency was calculated by taking the ratio of clear zone diameter to colony diameter.

Biochemical Analysis

For ligninolytic enzymes production, 100 ml of malt extract broth taken in 250 ml Ehrlenmeyer conical flask and inoculated with approximately 10^7 spores/ml from 7 days old slant of fungal isolates. The flasks were then incubated at $28\pm2^{0}C$ on rotary shaker at 140 rpm. The clear culture filtrate obtained by filtration was used for determining ligninolytic enzymes' activity.

Determination of Laccase Activity

Two ml of culture filtrate was taken in cuvette and placed in UV Spectrophotometer at 495 nm to adjust light absorbance at zero. The cuvette was taken out and dispensed with 2 ml of guaiacol solution (a known volume of 1.24 g of guaiacol and 500 ml of phosphate buffer) and immediately transferred back to spectrophotometer. Change in light absorption was recorded for every 30 seconds.

Determination of Manganese Peroxidase Activity

The culture filtrate, 2.0 ml, was put in cuvette and placed in UV Spectrophotometer at 465 nm to adjust light absorbance at zero. The cuvette was taken out and dispensed in a mixture of 2.0 ml buffered guaiacol (a known volume of 0.6207 g (0.55 ml) of guaiacol and 100 ml phosphate buffer), 0.2 ml of manganese sulfate and 0.2 ml H_2O_2. The change in light absorption was recorded for every 30 seconds.

Determination of Lignin Peroxidase Activity

The culture filtrate, 0.5 ml, was put in cuvette and placed in UV Spectrophotometer at 310 nm to adjust light absorbance at zero. The cuvette was taken out and dispensed in a mixture of phosphate citrate buffer 1.5 ml, veratryl alcohol 1.0 ml, H_2O_2. The change in light absorption was recorded for every 30 seconds.

For all these enzymes, the change in absorbance between 30 and 150 seconds was taken and results were expressed as change in absorbance/minute. An increase in activity by 0.001 in 60 seconds was taken as one unit of enzyme activity.

Morphological Characterization of Isolated Fungal Isolates

Wet mounts of isolated fungi were prepared in water and lactophenol blue and all the fungi were characterized on the basis of their hyphae and arrangement of spores.

Results and Discussion

Isolation and Screening of Fungal Isolates

Twenty four mutually distinct fungi were isolated from different soil samples collected from tropical areas of Haryana. Screening of all the fungal isolates having ligninolytic activity was done on malt extract agar media containing aniline blue dye (0.04%) and their zone of clearance was measured after seven days of incubation at $28\pm2^{O}C$. Out of 24 mutually distinct isolates, 16 were found to be ligninolytic and the zone of clearance varied from 1.03 to 1.20 **(**zone of clearance =A/B where A stands for total diameter (zone + colony) and B stands for colony diameter) (fig. 1). In a similar study Patrick *et al*., 2011 isolated a white-rot fungus *Pleurotus sajor-caju* from coastal Tanzania and screened for crude ligninolytic enzyme production using rhemazol brilliant blue R (RBBR) dye, 2,2-azino-bis (3-ethylbenzthiazoline)-6-sulfonate (ABTS) and guaiacol in a semi-solid medium.

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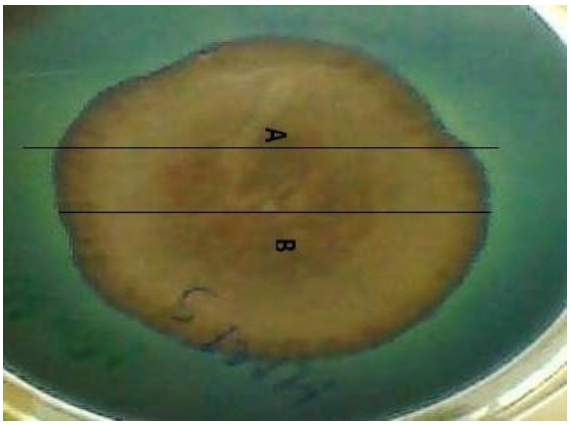

Fig. 1: Measurement of zone of clearance (A/B)

Activity of Lignin Degrading Enzymes (Ligninases) of Fungal Isolates

A total of five cultures were selected for measuring enzyme activity on the basis of their zone of clearance on malt extract agar medium.

Laccase Activity of Fungal Isolates

The capacity of fungal laccase to remove xenobiotic substances and produce polymeric products makes them a useful tool for bioremediation purposes. Laccase activity of the isolates ranged from 0.75 to15.5 U/ml. The isolate HST 9 showed highest laccase activity (15.5 U/ml) (Table 1). Similarly, Arul Diana Christie and Shanmugam in 2012 studied laccase production from four Ascomycetes species and observed that *Alternaria arborescence* showed maximum production of enzyme (800 U/l) at 30^{O} C in 4.5 pH followed by *Fusarium oxysporium* (JQ950134) with 600 U/l at 45^{O} C in 5 pH after 15 days of incubation.

Manganese Peroxidase Activity of Fungal Isolates

The presence of manganese peroxidase can increase the degree of dye decolorization. Manganese peroxidase activity of the isolates ranged from 0.75 to 4 U/ml. The isolate HST 9 had highest manganese peroxidase activity, 4U/ml (Table 1). In a similar study, Varshney *et al.* 2013 investigated the production of MnP by Phanerochaete chrysosporium NCIM 1197 by screening and optimizing media constituents and physiological factors. This strain was shown to produce 70.20 U/ml of MnP on the 8^{th} day of incubation.

Lignin Peroxidase Activity of Fungal Isolates

Lignin peroxidases are heme-containing glycoproteins and play a central role in the biodegradation of lignin. Lignin peroxidase activity of the isolates ranged between 5 to 21 U/ml. The isolate HST15 had highest lignin peroxidase activity (21.0 U/ml) whereas HST9 had lignin peroxidase activity of 5 U/ml (Table 1). A similar study was conducted by Sivakami *et. al.,* 2012. They isolated a ligninolytic fungus which was producing lignin peroxidase enzyme. They optimized conditions for the production of this enzyme.

Table 1

Ligninases Activity of Selected Fungal Isolates

S. No.	Fungal isolate	Laccase activity (U/ml)	Manganese peroxidase activity (U/ml)	Lignin peroxidase activity (U/ml)
1	HST9	15.50	4.00	5.0
2	HST11	2.00	3.00	10.0
3	HST14	0.75	0.75	5.0
4	HST15	6.00	1.60	21.0
5	HST16	8.50	1.50	13.0

Morphological characterization of selected fungal isolates

Morphological studies were on the basis of their hyphae, spores and sporangiophore (Table 2). Isolate 15 was identified to be an Ascomycete (fig. 2).

Table 2 Identification of Selected Fungal Isolates

S.No.	Fungal isolate	Fungi identified
1	HST9	Ascomycetes
2	HST11	Basidiomycetes
3	HST14	Basidiomycetes
4	HST15	Ascomycetes
5	HST16	Ascomycetes

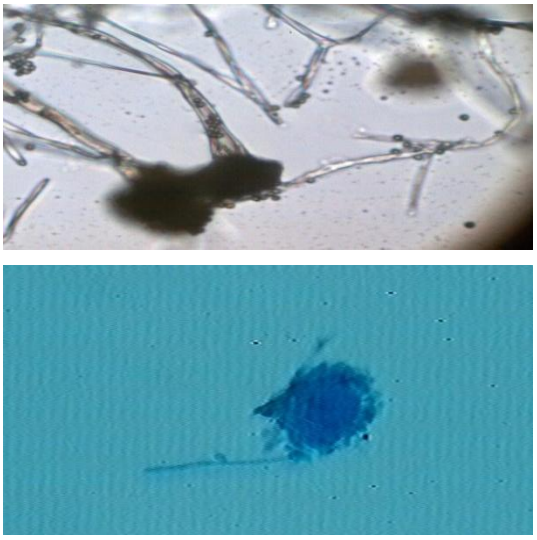

Fig 2: HST 15, Isolated from Garbage Dumping Site

Conclusion

This study concluded that the soil is a habitat for a diverse group of lignin degrading fungi. The isolate HST 15 showed high ligninolytic activity. Hence this isolate can be used for the production of industrially important ligninolytic enzymes and for the degradation of complex agricultural wastes so that dependence on environmentally harsh chemical degradation of lignin is reduced.

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