Isolation of Lignin Peroxidase- producing Plant Pathogenic Fungus from the effluent sample

Ira Chaudhary¹, Faria Fatima¹, Neelam Pathak¹, Smita Rastogi², Rolee Sharma¹

¹Department of Biosciences, Integral University, Kursi Road, Lucknow-226026
²Department of Biotechnology, Delhi Technological University, Delhi-110042

ABSTRACT

The study on lignin peroxidase is importance in industries as well as agriculture also. Fungal cultures from the effluent samples were screened by lignin peroxidase plate assay method using Azure B dye as an indicator. Thus a total of fourteen fungal isolates were obtained. Out of these, three (Alternaria sp., Fusarium sp. and Agaricomycetes sp.) showed appreciable lignin peroxidase activity. After ten days of growth the highest lignin peroxidase activity was showed by Agaricomycetes sp. (42.1 U/L), followed by Fusarium sp. and Alternaria sp. amounting to 28.7 U/L and 13.07 U/L at pH 6.0. The purified lignin peroxidase enzyme from Agaricomycetes sp. showed a single band of ~70 kD on the SDS PAGE. The DNA of fungal isolates were amplified with 18S rRNA showed the 500 bp DNA bands and were sequenced by chomas biotech. It was concluded that fungi, Agaricomycetes sp. were the most promising candidates for lignin peroxidase enzyme production.

Keywords: Agaricomycetes sp.; Lignin peroxidase; Alternaria sp.; Fusarium sp.
Introduction-
Lignin is a complex racemic aromatic heteropolymer containing hydrophobic network of phenylpropanoid units by oxidative polymerization of three types of hydroxycinnamyl alcohol precursors (monolignols) (4, 11, and 9). Fungal degradation is considered the main mechanism of biological cycling of lignin, causing oxidation of propyl chains, demethylation of 3- and 5-methoxyl groups and aromatic ring cleavage. White-rot basidiomycete fungi and actinomycetes have ability to degrade lignin (6) during the secondary metabolism or in the absence of nitrogen, carbon or sulphur.

The ligninolytic microbes exhibit a unique strategy for lignin degradation, which is based on unspecific one-electron oxidation of the benzenic rings catalyzed by synergistic action of extracellular hemeperoxidases and peroxide-generating oxidases.

The ligninolytic potential of the isolates was furthermore assessed by establishing their ability to decolourize synthetic, lignin-like dyes. The recalcitrant thiazine dye Azure B (AB) is particularly suited for this purpose. This dye is decolourized by high redox potential agents, specifically LiP’s [Arantes V and Ferreira Milagres AM (2007) Archibald FS(1992), Aguiar A and Ferraz A (2007)], whereas it cannot be oxidized by nonperoxidase alcohol oxidases, MnP’s or laccases alone [Archibald FS(1992), Arora DS and Gill PK (2001)].

The millions of tons of lignin and lignin-related compounds are produced as waste effluent from the pulping and paper industries (De los Santos Ramos W, et al; 2009). These amounts are expected to further increase in the near future as a result of the recent
developments aimed at replacing fossil feedstocks with lignocellulosic biomass for the production of fuels and chemicals. Generally, biorefinery processes only employ the (hemi-)cellulosic part; the lignin component remains as a low-value waste stream (Stewart D, 2008) that is commonly incinerated to generate heat and power (Himmel ME et, al; 2007, Zaldivar J et al; 2001). To date, less than 100 000 t a-1 of lignin obtained from the Kraft pulping process is commercially exploited (Gosselink RJA et al; 2004). Lignin peroxidase are used in industries, paper and dye industries (1), prevention of wine discoloration, and detoxification of environmental pollutants and production of chemicals from lignin (3). In view of its importance in large-scale application, the present work aims on screening and isolation of highly-efficient lignin peroxidase-producing fungi.

Materials and Methods

**Sample collection and screening** - Effluent samples from various locations were subjected to serial dilution in saline water and were plated for screening of lignin-degrading fungi. Screening was done on a medium comprising Olga et al. media (7) and 0.02% Azure B (indicator), pH 6.0. The plates were incubated for 6-12 days at 28°C. Positive cultures were screened by observing white coloration.

**Lignin peroxidase enzyme activity**- LiP enzyme activity was determined spectrophotometrically according to the protocol described by (8) Reaction mixture (3.0 ml) was prepared by mixing 50 mM sodium tartrate buffer (pH 4.5), 100 μM Azure B, 0.1 mM H₂O₂, 1.0 ml enzyme and 0.88 ml water. The increase in absorbance at 651 nm (millimolar extinction coefficient 48.8) was recorded for 1 min against reagent blank.
Enzymatic activity (U/L) = (ΔAbs×10^6) (εRT)^{-1}. The protein concentration was determined, Lowry et al. (6).

**Purification of the Lip activity of Agaricomycetes sp** - Supernatant from a 10-day-old culture was clarified by filtration and Proteins in the samples were precipitated with 85% ammonium sulfate, dissolved in 10 mM acetate acetate buffer (pH 5.0), and dialyzed for 12 h against the same buffer. Crude enzyme samples were loaded onto a column of DEAE-Cellulase, equilibrated with 10 mM sodium acetate, pH 4.5. Proteins were eluted with a linear gradient of 0–1 M NaCl in the same buffer. The eluate was monitored for absorbance at 280 nm and lip activity. The fractions with enzymatic activity were pooled and stored at 70°C.

**Isolation of Genomic DNA and its amplification** - The genomic DNA was isolated from the fungus culture grown at 28°C for 10 days in LMM broth by using a CTAB extraction method and amplified with 18S rRNA (F- 5’ATTGGAGGGCAAGTCTGGTG 3’, R- 5’CCGATCCCTAGTCGGCAG3’) and Analysis of DNA sequence.

**Results and discussion**

The screening of lip-producing fungi from effluent samples showed that out of fourteen positive species three formed white colony on media due to oxidative polymerization of azure B (Fig. 1).

Maximum lip production by fungi was also observed at the 10th day of incubation. The extinction coefficient values for lip, as estimated by the standard protocol (2) were found to be Agaricomycetes sp (42.1 U/L), followed by Fusarium sp. and Alternaria sp. amounting to 28.7 U/L and 13.07 U/L at pH 6.0 (figure 2).
The purification of the isozyme patterns of various lignin-degrading enzymes by DEAE-cellulose chromatography (figure 3 and table 1) On the SDS protein the single major band of 70 kD was observed.

The sizes of the fungal genomic DNA was 500Kbp (figure 4) and the accession no was *Agaricomycetes sp* (HM167516.1), *Fusarium oxysporum* (GU724514.1), *Alternaria brassicae*. (JN108900.1 )

These fungal strains, therefore, offer promise for use in several bioprocesses involving laccase applications, such as biopulping, biobleaching, bioremediation, food technology and treatment of industrial waste water (5) and (10).

**Acknowledgement**-Financial support in the form of research project from CST (UP), India is greatly acknowledged.

**References**


**Figure Captions:**

Ira,Faria,Nelam,Smita,Rolee
Fig 1. Lip positive plant pathogenic fungi, ((a) *Agaricomycetes* sp, (b) *Fusarium* sp. and (c) *Aletnaria* sp.) on agar plates (Olga medium).

Fig 2. Lip activity in fungal culture filtrates of the *Aletnaria* sp., *Fusarium* sp. and *Agaricomycetes* sp various incubation periods (days). Values are means for three replicates.

Fig 3. Purification of lip from *Agaricomycetes* sp fungus.

Fig 4. Amplified genomic DNA.

Table 1- Lignin peroxidase was partially purified by ammonium sulphate fractionation and DEAE (diethyl aminoethyl)-cellulose column chromatography.

**List of Figures**

**Fig.1-** A  B  C

**Fig.2-**

**Fig.3-**
SDS gel with coomassie staining after purification. Lane 1: Marker, Lane 2, 3: Dialysis, Lane 4: pooled fraction after DEAE cellulose, Lane 5: ASP, Lane 6: Crude, Lane 7: Lyophilized sample, Fig. B: in gel activity of LiP

Fig. 4-

Table-

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total Activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Recovery</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>4195</td>
<td>104.8</td>
<td>40.02</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>Ammonium sulphate fraction (Dialyzed)</td>
<td>1946</td>
<td>24.56</td>
<td>79.23</td>
<td>46.39</td>
<td>1.97</td>
</tr>
<tr>
<td>DEAE</td>
<td>848.6</td>
<td>9.41</td>
<td>90.1</td>
<td>20.23</td>
<td>2.25</td>
</tr>
</tbody>
</table>