Chapter 1
ISOLATION AND SCREENING FOR LACCASE PRODUCING PLEUROTUS FLORIDA ISOLATES

1.1 Introduction

The white rot fungi (WRF) seem to be the unique microorganisms, which are capable of degrading and mineralizing lignin and a series of organic pollutant compounds, which are highly toxic and recalcitrant. This ability is, at least in some extent, caused by non-specific enzymatic system produced by these fungi during the lignin degradation, includes several isoenzymes of Lignin Peroxidase (LiP, EC 1.11.1.14), Manganese dependent Peroxidase (MnP, EC 1.11.1.13), laccases (EC 1.10.3.2) as well as H2O2-producing oxidases.

*Phanerochaete chrysosporium* has been widely used as a model system to understand the process of lignin and some environmental pollutants biodegradation. However, there is a great diversity of basidiomycetes with different ligninolytic enzymes pattern, which also has great differences in their ability in xenobiotic degradation. The ligninolytic systems of other basidiomycetes and several methods involving dyes have been reported as screening methodologies for the selection of xenobiotic compounds degrading microorganisms.

In this study, laccase producing fungi were screened from various environmental samples by plate tests using the indicator compounds like guaiacol, tannic acid and the polymeric dyes such as Remazol brilliant blue R and Poly R-478. A total of 20 positive strains were isolated, and their ability to produce laccase was studied in liquid media. The fungal strains, which produce high amount of laccase, were characterized. The most promising positive fungal strains were found to be *Pleurotus florida* by the Tamilnadu Agricultural University, Mushroom division, Coimbatore, Tamilnadu, India.
1.2 Materials and methods

1.2.1 Fungi Isolation

White-rot fungi isolated from the foothills of Western Ghats, TamilNadu and Karnataka, India (samples collected from Crude cork material, Municipal biowaste and soiled pulp from coffee plantations) was cultivated on basal medium. The basal medium contained (per liter of distilled water): 0.5 g of KH$_2$PO$_4$, 0.2 g of MgSO$_4$.7H$_2$O, 0.1 g of NH$_4$NO$_3$, 0.1g of KCl, 0.02g of FeSO$_4$.7H$_2$O, 0.05g of Ca(NO$_3$)$_2$.4H$_2$O, 2.0 g of Yeast malt extract (HiMedia chemicals)and 15 g of agar (Himedia Chemicals). Basal medium was autoclaved in flasks with magnetic stir bars and cooled to 55°C, when the following were added aseptically: 5 ml of 1 M KOH; 0.4 ml of guaiacol;1.0g of indulin AT(alkali lignin;Sigma)which was suspended and partially dissolved in 10 ml of dioxane; 60 mg of chlorotetracycline-HCL;30 mg of streptomycin sulphate;30 mg of Penicillin G (Na salt); 4mg of benomyl(as Benlate 50 WP) in 2 ml of 1:1 acetone-70% ethanol. The medium was stirred and poured into petridishes. Potato dextrose agar medium (PDA) and Malt extract agar medium(MEA) was used for the first subculture of chosen isolate and the plates were examined by light microscopy to check the absence of bacteria and the unique fungi isolation.

1.2.2 Primary screening method on solid medium

As an initial screening method for detecting the ability of the fungal strains to produce lignin-modifying enzymes, the dye decolourising method is used. Screening was performed in Petri dishes (60 mm diameter) with 15 ml of (Malt extract agar (MEA) medium and Potato dextrose agar (PDA) medium from Himedia media, Mumbai, India), containing indicator compounds such as 0.04 % (w/v) of Remazol Brilliant Blue-R (RBB-R) and PolyR-478, 0.01% (v/v) of guaiacol and tannic acid 0.05% (w/v). Guaiacol (Sigma) RBBR (Sigma) and Poly R-478 (Sigma) were added to the media after autoclaving as sterile-filtered solutions. Tannic acid (Merck chemicals Ltd., UK) was autoclaved separately before addition to the media. Guaiacol is a sensitive substrate that allows a rapid screening of fungal strains producing extracellular guaiacol oxidizing enzymes by means of a colour reaction. The correlation between positive reactions with different indicators was investigated by cross-cultivating positive strains on all different
indicator plates. The white-rot fungus *Trametes hirsuta* that produces laccase (Rittstieg et al., 2002), manganese peroxidase and lignin peroxidase was used as a positive control. Subsequently, chloramphenicol and chlorotetracycline (Sigma) were added at a concentration of 0.01% (w/v) to the media in order to inhibit the growth of bacteria. In addition, 1% (w/v) Benomyl, a benzimidazole fungicide was added in order to select the wood decaying fungi (Maloy 1974). Benomyl was autoclaved together with the media, whereas chloramphenicol and chlorotetracycline were added to the media after autoclaving. White rot fungi producing laccase in plate test were grown in different types of liquid media and laccase production was monitored with activity measurements.

1.2.3 Screening in Liquid Media

Decolourizing Poly R 478, RBBR, guaiacol and tannic acid oxidizing strains were screened on liquid cultures for Lignin peroxidase, Manganese peroxidase and laccase activities. For laccase activity, the isolated fungi were grown at 25 °C for 12 days with rotary shaking (150 rpm) in 500-ml baffled Erlenmeyer flasks containing 50 ml of Potato dextrose medium.

For Lip, and MnP the isolated fungi were grown at 25 °C for 12 days in static conditions in 500-ml baffled Erlenmeyer flasks containing 50 ml of Potato dextrose medium.

1.2.4 Enzyme assay

Enzyme activity was assayed by using 50μl of crude filtrate, 950μl of 10mM guaiacol in 0.1 M acetate buffer containing 10% (v/v) acetone, pH 5.0 (Palmieri, G, 1993). The enzyme blank consists of 50μl of crude filtrate and 950μl of 0.1M acetate buffer and the substrate blank consists of 50μl 10mM guaiacol in 0.1 M acetate buffer containing 10% (v/v) acetone and 950μl of 0.1M acetate buffer. The contents were mixed well and incubated at 30°C for 5 minutes (Hosoya, T. 1960 and Mliki, A., and W. Zimmermann, 1992). The brown colored formed was spectrophotometrically read at 460nm using Beckman DU-530 Spectrophotometer. Enzyme activity was expressed in...
units/ml. One unit of laccase activity was defined as the amount of enzyme required to oxidise 1μmol guaiacol per minute.

In order to rule out the role of peroxidase oxidation and prove the oxidation only by laccase, the enzyme was pre incubated with catalase (1000 units ml⁻¹) (E.C.1.11.1.6 from *Aspergillus niger*, Sigma C3515) for 30 min at 30° C prior to assay to remove any endogenous hydrogen peroxide (Pointing *et al.*, 2000). Similarly, manganese independent peroxidase activity was measured by adding hydrogen peroxide (1mM final concentration) to the laccase assay mixture and to subtract the activity due to laccase alone (Pointing *et al.*, 2000). Also, lignin peroxidase activity was determined by measuring the production of veratraldehyde from veratryl alcohol at 310 nm in glycine-HCl buffer (pH 3.0) at 30° C, upon addition of hydrogen peroxide (1 mM concentration) (Kirk *et al.*, 1998).

Further, aryl alcohol oxidase activity was assayed under the same conditions without the addition of hydrogen peroxide and manganese dependent peroxidase activity was measured by the oxidation of phenol red at 431 nm in the presence of 100 μM MnSO₄.5 H₂O in glycine-HCl buffer (pH 3.0) at 30° C, upon addition of hydrogen peroxide (0.5 mM final concentration) (Pointing *et al.*, 2000).

1.3 Results and Discussion

1.3.1 Isolation of Fungi

The laccase producing fungal species were identified by confirming brown color development surrounding the fungal colonies. Any colony that turned yellow or decolourised the Poly R-478 was considered as ligninolytic positive and selected. Altogether 20 fungal strains showing positive reactions for laccase production on indicator plates were isolated from the collected samples. Positive strains were found from all the sources explored. The crude cork material and soiled pulp from coffee plantations from Western Ghats proved to be the best source, as altogether 10 positive strains were isolated from it. As for the test compost of municipal biowaste, three positive strains were isolated only at the late maturation phase. Of the 20 positive fungal strains,
11 were isolated from plates containing the polymeric indicators RBBR or Poly R-478, and nine strains from the guaiacol plates. Tannic acid gave relatively weak positive reactions with many samples. However, the ability of most positive microbes to form brown colour on tannic acid weakened during subculturing, whereas with other indicators the same effect was not observed. Only two fungal strains (WLP11 and WLP16) were strongly positive on tannic acid plates that they were considered as laccase positives. Potato dextrose agar proved to be the best medium for isolating laccase-producing fungi. The most promising positive fungal strains were identified to be *Pleurotus florida* (*P.*florida) by the Tamilnadu Agricultural University, Mushroom division. Coimbatore, Tamilnadu, India.

1.3.2 Specificity of the indicators

Reactions with the four different indicators, RBBR, Poly R-478, guaiacol and tannic acid, were tested with the fungal strains WLP1-WLP20 on Potato dextrose agar plates. Tannic acid seemed to be less specific, as it failed to give positive results with 10 strains that were positive on other indicators. In addition, there was one strain, WLP11, that was positive only on tannic acid.

1.3.3 Production of laccases in liquid media

Laccase production by the positive strains found in the screening was studied in liquid cultures. Detectable laccase production was observed with nine isolated fungal strains: WLP1-5, WLP12-13, WLP15, and WLP 20. (Table.1.1)
Table 1.1 Lac, MnP and Lip activities and identification of highest laccase producers screened in liquid media

<table>
<thead>
<tr>
<th>Strain Ref</th>
<th>Lac (U/ml)</th>
<th>MnP(U/ml)</th>
<th>LiP(U/ml)</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>WLP 1</td>
<td>1.4</td>
<td>1.0</td>
<td>13.5</td>
<td><em>Polyporus sp.</em></td>
</tr>
<tr>
<td>WLP 2</td>
<td>2.5</td>
<td>3.0</td>
<td>12.0</td>
<td><em>Ganoderma sp.</em></td>
</tr>
<tr>
<td>WLP 3</td>
<td>2.1</td>
<td>3.5</td>
<td>11.5</td>
<td><em>Ganoderma sp.</em></td>
</tr>
<tr>
<td>WLP 4</td>
<td>1.6</td>
<td>5.6</td>
<td>-</td>
<td><em>Microporus sp.</em></td>
</tr>
<tr>
<td>WLP 5</td>
<td>1.1</td>
<td>4.5</td>
<td>2.5</td>
<td><em>Phellinus sp.</em></td>
</tr>
<tr>
<td>WLP 12</td>
<td>6.4</td>
<td>1.8</td>
<td>-</td>
<td><em>Pleurotus sp.</em></td>
</tr>
<tr>
<td>WLP 13</td>
<td>8.5</td>
<td>2.5</td>
<td>5.5</td>
<td><em>Pleurotus florida</em></td>
</tr>
<tr>
<td>WLP 15</td>
<td>5.6</td>
<td>2.0</td>
<td>3.5</td>
<td><em>Pleurotus sp.</em></td>
</tr>
<tr>
<td>WLP 20</td>
<td>4.8</td>
<td>2.4</td>
<td>-</td>
<td><em>Pleurotus sp.</em></td>
</tr>
</tbody>
</table>

Considerable laccase activities were measured with strains WLP12, WLP13, WLP15 and WLP20, whereas other positive strains produced very low activity levels. Laccase production proved to be very much dependent on the medium, and the highest laccase activities were detected on Potato dextrose medium. Laccase production by fungi has previously been shown to depend solely on the composition of the cultivation medium; for example carbon source, nitrogen source 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). Induction of laccase production by high medium nitrogen content has recently been detected in the Basidiomycte 1-62 (CECT 20197) (Mansur *et al.*, 1998), in *Pleurotus sajor-caju* (Soden and Dobson, 2001) and in *Trametes trogii* (Colao *et al.* 2003). With the strains WLP17 and WLP18, laccase production was enhanced by the addition of wood chips, whereas the strain WLP19 produced highest laccase activities when glucose concentration was decreased to 5 g\(^{-1}\) and the medium was supplemented with 5 g\(^{-1}\) bacto peptone.
1.3.4 Characterization of novel laccases

The highest laccase production was detected with the strains WLP12, WLP13, WLP15 and WLP 20, and the laccases from these strains were further analysed. The pH optima were determined with o-dianisidine and guaiacol as substrates. All novel laccases discovered in this screening had their pH optima at acidic pH values (shown in figure 1-8).

1.4 Conclusion

Plate-test screening with laccase indicator compounds has been reported by many groups (Nishida et al., 1988; Barbosa et al., 1996; Gonclaves and Steiner 1996; Chefetz et al., 1998; Raghukumar et al., 1999), but most of the laccases found in the screening studies have not been purified or characterized. But Gonclaves and Steiner (1996) and Chefetz et al., (1998) reported the enzyme characteristics of *Polyponis* sp., and *Chaetomium thermophilum* respectively. In addition, the use of indicator plates facilitated isolation of the basidiomycete fungi *Flavodon flavus* that has the potential in bioremediation (Raghukumar et al., 1999). In this study, screening for laccase-producing microbes on plates containing coloured indicators resulted in isolation of 20 fungal strains, but Crude cork material and soiled pulp from coffee plantations proved to be highly interesting sources for laccase producers. This is presumably because of the presence of relatively high concentration of laccase substrates in these environments. Comparison of the reactions with different indicators showed that the polymeric dyes RBBR and Poly R-478 gave very similar results, thus either one can be chosen as an indicator in future screening procedures. Reactions with guaiacol also correlated well with reactions on the polymeric dyes. However, tannic acid deviated substantially from the polymeric dyes and guaiacol. Tannic acid is one of the traditional screening reagents for laccases. Our results suggest, however, that colour reactions with synthetic dyes and guaiacol are more easily detectable, detect more laccase-positives, and these compounds can thus reliably be used for laccase activity screening.
Screening of laccase using tannic acid as substrate
Screening of laccase using guaiacol as substrate