Keywords. Ligninases; Fungal Strain; Pleurotus ostreatus; P. sanguineus; Submerged Fermentation; FPLC; MnP; LiP; Lcc; Time course studies

Introduction

Among microorganisms that colonize living wood, white-rot fungi are regarded as considerable lignin degraders. They produce extracellular enzymes, such as MnP, LiP and laccase, which play important roles in lignin biodegradation [1]. Pleurotus ostreatus is a white rot fungus belonging to the basidiomycetes and it is also considered to be a cholesterol reducing mushroom [2]. It was also noticed that the deficiency of ligninases started in some genera of Basidiomycetes, such as Pleurotus spp. [3], especially ligninolytic laccases involved in the degradation of lignin. The combined action of laccase and aryl alcohol oxidase decreases the molecular weights of soluble lignosulphonates secreted by P. ostreatus [3]. Ligninolytic system configuration is complicated and species restricted [4]. White-rot fungi produce ligninases, including MnPs, LiPs and Lcc, which are possible contributors to fungal ligninolysis. White-rot fungi were mainly found to produce consistent products following the ligninolysis of model lignin compounds [5].

MnP belongs to the family of peroxidases, and the methodical name of this enzyme is Mn(II):H₂O₂ oxidoreductase. According to protein databank, the MnP containing cofactor is protoporphyrin IX having Fe(C₃₄H₃₂FeN₄O₄) incorporated with Mn²⁺ and Ca²⁺ ions required for enzyme activity.

Eventually, peroxidases oxidize phenolic compounds and reduce molecular oxygen to water [1]. Ligninases oxidize several environmental pollutants such as polycyclic aromatic hydrocarbons, dyes and chlorophenols. Heme containing enzymes such as LiP and MnP also have catalytic cycles characteristic of other peroxidases. LiP has an ability to oxidize many aromatic compounds, whereas MnP oxidizes Mn (II) to Mn (III) [6, 7]. Laccases (benzenediol: oxygen oxidoreductase, EC 1. 10. 3. 2) are multi-copper blue oxidases that oxidizes Mn (II) to Mn (III) [6, 7]. Laccases have significant importance in many industrial areas due to their remarkable catalytic properties. Potential applications include immunoassay bio-labeling, biosensors, biocatalysts, and advancement of oxygen cathodes in biofuel cells. In addition, they have good prospects in the environmental sector, including use in textile dye bleaching, pulp delignification and xenobiotic compound degradation due to their wide-ranged substrate specificity [6, 8]. The present applications of this enzyme motivated us to do new basic research.

The current research activity of ligninases includes utilizing the local lignin sources (Eucalyptus and sugarcane bagasse) and checking their delignification pattern. The characterization of ligninases (MnPs, LiPs and Lcc.) from Brazilian fungal isolates, with respect to production, partial purification and time course studies, is reported in this study.

Materials and Methods

Strain isolation

The unknown fungal strain was isolated from the Northeast part of Brazil called Caatinga. Caatinga has a semi-arid climate and covers an area of nearly 735,000 km², although < 1% of this semi-arid zone is preserved [9].

Substrate collection and preparation

Sugarcane bagasse and Eucalyptus lignin were used for delignification. Sugarcane bagasse and Eucalyptus were collected from LWARD Química, Brazil. All substrates were dried in an oven at 80°C to constant weight and were powdered using an electric grinder and stored in airtight glass containers to keep out moisture.
Fermentative organism and cultivating conditions

*P. ostreatus* and *P. sanguineus* were collected from the Mycology Collection Lab, Department of Plant Protection, UNESP, Botucatu, SP, whereas the local fungal strain was isolated from the Northeast part of the country in the Caatinga forest. All fungal strains were plated on malt extract agar medium (malt extract 25 g; agar 20 g; distilled water 1 L) containing 0.05% of the dye Remazol Brilliant Blue R (RBBR). Plates were incubated in the dark for 14 days at 25°C. Ligninolytic activity was assessed by scoring for the presence of a halo of decolorized dye surrounding the fungal colonies.

Quantification of dye Remazol Brilliant Blue R (RBBR) oxidation in liquid culture medium

Out of ten strains, seven exhibiting ligninolytic activity were then grown in broth culture media containing (2.5% malt extract and 0.05% RBBR). All the cultures were kept in the dark and incubated at 30°C under continuous shaking. The presence of ligninases activity, as evidenced by decolorization following oxidation of dye, was quantified by a decrease in the absorption peak as monitored at 595 nm using a Shimadzu UV-1601 PC spectrophotometer. Cell biomass was measured gravimetrically by oven-drying at 70°C. All shake flask experiments were tested for ligninolytic activity in replicates of three.

Growth Media preparation

Growth media for *P. ostreatus* contained: Glucose 10g/L, L-Asparagine monohydrate 3g/L, MgSO4.7H2O 0.05g l-1, KH₂PO₄ 300mg; reactions were terminated by the addition of 40 μl of 2N NaOH reaction mixture was kept in a water bath for 5 min and incubated at 300°C in a shaker (130 rpm) with continuous shaking.

Enzymatic analysis

MnP activity was measured at 610 nm (ε = 4460 M⁻¹cm⁻¹) in submerged fermentation using the methodology described by Kuwahara [10]. The reaction mixture / ml contained: 500 μl of culture medium supernatant; 100 μl of phenol red (substrate); 100 μl of 250-mM sodium lactate solution; 200 μl of 0.5% bovine serum albumen (BSA); 50 μl of 2-mM MnSO₄; and 50 μl of H₂O₂ from 2-mM sodium tartrate buffer pH-3.0; 125 μl of 4 mM-veratryl alcohol (substrate); 50 μl of 10 mM-H₂O₂; 450μl distilled water and 250 μl of culture medium supernatant for a final volume of 250 μl.

During fermentation, LiP activity was assayed by measuring the oxidation of veratryl alcohol to veratryl aldehyde by a UV spectrophotometer at 310 nm (ε = 9300 M⁻¹ cm⁻¹) as reported by Tien and Kirk [11]. The reaction mixture contained: 375μl of 0.33-M sodium tartrate buffer pH-3.0; 125 μl of 4 mM-veratryl alcohol (substrate); 50 μl of 10 mM-H₂O₂; 450μl distilled water and 250 μl of culture medium supernatant for a final volume of 1250 μl.

The oxidation of o-dianisidine at absorbance 525 nm (ε = 65,000 M⁻¹ cm⁻¹), which is indicative of laccase activity, was measured spectrophotometrically according the method used by Szklarz et al. [12]. The reaction mixture contained / mL: 200 μl of 0.5-M citrate–phosphate buffer pH-5.0; 100 μl of 1-mM o-dianisidine solution (substrate); 600 μl of supernatant from culture medium and 100 μl of H₂O₂. Boiled culture medium was used as a control. For all peroxidases, the unit activity is defined as the amount of enzyme required to oxidize 1 μmol of substrate / minute. The unit of specific activity was stated as U/mg of protein [13]. The given values in Table 1 are the mean of three replicates of each.

Ligninases cocktail partial purification and characterization

The total proteins in 2L of broth culture filtrate were precipitated by the addition of ammonium sulfate (80% saturation); all the steps were carried out at 4°C. The ammonium sulfate precipitate was collected by centrifugation (5000 × g 30 min) and dissolved in 50 mM potassium phosphate buffer, pH 6.5 (buffer A). The dissolved precipitate was dialyzed overnight in Buffer A and then loaded onto a DEAE Sepharose Fast Flow column (10 × 300 mm) equilibrated with buffer A. The column was washed with buffer A and then eluted at a flow rate of 0.5 ml/min with a 0 to 1.0 M linear gradient of sodium chloride in buffer A. MnP fractions were collected, assayed, and dialyzed overnight against buffer A and then loaded onto a Superdex 75 column (10 × 300 mm), which was also equilibrated with buffer A.

30 ml MnP fractions were collected and concentrated to 5 ml by ultrafiltration with a Centricon-3 (3 kDa cut-off, Amicon). The concentrated MnP fraction was adjusted to 100 mM sodium chloride and loaded onto a Sephadex G-100 column equilibrated with buffer A, in a fast protein liquid chromatography (FPLC) system (Pharmacia, AKTA purifier). Fractions were observed by a UV detector using Unicorn 5.11 software of Pharmacia. At this phase, the enzyme activity corresponded to a peak of absorbance observed at 280nm and eluted as a single peak. The purified and concentrated enzyme was preserved at -20°C and did not exhibit any noteworthy loss of enzymatic activity over several months [14].

Scanning electron microscopy (SEM)

Sugarcane bagasse and black liquor of eucalyptus samples were kept in an oven and dried at 50°C for 1h and thick layers of the samples were spread on a carbon ribbon in the sample holder. Until analysis, the sample assembly was maintained in a vacuum desiccator. The SEM analysis of lignin samples before and after fungal treatment was observed using a Jeol model [SM-6360LV microscope [15].

Determination of total proteins

Total proteins were quantified by absorbance at 595 nm according to the Bradford assay method [16] using BSA as a standard.

Gel electrophoresis and staining

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was performed as described by Höfer [17] to determine the molecular weight of proteins. Gels were stained with Coomassie brilliant blue R-250 and commercially available low molecular weight size markers (Bio-Rad, Munich, Germany) were used as standards.

MS Analysis to determine the composition of lignin monomers

Autoflex III Series MALDI-TOF, (Bruker Daltonic Leipzig, Germany) was used to determine the composition of lignin monomers with pulsed ion extraction of 130 ns. The specification of the mass spectrometer was set up with Nd : YAG laser (337 nm, 3 ns pulse width with pulse energy of 200 μJ). The acceleration voltage of ion source-1=19.18 kV; ion source-2=18.2 kV, Lens: 6.5 kV Reflector: 21 kV Reflector-1= 9.7 kV was 19 kV and reflectron voltage was 15.1 kV. α-cyano-4-hydroxy
cinnamic acid (Sigma-Aldrich) was used as the matrix compound. The matrix compound (10mg/ml) was dissolved 1:1 (v/v) in acetonitrile/Milli-Q-water with a concentration of 2.5% trifluoroacetic acid reagent. The samples were diluted 9:1 (v/v) in acetonitrile/Milli-Q-water with a concentration of 0.1–0.5% (v/v) and mixed 1:1 (v/v) with the matrix compound solution used for analysis. The MS measurements were performed in the reflector mode [18].

**Statistical analysis**

The least significant difference among the experimental values was calculated by ANOVA using Assistat-Statistical Assistance version 7.6 beta software.

**Results and Discussion**

Due to broad substrate specificity, the ligninases potential of white rot fungi has been reported as liable for the alteration and mineralization of organic pollutants that are structurally similar to lignin. Ligninases are the combination of three peroxidases, MnP, LiP and laccases, which are characterized as lignin degrading enzymes. The distribution of MnP and laccases are very frequent in white rot fungi, while LiP is not [19].

Degradation and decolorization of many organic pollutants by white rot fungi have been reported by many researchers [20], but MnP and LiP are noted mainly for the degradation of polymeric dyes [21]. The white rot fungi have the ability to decolorize polymeric dyes because of existing ligninolytic enzymes. An anthracene derivative like Remazol Brilliant Blue R (RBBR), which is considered to be an organic pollutant and is similar to the lignin polymers, has been used as a model compound by many researchers to measure ligninases activity [21]. Therefore, we developed a petri plate assay to identify ligninases-producing fungal strains, based on their ability to decolorize RBBR. Using this system, we determined that the fungal strains *Pleurotus ostreatus*, *Pycnoporus sanguineus* and the new Brazilian isolate all exhibited ligninolytic activity (Figure 1).

**Ligninases production during time course studies**

To better quantify ligninases production, the ligninolytic activity of the aforementioned strains was measured in liquid cultures. During the time course study, triplicate fermentation flasks were harvested after the aforementioned strains was measured in liquid cultures. During the time course study, triplicate fermentation flasks were harvested after increasing fermentation time for 10 days, but a further increase (12 days) in fermentation time (Table 1) decreased ligninase activities. However, the specific activities of MnP and LiP constantly increased [22].

**Purification of MnP**

MnP purification mainly consists of two steps, ammonium sulfate precipitation and size exclusion chromatography. Following chromatography, protein fractions eluted in two peaks as shown in Figure 2A. The MnP activity eluted as a single peak at A_{280} following size exclusion chromatography (Figure 2B). Under reducing and non-reducing conditions, total proteins were run on native-PAGE shown in (Figure 3A). Finally, to determine the molecular weight of the purified enzyme, MnP was separated on SDS-PAGE and stained with Coomassie Blue R-250 (Figure 3B). The purification procedure is summarized in Table 2. The molecular mass of purified MnP was 37kDa as shown in (Figure 3B) and the specific activity of the purified enzyme was 3.22-fold.

**Electron microscopy**

Scanning electron microscopy (SEM) was performed to determine

**Figure 1: Potential screening of three fungal strains out of ten based on the ligninases production and degradation of RBBR dye.**

### Table 1: Measurement of ligninases activities during the time course study in three different fungal strains.

<table>
<thead>
<tr>
<th>Isolated fungal strain</th>
<th>Days</th>
<th>Laccase (IU l(^{-1}))</th>
<th>LiP (IU l(^{-1}))</th>
<th>MnP (IU l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pleurotus ostreatus POS97/14</strong></td>
<td>2</td>
<td>0.14 ± 0.01(^{\text{e}})</td>
<td>0.82 ± 0.07(^{\text{d}})</td>
<td>5.17 ± 0.11(^{\text{d}})</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.18 ± 0.02(^{\text{d}})</td>
<td>2.93 ± 0.05(^{\text{d}})</td>
<td>7.62 ± 0.09(^{\text{d}})</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.31 ± 0.01(^{\text{d}})</td>
<td>4.03 ± 0.07(^{\text{d}})</td>
<td>10.09 ± 0.16(^{\text{d}})</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.56 ± 0.01(^{\text{d}})</td>
<td>5.84 ± 0.04(^{\text{d}})</td>
<td>13.30 ± 0.14(^{\text{d}})</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.80 ± 0.01(^{\text{d}})</td>
<td>6.77 ± 0.09(^{\text{d}})</td>
<td>17.94 ± 0.45(^{\text{d}})</td>
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<tr>
<td></td>
<td>12</td>
<td>0.54 ± 0.01(^{\text{d}})</td>
<td>6.58 ± 0.06(^{\text{d}})</td>
<td>12.90 ± 0.14(^{\text{d}})</td>
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</tbody>
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<table>
<thead>
<tr>
<th><strong>Pycnoporus sanguineus</strong></th>
<th>Days</th>
<th>Laccase (IU l(^{-1}))</th>
<th>LiP (IU l(^{-1}))</th>
<th>MnP (IU l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>0.31 ± 0.01(^{\text{d}})</td>
<td>0.67 ± 0.04(^{\text{d}})</td>
<td>1.09 ± 0.14(^{\text{d}})</td>
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<td>0.79 ± 0.02(^{\text{d}})</td>
<td>1.73 ± 0.13(^{\text{d}})</td>
<td>6.11 ± 0.11(^{\text{d}})</td>
</tr>
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<td>6</td>
<td>1.07 ± 0.01(^{\text{d}})</td>
<td>2.36 ± 0.05(^{\text{d}})</td>
<td>9.79 ± 0.14(^{\text{d}})</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1.13 ± 0.00(^{\text{a}})</td>
<td>2.77 ± 0.08(^{\text{a}})</td>
<td>10.93 ± 0.14(^{\text{a}})</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.15 ± 0.01(^{\text{a}})</td>
<td>3.41 ± 0.09(^{\text{a}})</td>
<td>13.21 ± 0.14(^{\text{a}})</td>
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<td>12</td>
<td>1.12 ± 0.01(^{\text{a}})</td>
<td>2.06 ± 0.13(^{\text{a}})</td>
<td>1.66 ± 0.20(^{\text{a}})</td>
</tr>
</tbody>
</table>
the surface morphology of lignin samples of sugarcane bagasse and black liquor of *Eucalyptus* that were treated with the isolated fungal strain (Figure 4). Abundant fungal growths were seen on bagasse and black liquor fibers, which indicated the decay of lignin samples. After fungal modification, the fiber structure of bagasse specifically shows the presence of bore holes appeared, indicated by arrows in Figure 4 (C and D).

**MALDI-TOF MS**

The MALDI-TOF method provides information regarding the interpretation of lignin composition. This method also helps to determine the lignin structure. After biological modification, it illustrates the effectiveness of fungal attack on the lignin bio-molecule and the impact on its compositional arrangement. The fungal attack generally affected phenolic units of the lignin molecule, as observed by the reduction in the relative frequency of β-O-4-linked H units, which are mainly terminal units with free phenolic groups [23]. After delignification, the complete structures of molar mass distribution of lignophenols by MALDI-TOF-MS spectra are shown in (Figure 5). In delignification spectra, the lignin monomers specified the dominant signals at m/z 171, 188 (coniferyl aldehyde), m/z 227, 229, 233 (syringyl propene), m/z 334, 378, 397 (phenyl coumaran), m/z 453 (resinol), m/z 655, 715 (dimethoxyphenol) reported by Rolf [18]. Most monomers were by-products from guaiacol and syringol. Most dimers were allocated to phenylcoumaran structure. Biphenyl and resinol were infrequent.
Figure 4: Scanning electron microscopy of sugarcane bagasse (SCB) and Black liquor (BL) of Eucalyptus. (A & B) Control SCB (magnification, x 400); (C & D) bagasse treated with local fungal isolate grown after 2 weeks (magnification, x 2k); (E & F) Control BL of Eucalyptus fibers (magnification, x 10k); (G & H) BL treated with local fungal isolate grown after 2 weeks (magnification, x 5k); indicates the fungal growth where k denoted by (x1000 magnification).
Figure 5: MW distribution to determine the lignin monomers by using MALDI-TOF-MS where α-cyano HCA was used as matrix compound.
Conclusions

With the increasing global anxiety over fossil fuel use and its environmental footprint, there is a strong interest in using biorenewable materials as substitute feedstocks for making more environmentally-friendly biomaterials. The results of the present study specify the screening of potential fungal strains for ligninases production, partial purification of MnP and the degradation pattern of local lignin resources found in Brazil. It allows a deeper insight into the mechanism of the delignification process. The industrial and biotechnological application of ligninases is constantly increasing due to their multiple uses and applications in a diversity of processes. Their ability to remove xenobiotic pollutants and produce polymeric products makes them a beneficial tool for bioremediation purposes. The unknown isolated fungal strain has the potential for delignification. Nevertheless, further molecular biology studies are needed to identify the species by 18s-rDNA.

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References