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Ligninases Production and Partial Purification of Mnp from Brazilian Fungal Isolate in Submerged Fermentation

Ferhan M[1, 2, 3]*, Leao AL[2], Itamar S de Melo[3], Yan N[1] and Sain M[1]

[1]*Center for Biocomposites and Biomaterials Processing, Faculty of Forestry, University of Toronto, Toronto, ON, Canada*
[2]*Department of Environmental Sciences, Sao Paulo State University, UNESP, Botucatu, SP, Brazil*
[3]*Department of Microbiology, Embrapa Environment, Jaguariúna, SP, Brazil*

Abstract

The potential of ligninases as a green tool for effective valorization of lignin can be shown through enzymatic cocktails containing different lignin degrading enzymes. The present study deals with the screening of potential fungal strains useful for the liquefaction of bark containing lignin. Three different local isolates (*Pleurotus ostreatus POS97/14, Pycnoporus sanguineus* and the local isolated fungal strain) were selected out of ten different strains for ligninases production. Maximum production of enzymes was observed in the local isolated fungal strain after ten days in submerged fermentation.The isolated fungal strain produces ligninases mainly for manganese peroxidase (MnP). The enzyme oxidized a variety of the usual MnP substrates, including lignin related phenols. Furthermore, the partial purification for MnP was determined by FPLC and the molecular weight was evaluated by SDS-PAGE.

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):H_2O_2 oxidoreductase. According to protein databank, the MnP containing cofactor is protoporphyrin IX having Fe ($C_{34}H_{32}FeN_4O_4$) incorporated with Mn^{+2} and Ca^{+2} 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 cholorophenols. 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 commonly found in higher plants, several bacteria and some insects. Nevertheless, the well-characterized laccases are fungal in origin [6]. 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 LWART 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.

***Corresponding author:** Ferhan M, Center for Biocomposites and Biomaterials Processing, Faculty of Forestry, University of Toronto, Toronto, ON, Canada M5S 3B3; E-mail: muhammad.ferhan@utoronto.ca

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Fermentative organism and culturing 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 1L) containing 0.05% of the dye Remazol Brilliant Blue R (RBBR). Plates were incubated in the dark for 14 days at 25^{0}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^{0}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 595nm using a Shimadzu UV-1601PC spectrophotometer. Cell biomass was measured gravimetrically by oven-drying at 70^{0}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, $MgSO_4.7H_20$ 0.05g l^{-1}, KH_2PO_4 0.5g/L, K_2HPO_4 0.6g l^{-1}, $CuSO_4.5H_20$ 0.4mg l^{-1}, $MnCl_2.4H_20$ 0.09mg l^{-1}, H_3BO_3 0.07mg l^{-1}, $NaMoO_4.2H_20$ 0.02mg l^{-1}, $FeCl_3$ 1mg l^{-1}, $ZnCl_2$ 3.5mg l^{-1}, Thiamine- HCl 0.1mg l^{-1}, Biotin 5µg l^{-1}. The medium was dispersed into 250 mL Erlenmeyer flasks at a rate of 50 mL of medium per flask adjusted pH to 6.5 with 1N NaOH and autoclaved at (121°C) for fifteen minutes. A loop with fungal strains was transferred to the sterilized growth medium under sterile conditions and the flasks were incubated at 30^{0}C in a shaker (130 rpm) with continuous shaking.

Enzymatic analysis

MnP activity was measured at 610 nm (ε =4460 $M^{-1}cm^{-1}$) 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 $MnSO_4$; and 50 µl of H_2O_2 from 2- mM sodium succinate buffer prepared from 20-mM, pH-4.0 stock solution. The reaction mixture was kept in a water bath for 5 min and incubated at 30^{0}C; reactions were terminated by the addition of 40 µl of 2N NaOH solution.

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-H_2O_2; 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 H_2O_2. 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^{0}C. The ammonium sulfate precipitate was collected by centrifugation (5000 g x 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 x 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 x 300 mm), which was also equilibrated with buffer A.

30 ml MnP fractions were collected and concentrated to 5 ml by ultrafiltration with a Centriprep-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^{0}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^{0}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 JSM-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 N_2 laser (337 nm, 3 ns pulse width with pulse energy of 200 mJ). The acceleration voltage of ion source-1=19.48 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

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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 acetone/Milli-Q-water with a concentration of 0.1–0.5% (w/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 lingninases-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 every 24 hour and the culture supernatants were analyzed for ligninases activity and cell biomass was also noted. The maximum production of ligninolytic activity by the newly isolated Brazilian fungal strain, MnP (64 IU l^{-1}), LiP (26.35 IU l^{-1}) and laccase (5.44 IU l^{-1}), respectively, was attained in 10 days. In contrast, the ligninolytic activity of *Pleurotus ostreatus,* and *Pycnoporus sanguineus* (Table 1) had minimal remazol decolorization efficiency and surprisingly did not show any peroxidase activity. The low peroxidase activity and decolorization efficiency, especially as compared to that which has been reported for other strains (*Ganoderma spp, Stereum ostrea,* and *Trametes versicolor*) [22] raises the possibility that the growing conditions or the growth medium were not optimal for these fungal strains.

It was also observed that cell biomass increased with increasing fermentation time. In addition, ligninases activity also increased with increasing fermentation time for 10 days, but a further increase (12 days) in fermentation time (Table 1) decreased ligninase activities (Table 1). The local isolated fungal strain was the best producer of MnP (64 IU l^{-1}) as the main enzyme activity, followed by LiP (26.35 IU l^{-1}) and laccase (5.44 IU l^{-1}), respectively, as evidenced by the high percentage (94%) of RBBR decolorization in the broth medium. During exponential phase, the increase of cell biomass resulted in an increase in secretory proteins in the growth medium. Increase of cell biomass yield was associated with increased extracellular secretory protein in the fermentation medium up to the 12th day, when biomass started to decrease. 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

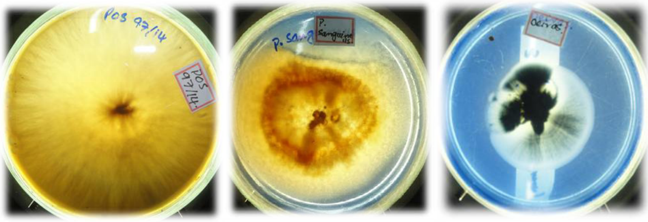

Figure 1: Potential screening of three fungal strains out of ten based on the ligninases production and degradation of RBBR dye.

Isolated fungal strain

Days	Laccase (IU l^{-1})	LiP (IU l^{-1})	MnP (IU l^{-1})
2	$1,42 \pm 0,01^{f}$	$17,61 \pm 0,05^{e}$	$16,7 \pm 0,21^{f}$
4	$3,17 \pm 0,01^{e}$	$19,70 \pm 0,09^{d}$	$36,8 \pm 0,09^{d}$
6	$4,45 \pm 0,00^{d}$	$21,88 \pm 0,03^{c}$	$57,0 \pm 0,09^{c}$
8	$5,3 \pm 0,01^{b}$	$23,80 \pm 0,09^{b}$	$59,3 \pm 0,09^{b}$
10	$5,44 \pm 0,01^{a}$	$26,35 \pm 0,07^{a}$	$64,0 \pm 0,12^{a}$
12	$5,19 \pm 0,01^{c}$	$14,90 \pm 0,10^{f}$	$30,9 \pm 0,14^{e}$

Pleurotus ostreatus POS97/14

Days	Laccase (IU l^{-1})	LiP (IU l^{-1})	MnP (IU l^{-1})
2	$0,14 \pm 0,01^{d}$	$0,82 \pm 0,07^{f}$	$5,17 \pm 0,11^{e}$
4	$0,18 \pm 0,02^{d}$	$2,93 \pm 0,05^{e}$	$7,62 \pm 0,09^{d}$
6	$0,31 \pm 0,01^{c}$	$4,03 \pm 0,07^{d}$	$10,09 \pm 0,16^{c}$
8	$0,56 \pm 0,01^{b}$	$5,84 \pm 0,04^{c}$	$13,30 \pm 0,14^{b}$
10	$0,80 \pm 0,01^{a}$	$6,77 \pm 0,09^{a}$	$17,94 \pm 0,45^{a}$
12	$0,54 \pm 0,01^{b}$	$6,58 \pm 0,06^{b}$	$12,90 \pm 0,14^{b}$

Pycnoporus sanguineus

Days	Laccase (IU l^{-1})	LiP (IU l^{-1})	MnP (IU l^{-1})
2	$0,31 \pm 0,01^{d}$	$0,67 \pm 0,04^{f}$	$1,09 \pm 0,14^{f}$
4	$0,79 \pm 0,02^{c}$	$1,73 \pm 0,13^{e}$	$6,11 \pm 0,11^{d}$
6	$1,07 \pm 0,01^{b}$	$2,36 \pm 0,05^{c}$	$9,79 \pm 0,14^{c}$
8	$1,13 \pm 0,00^{a}$	$2,77 \pm 0,08^{b}$	$10,93 \pm 0,14^{b}$
10	$1,15 \pm 0,01^{a}$	$3,41 \pm 0,09^{a}$	$13,21 \pm 0,14^{a}$
12	$1,12 \pm 0,01^{a}$	$2,06 \pm 0,13^{d}$	$1,66 \pm 0,20^{e}$

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

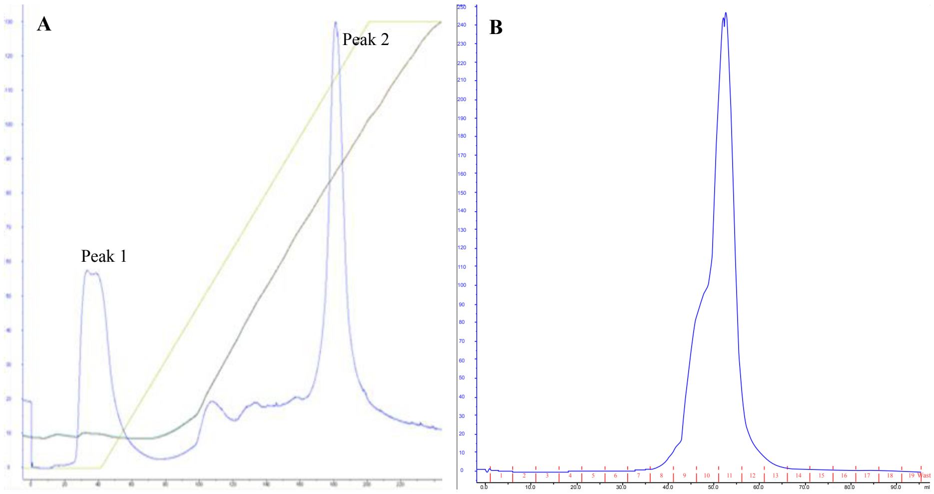

Figure 2: Size exclusion chromatography of MnP from isolated fungal strain (A): DEAE fractionations from isolated fungal strain (B): MnP activity was eluted as a single peak.

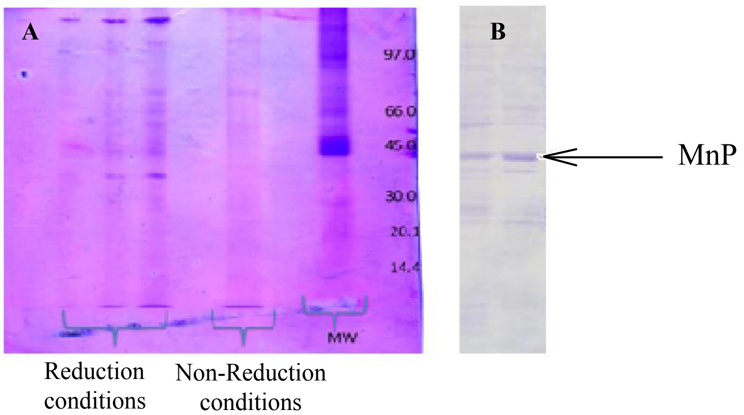

Figure 3: Polyacrylamide gel electrophoresis (A): Ligninases with reduction and non-reduction conditions (B): Purified MnP from isolated fungal strain stained with Coomassie Blue R-250 showed a molecular mass of 37 kDa.

Purification steps	***Activity/L***	***Protein content (U/mg)***	***Specific activity (U/mg)***	***Purification fold***
Crude enzyme	4468,00	228,95	98,6	1
Ammonium Sulphate	1635,00	157,8	182,07	1,84
Dialysis	1443,00	101,2	190,1	1,92
1st DEAE- Sepharose Fast Flow column (10 x 300 mm)	1382,00	89,0	220	2,23
2nd DEAE Superdex 75 (10 x 300 mm)	1254,00	20	294	2,98
Sephadex G-100	1086,00	5,6	318	3,22

Table 2: Partial purification summary of MnP produced during submerged fermentation by isolated fungal strain under optimized conditions.

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.

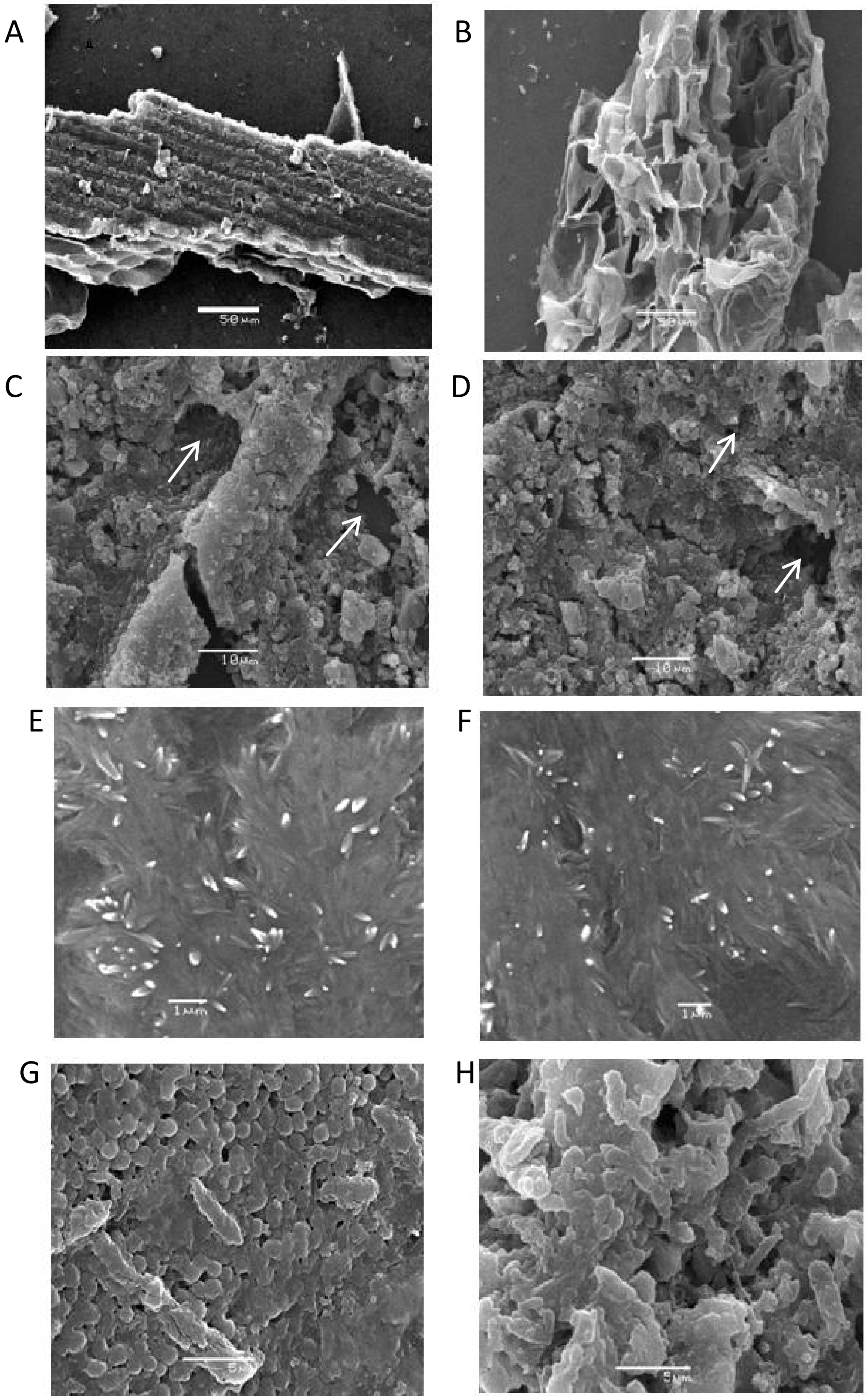

Figure 4: Scanning electron microscopy of sugarcane bagasse (SCB) and Black liquor (BL) of Eucalyptus. (A & B) Control SCB (magnification, x 400); (C & D) bagasse treated with local fungal isolate grown after 2 weeks (magnification, x 2k); (E & F) Control BL of Eucalyptus fibers (magnification, x 10k); (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).

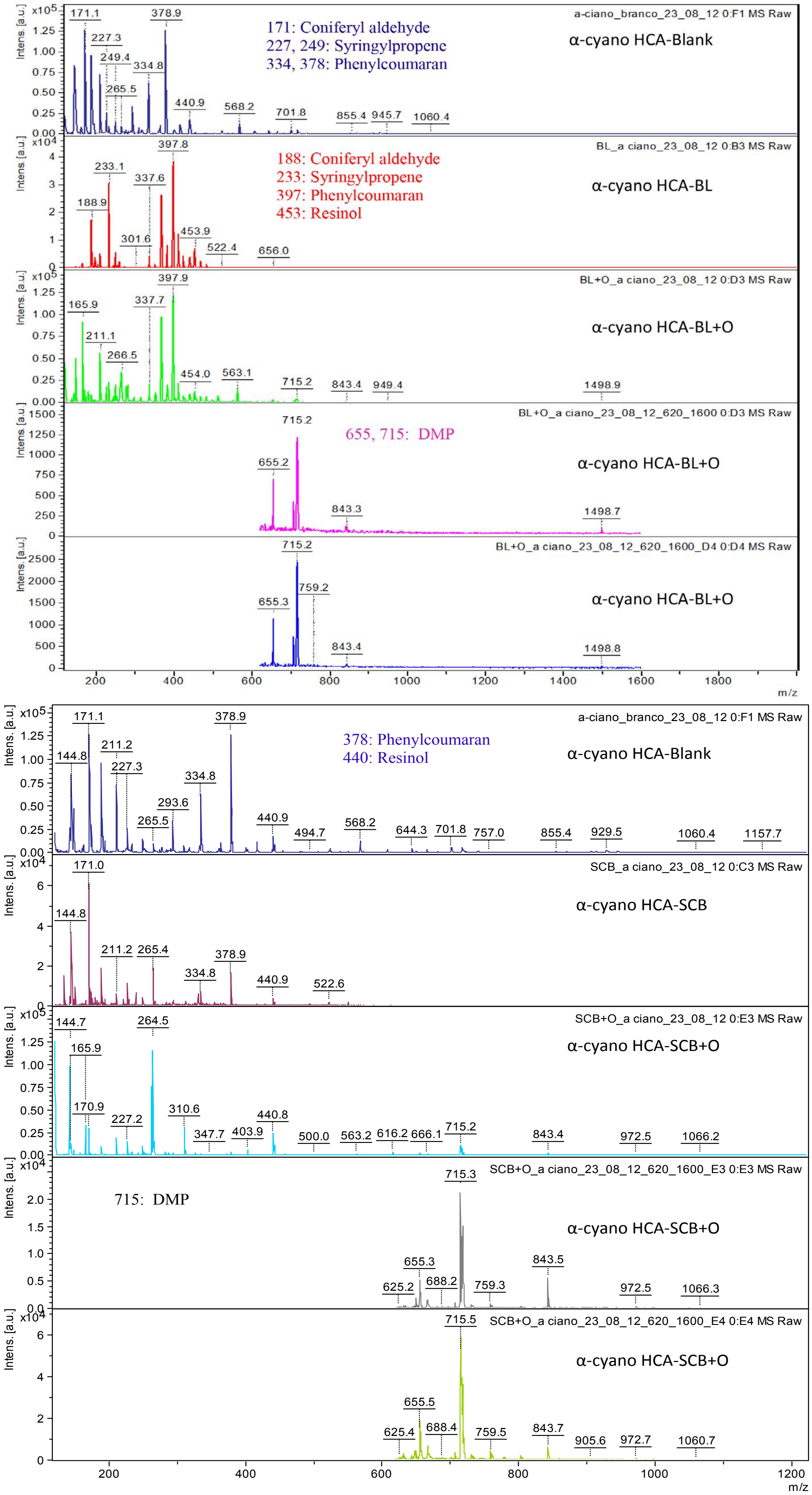

Figure 5: MW distribution to determine the lignin monomers by using MALDI-TOF-MS where α-cyano-HCA was used as matrix compound.

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 environmental-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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