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

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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, Fast protein liquid chromatography; Manganese peroxidase; Lignin peroxidase; Lcc; Time course studies

Introduction

The considerable lignin degraders are white-rot fungi regarding wood living microorganisms. It produces extracellular enzymes, that is, MnP, LiP and laccase which play important role in lignin biodegradation [1]. Pleurotus ostreatus is a white rot fungus belongs to basidiomycetes and it also considered as cholesterol reducing mushroom [2]. *Pleurotus spp.*, were discovered to drought lignin peroxidases [3], presenting the ligninolytic enzymes mainly laccases are involved for the degradation of lignin. The combined action of laccase and aryl alcohol oxidase makes important decrease in molecular weight of soluble lignosulphonates secreted by *P. ostreatus* [3]. Ligninolytic system configuration is complicated and species restricted [4]. White rot fungi contains ligninases include MnPs, LiPs and Lcc which are possible providers to fungal ligninolysis. White rotted lignin and depolymerized lignin were mainly found after breakdown of lignin model compounds to produce consistent products [5].

MnP belongs to the family of oxidoreductases, to be specific of those acting on peroxide as acceptor (peroxidases). The systematic name of this enzyme class is Mn (II):hydrogen-peroxide oxidoreductase. Other names in common use includes peroxidase-M2, and Mn-dependent (NADH-oxidizing) peroxidase. This enzyme needs Ca^{2+} for activity. Eventually, peroxidases oxidize phenolic compounds and decreased molecular oxygen to water [1]. Ligninases oxidized several environmental pollutants such as polycyclic aromatic hydrocarbons, dyes and cholorophenols. Heme containing enzymes like (LiP and MnP) having characteristic catalytic cycles include certain of other peroxidases. LiP has an ability to oxidize many aromatic compounds whereas MnP about entirely oxidized Mn (II) to Mn (III) [6,7]. Laccases (benzenediol: oxygen oxidoreductase, EC (1. 10. 3. 2) are multi-copper blue oxidases commonly divided in higher plants, few bacteria and in some insects. Nevertheless, the well-known laccases produced from fungal origin [6]. Laccases got significant importance in many industrial areas due to remarkable existing catalytic properties. Most investigated applications comprised immunoassay bio-labelling, biosensors, biocatalyst, and advancement of oxygen cathodes in biofuel cells. In addition, also had good prospective in environmental sector includes textile dye bleaching, pulp delignification and xenobiotic compound degradation due to the wide-ranged substrate specificity [6,8]. The present applications of this enzyme motivate us to do new basic research.

The current research activity of ligninases includes by utilizing the local lignin sources (eucalyptus and sugarcane bagasse) and checked their delignification pattern. The characterization of ligninases (MnPs, LiPs and Lcc,) from the 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 covers about 735,000 km^2. It is the most degraded vegetation type in the semi-arid, and has less than 1% of it protected in permanent reserves [9].

Substrate collection and preparation

Lignocellulosic materials like sugarcane bagasse, eucalyptus were used for delignification. Sugarcane bagasse and eucalyptus were collected LWART Química, Brazil. All the substrates were dried in an oven at 80°C to constant weight and were grounded in an electric

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grinder to powder form and stored in airtight plastic jars to keep the substrate free of moisture.

Fermentative organism and culturing conditions

Cultures of *P. ostreatus* and *P. sanguineus* were collected from Mycology Collection Lab, Department of Plant Protection, UNESP, Botucatu, SP while local fungal isolate fungal strain was isolated from Northeast part of the country named Caatinga forest. Each fungus were plated on the center of a petri dish containing malt extract (malt extract 25 g; agar 20 g; distilled water 1l), supplemented or not with 0.05% Remazol Brilliant Blue R (RBBR). The cultures were incubated in the dark for 14 days at 25°C. Then, the strains cultured in the presence of the dye were evaluated with regard to their ligninolytic potential by decolorization of the dye.

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

Seven strains that showed the best ligninolytic potential were grown in liquid culture medium (2.5% malt extract; 0.05% RBBR and water 100%). The cultures were incubated at 30°C, in the dark, under constant agitation. Decolorization, which is associated with dye oxidation, was determined at 10 days by monitoring the decrease in the absorbance peak at 595 nm, using a Shimadzu brand UV-1601PC spectrophotometer. The mycelial biomass was separated by filtration and weighed; quantification was performed through gravimetry, by oven-drying at 70°C until constant weight was achieved. The experimental design was completely randomized and consisted of three replicates.

Growth media preparation

Growth media for *P. ostreatus* contained: Glucose 10 g/L, L-Asparagine monohydrate 3 g/L, $MgSO_4.7H_20$ 0.05 gl^{-1}, KH_2PO_4 0.5 g/L, K_2HPO_4 0.6 gl^{-1}, $CuSO_4.5H_20$ 0.4 mgl^{-1}, $MnCl_2.4H_20$ 0.09 mg l^{-1},H_3BO_3 0.07 mg l^{-1}, $NaMoO_4.2H_20$ 0.02 mg l^{-1}, $FeCl_3$1 mg l^{-1}, $ZnCl_2$3.5 mgl^{-1}, Thiamine- HCl 0.1 mgl^{-1}, Biotin 5 μgl^{-1}. The medium was dispersed into 250 mL erlenmeyer flasks at a rate of 50 mL of medium per flask adjusted pH to 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°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 reactive mixture (1 ml) contained: culture medium (500 µl); phenol red (100 µl); sodium lactate 250 mM (100 µl); bovine albumin 0.5% (200 µl); manganese sulfate 2 mM (50 µl) and hydrogenperoxide 2 mM (50 µl) prepared in a sodium succinate buffer 20 mM, pH 4.0. The reactions occurred at 30°C for 5 min and were interrupted by the addition of 2 N NaOH (40 µl).

The lignin peroxidase activity was evaluated by UV spectrometry of the veratryl aldehyde produced (ε_{310} =9300 M^{-1} cm^{-1}) during veratryl alcohol oxidation. The reactive mixture contained 375 µl sodium tartrate buffer 0.33 MpH; 125 µl veratryl alcohol 4 mM; 50 µl hydrogen peroxide 10 mM; 450 µl distilled water and 250 µl culture medium for a final volume of 1250 µL.

The laccase activity was also measured by spectrophotometry, as o-dianisidine oxidation at 525 nm (ε_{525}=65,000 M^{-1} cm^{-1}). The reactive mixture contained per 1 mL: citrate–phosphate buffer, 0.5 M with pH 5.0 (200 µl); 1 mM o-dianisidine solution (100 µl), culture medium (600 µl) and hydrogen peroxide (100 µl). The boiled culture medium was used as a control. For all enzymes under evaluation, one activity unit was defined as the amount of enzyme necessary to oxidize 1 µmol of substrate per minute. The specific activity was expressed as units per microgram of protein [11]. Each value presented here represents the mean of three replicates.

Ligninases cocktail partial purification and characterization

All steps were carried out at 4°C. Proteins in 2 L of culture filtrate were precipitated by addition of ammonium sulfate (80% saturation). The precipitate was collected by centrifugation (5000 g×30 min) and dissolved in 50 mM potassium phosphate buffer, pH 6.5 (buffer A). The enzyme solution was dialyzed overnight against buffer A, then loaded onto a DEAE Sepharose Fast Flow column (10×300 mm) previously equilibrated with buffer A. The column was washed with buffer A and eluted with 220 ml of 0 to 1.0 M linear gradient of NaCI in buffer A at a flow rate of 0.5 ml/min. Fractions containing MnP activity were collected. Fractions with MnP activity were pooled and dialyzed overnight against buffer A, then loaded onto a Superdex 75 (10×300 mm), previously equilibrated with buffer A.

The active fractions (30 ml) were collected and concentrated to 5 ml by ultrafiltration with a Centriprep-3 (3 kDa cut-off, Amicon). The concentrated supernatant was loaded onto a Sephadex G-100 column equilibrated with buffer A, containing 100 mM NaCl in a fast protein liquid chromatography (FPLC) system (Pharmacia, AKTA purifier). The samples were detected by UV detector by using Unicorn 5.11 software of Pharmacia. At this step, MnP activity was eluted as a single peak corresponding to a peak of absorbance at 280 nm. The purified and concentrated enzyme was kept frozen at -20°C without significant loss of activity for several months [12].

Scanning electron microscopy (SEM)

Samples of sugarcane bagasse and black liquor of eucalyptus were oven-dried at 50°C for 1 h and thick layer of samples were supported in the sample-holder fixed on a carbon ribbon. This assembly was maintained in a vacuum- desiccator until the analysis. The SEM with a Jeol model JSM-6360LV microscope was used for observing the lignin samples before and after fungal treatment [13].

Determination of total proteins

The quantification of total proteins was performed by means of a standard curve obtained from solutions containing bovine serum albumin (BSA) at known concentrations (0; 0.005; 0.010; 0.015; 0.020; and 0.025 mg ml^{-1}), Bradford's reagent (0.20 mL), and sufficient water to complete a final volume of 1 ml. The samples to be analyzed contained 800 µl of the enzymatic mixture and 200 µl of Bradford's reagent. After agitation, the absorbance was measured at 595 nm.

Gel electrophoresis and staining

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Höfer [14]. Gels were stained with Coomassie brilliant blue R-250. Molecular weights of proteins were determined with commercial molecular weight markers (Bio-Rad, Munich, Germany).

MS Analysis to determine the lignin monomers

MALDI-TOF-MS spectra were acquired on Autoflex III Series MALDI-TOF, (Bruker Daltonic Leipzig, Germany) with pulsed ion

extraction of 130 ns. The mass spectrometer was equipped with N_2 laser (337 nm, 3 ns pulse width with Pulse energy of 200 mJ). Acceleration voltage 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 used. The α-cyano-4-hydroxy cinnamic acid (Sigma-Aldrich) was used as matrix compound α-cyano-HCA 10 mg/ml dissolved in acetonitrile/Milli-Q-water 1:1 (v/v) with a concentration of 2.5% TFA. The samples were diluted in acetone/Milli-Q-water 9:1 (v/v) with the concentration of 0.1–0.5% (w/v) and mixed 1:1 (v/v) with the matrix solution for the analysis. The MS measurements were done in the reflector mode [15].

Statistical analysis

Treatment effects were compared by the protected least significant difference methods with ANOVA using Statistical Assistance version 7.6 beta software.

Results and Discussion

Due to broad substrate specificity, the white rot fungi had ligninases potential has been reported as liable for alteration and mineralization of organic pollutants which is structurally resemble to lignin. Ligninases are the combination of three peroxidases contains MnP, LiP and laccases which characterized as lignin degrading enzymes. Distribution of MnP and laccases are very frequent in white rot fungi but not LiP [16].

Degradation and decolorization of many organic pollutants by white-rot fungi have been reported by many researchers [17] but mainly MnP and LiP were reported for the degradation of polymeric dyes [18]. The white rot fungi had ability to decolorization of polymeric dyes because of existing ligninolytic enzymes (Figure 1). To select the best ligninases produced microorganism on petri-plate, used polymeric dyes like Remazol Brilliant Blue R (RBBR) which is similar to lignin polymer. An anthracene derivative like RBBR considered as organic pollutant. Many researchers used as a model compound and measured the ligninases activity based on their decolorization efficiency [18].

Ligninases production during the time course studies

After every 24 h, the triplicate fermentation flasks were harvested and culture supernatants were analyzed for ligninases and dry weight of biomass (residue) was also recorded. The results of isolated fungal strain during time course study showed that maximum production of MnP (64 IUl^{-1}), LiP (26.35 IUl^{-1}) and laccase (5.44 IUl^{-1}) were achieved in 10 days from local isolated fungal strain as compared to other two strains (*Pleurotus ostreatus, Pycnoporus sanguineus*) The enzymatic pattern for isolated fungal strain was differentiated among the known strains (Table 1); *P. ostreatus, P. sanguineus* showed ligninolytic activities were distinctive as compared to other strains (*Ganoderma spp*, *Stereum ostrea,* and *Trametes versicolor*) had the least remazol decolorization efficiency and did not show any peroxidase activity. The negative peroxidase test suggests that the fungi did not produce the enzymes at levels significant enough to be detected, or the production of ligninases requires different growing conditions for those strains.

As the incubation time increased, the biomass weight also increased. It was observed that ligninases production steadily increased with an increasing fermentation time and further increase in fermentation time showed a decrease in ligninases activities (Table 1). It was also important to note that local isolate is the best producer of MnP (64 IUl^{-1}) as the major enzyme activity, followed by LiP (26.35 IUl^{-1}) and laccase (5.44 IUl^{-1}) respectively. The isolated fungal strain had the highest percentage of RBBR decolorization (94%) in liquid medium. As a control, the fungi were grown in liquid culture medium without supplementation. During the development period, a follow-up was conducted for biomass and protein yield in the medium. The concentration of proteins was parallel to the biomass yield, with the exception of the 12th day, when biomass began to decrease. The specific activities of MnP and LiP however continued to increase [19].

Purification of MnP

MnP purification mainly consists of two steps, including ammonium sulfate precipitation and size exclusion chromatography. The protein peak was determined at (A280) and the DEAE fractionations were collected shown in (Figure 2A). The MnP activity was eluted as a single peak in size exclusion chromatography (Figure 2B). Under reduced and non-reduced conditions, the total proteins were run on native-PAGE shown in (Figure 3A). Finally to determine the molecular weight of purified enzyme MnP was separated on SDS-PAGE and stained with Coomassie Blue R-250 (3B). The purification procedure is summarized in Table 2. The molecular mass of purified MnP was 37 kDa shown in Figure 3B and the specific activity of purified enzyme was 3.22-fold.

Analysis of the effect of isolated fungal strain treatment on sugarcane

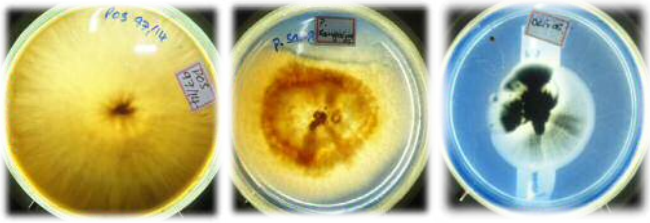

Figure 1: Potential screening of three fungal strains out of ten based on the ligninases production and degradation of RBBR dye.

Isolated fungal strain

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

Pleurotus ostreatus POS97/14

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

Pycnoporus sanguineus

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

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

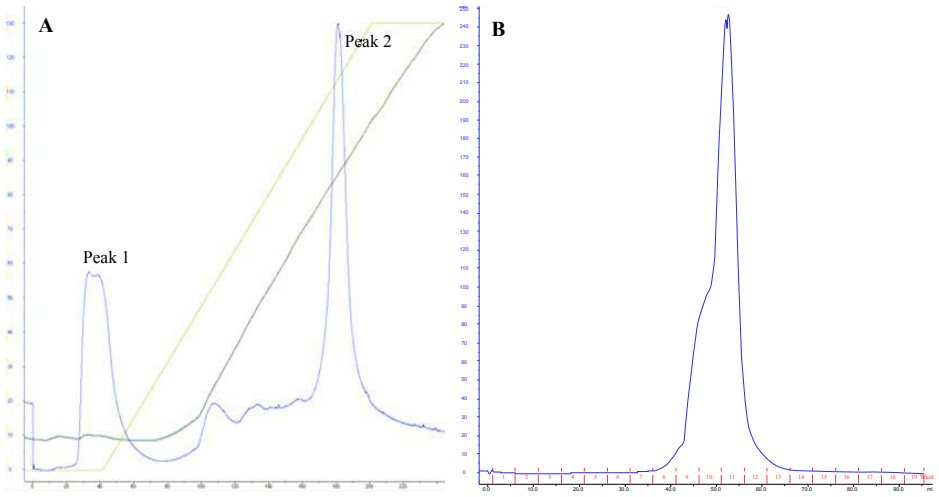

Figure 2: Size exclusion chromatography of MnP from isolated fungal strain (A): DEAE fractionations from isolated fungal strain (B): MnP activity was eluted as a single peak.

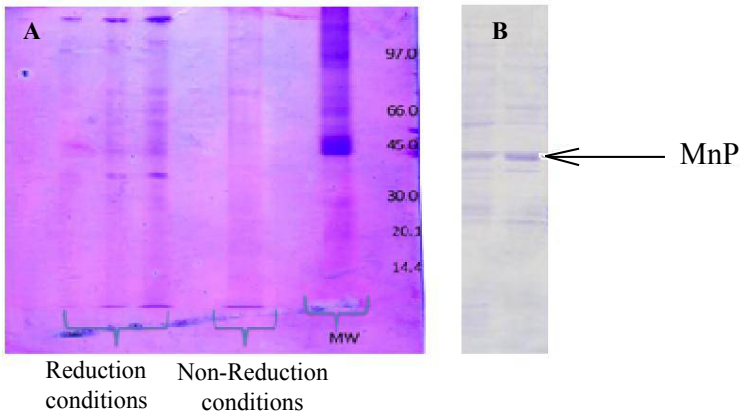

Figure 3: Polyacrylamide gel electrophoresis (A): Ligninases with reduction and non-reduction conditions (B): Purified MnP from isolated fungal strain stained with Coomassie Blue R-250 showed a molecular mass of 37 kDa.

Purification steps	*Activity/L*	*Protein content (U/mg)*	*Specific activity (U/mg)*	*Purification fold*
Crude enzyme	446800	2289.5	98.6	1
Ammonium Sulphate	163500	1578	182.07	1.84
Dialysis	144300	1012	190.1	1.92
1st DEAE- Sepharose Fast Flow column (10x300 mm)	138200	890	220	2.23
2nd DEAE Superdex 75 (10x300 mm)	125400	20	294	2.98
Sephadex G-100	108600	5.6	318	3.22

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

bagasse and black liquor of Eucalyptus by scanning electron microscope (SEM) was carried out (Figure 4). When the SEM technique was used, an abundant fungal growth on the bagasse and black liquor fibers were observed which indicates the decay of lignin samples. The fiber surface of bagasse sample clearly shows that modifications resulting from the fungus action on the fiber structure with the presence of bore holes occurred, indicated by arrows in Figure 4 (C and D).

The matrix compound method gives a contribution to the elucidation of the lignin constitution. The interpretation of matrix results leads to an understanding of the lignin structure. To evaluate the effect of the fungal treatment on the lignin, to characterized the lignin. It appeared that the fungal attack mainly targeted the phenolic units of the lignin, since there was only a decrease in the relative frequency of β-O-4-linked H units observed and as these units are essentially terminal units with free phenolic groups [20]. MALDI-TOF-MS spectra showed in (Figure 5) detailed structures of molar mass distribution of lignophenols after delignification. In delignification spectra, lignin monomers showed 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 [15] Most monomers were derivatives from guaiacol and syringol. Most dimers were assigned to phenylcoumaran structure. Biphenyl and resinol were less frequent.

Conclusions

With the increasing global concern on fossil fuel depletion and

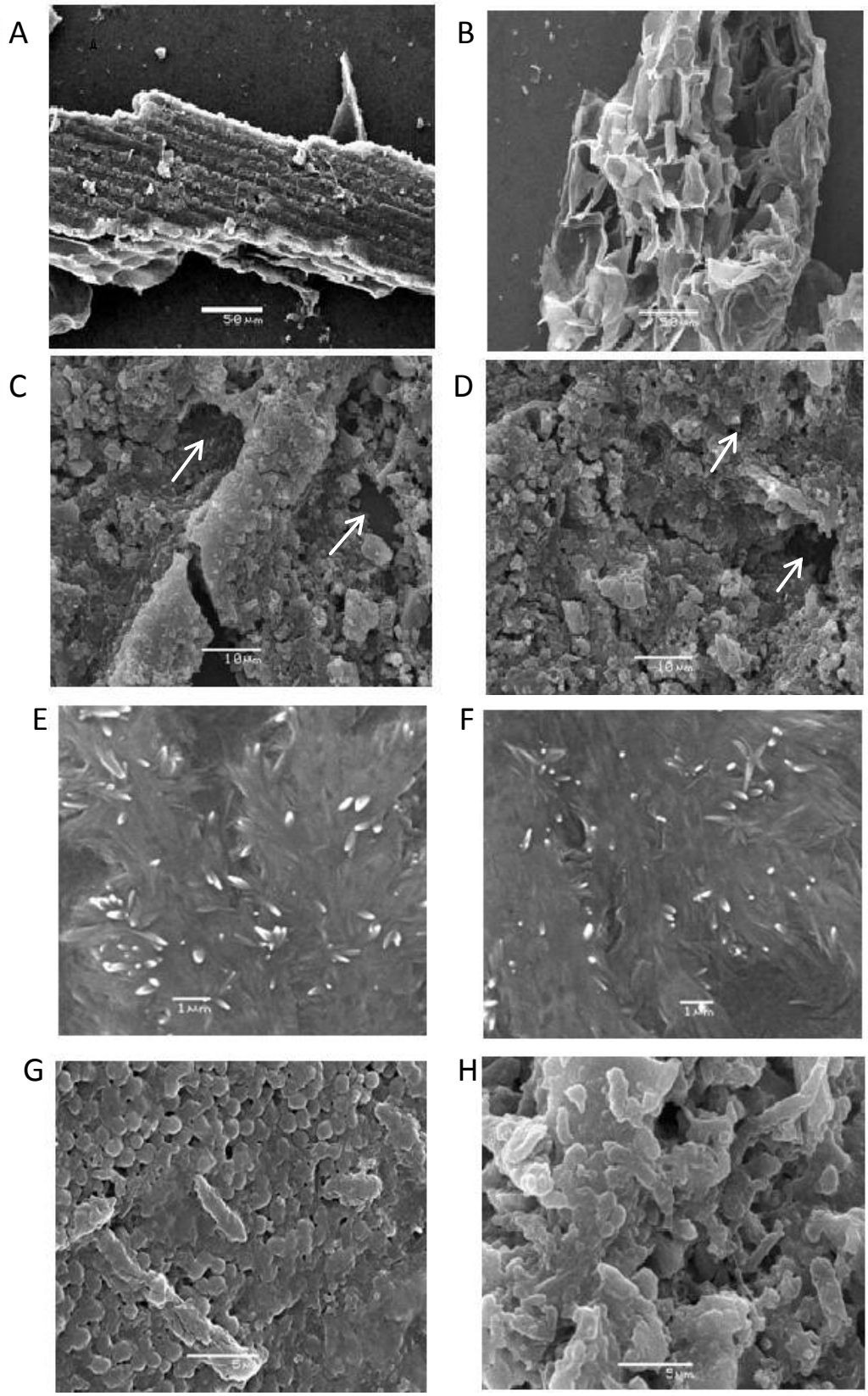

Figure 4: Scanning electron microscopy of sugarcane bagasse (SCB) and Black liquor (BL) of Eucalyptus. (A & B) Control SCB (magnification, x 400); (C & D) bagasse treated with local fungal isolate grown after 2 weeks (magnification, x 2k); (E & F) Control BL of Eucalyptus fibers (magnification, x 10k);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

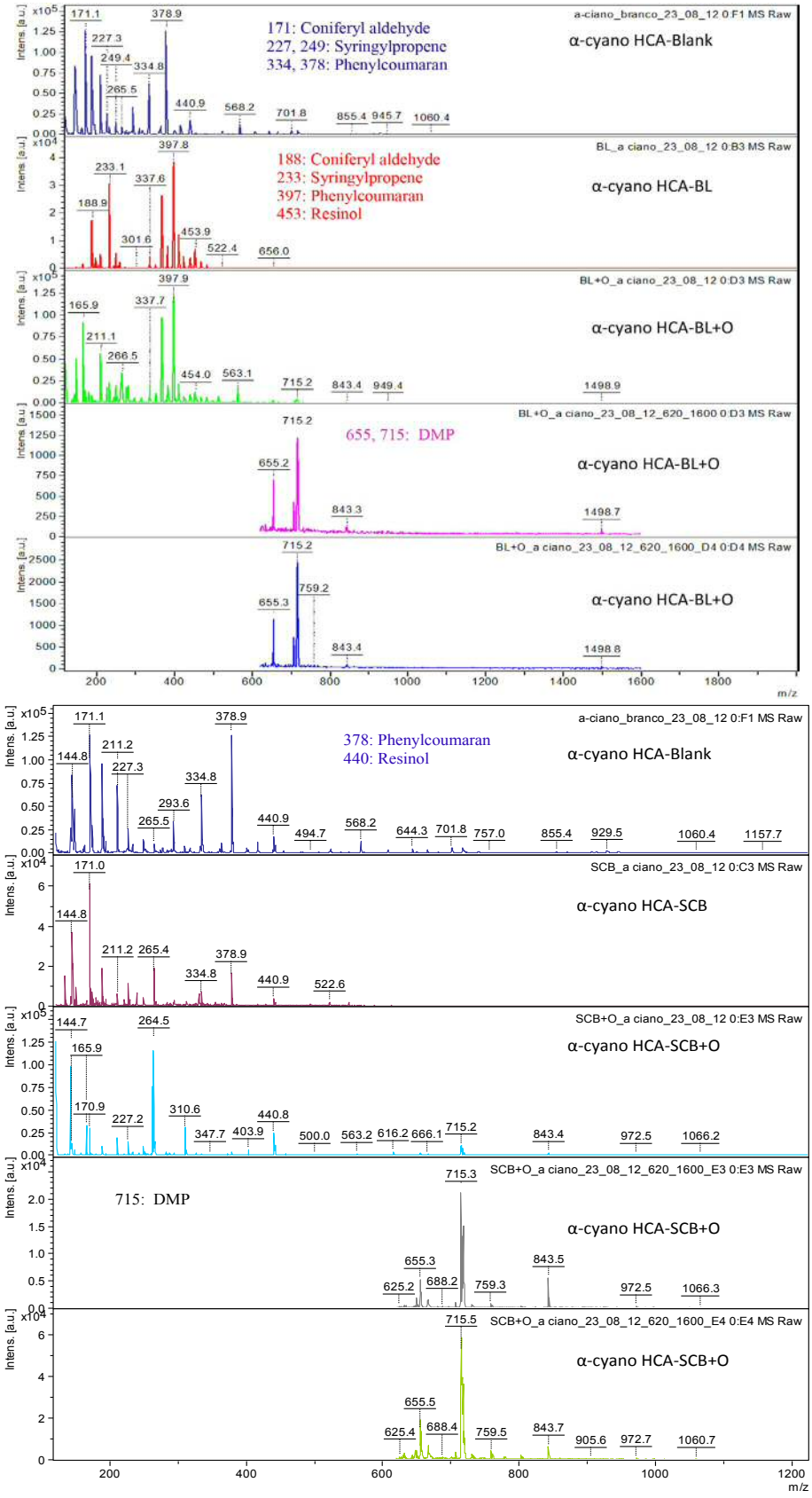

Figure 5: MW distribution to determine the lignin monomers by using MALDI-TOF-MS where α-cyano-HCA was used as matrix compound.

environmental footprint, there is a strong interest in exploring biorenewable resources as alternative feedstock for making more environmental-friendly biomaterials. The results of the present study indicate the screening of potential fungal strain for ligninases production, partial purification of MnP and the degrading pattern of local lignin resources found in Brazil. It allows a deeper insight into the mechanism of delignification process. The demand for application of ligninases in industry and biotechnology is ever increasing due to their use in a variety of processes. Their capacities to remove xenobiotic substances and produce polymeric products make them a useful tool for bioremediation purposes. The unknown isolated fungal strain has a potential for delignification. Nevertheless, further molecular biology studies are needed to identify the specie by 18S-rDNA.

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