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Comparative studies of loblolly pine biodegradation and enzyme production by Argentinean white rot fungi focused on biopulping processes

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Abstract

The ability of eight white rot fungi: *Coriolopsis rigida*, *Coriolus versicolor* var. *antarcticus*, *Peniophora* sp., *Phanerochaete sordida*, *Pycnoporus sanguineus*, *Steccherinum* sp., *Trametes elegans* and *Trametes villosa* to selectively delignify loblolly pine (*Pinus taeda*) chips was studied. They were selected among 34 basidiomycetes from Argentina because of their capacity to decolorize Poly R-478 and Azure B. Fungal pretreatment caused changes in wood chemical composition as well as in physical structure. The present study allowed the identification of a new strain, potentially a candidate for use in softwoods biopulping processes. Results showed that *P. sanguineus* was able to reduce lignin content in 11% in 14 days of treatment, but also that *P. taeda* wood suffered notable structural changes of lignin and hemicelluloses during the treatment, as revealed from ^{13}C CP-MAS NMR spectra. An increase of 15% in porosity of decayed wood confirmed physical changes due to fungal attack.

Keywords: Biopulping; Lignocellulolytic enzymes; Loblolly wood degradation; White rot fungi

1. Introduction

Wood cell walls are made up mostly of cellulose, hemicellulose and lignin. The tensile strength of wood fibers is primarily determined by cellulose and hemicelluloses, while lignin mediates adhesion among the fibers. White rot fungi (WRF) have all the necessary enzymes for the complete degradation of wood components. Some fungal species remove lignin more efficiently than other wood components; such degradation pattern is known as selective lignin degradation or delignification [1]. This effect decreases the dependence on chemicals in the pulping processes and it is the most useful for biopulping. Under particular conditions extensive differential degradation of lignin was observed, an interesting example of such process is ''palo podrido'', where up to 90% of lignin degradation was achieved [2].

The high capability of WRF to degrade all wood components is based principally on the activity of different complexes of extracellular enzymes. These fungi secrete hydrolytic enzymes such as cellulases, pectinases and xylanases, which are typically induced by their substrates. On the other hand, lignin (a polymer of phenylpropane units connected by different C–C and C–O–C linkages) is oxidized and degraded by a ligninase system made up of at least three enzyme activities: lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. In addition, several findings provided information about low weight molecules that participate in the initial attack of lignocellulose. Fenton reaction produces $^{\bullet}$OH, this radical is the strongest oxidant found in white rot fungi [3,4]; another reactive molecules involved in lignin attack are the peroxyl radicals rendered from lipid peroxidation [5]. The same unique non-specific enzymes and mechanisms that give these fungi the ability to degrade lignin also allow them to degrade a wide range of pollutants. Among such pollutants a wide range of dyes are substrates of ligninases rendering as a product a colourless substance. Thus, dye decolorization may be used as a screening method to select potentially promissory

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strains. Poly R-478 and Azure B were used in many previous reports as useful tools to detect ligninolytic activity in WRF [6]. Cellulose is a linear polymer of glucose units, which can be hydrolyzed by the action of endoglucanases, cellobiohydrolases and β-glucosidases. Hemicellulose is a heterogeneous, branched polymer. The backbone of the polymer is built up by sugar monomers like xylose, in this case, the enzymes involved in its degradation are named xylanases. Since the structure of xylans is more complex and variable, a more complex assembly of enzymes is required than for cellulose hydrolysis. Similar to cellulases, the xylanases can act synergistically to achieve hydrolysis, predominant enzymes within this system are endo 1,4-β-xylanases which attack the polysaccharide backbone, and β-xylosidases, which hydrolyze short xylooligosaccharides to xylose [7].

WRF and their enzymes (particularly ligninases and xylanases) are considered for the treatment of wood chips prior to pulping. While ligninases attack the lignin, xylanases degrade hemicelluloses and make the pulp more permeable for the removal of residual lignin. Termed ''biopulping'', this process removes not only lignin but also some of the wood extractives, thus reducing the pitch content and effluent toxicity. When biological treatment is followed by mechanical pulping, there is as much as 30% energy saving, whereas when it is followed by sulfite or kraft pulping, the cooking time is dramatically reduced. The paper strength properties have also been found to improve after biopulping. However, this process is still in its beginnings and no full-scale biopulping mills are in operation at the moment [8,9].

The studies on enzyme production by wood rot fungi are usually carried out using chemically defined liquid medium, under conditions able to induce the production of a particular enzyme [10–12]. However, in natural environments and in solid-state fermentation, these fungi grow on woody substrates under quite different conditions from those of submerged cultures, the patterns of enzyme production being consequently different. Much research work is still necessary to fully understand the degradation process, and particularly the enzymes and other metabolites secreted by the fungi during wood decay. Additional findings on this field could help to elucidate the biochemical mechanisms of wood decay by fungi and as a result facilitate the fungal strain selection for biopulping and for other industrial applications [13–15].

Many previous studies have focused on the lignin-degrading enzymes of *Phanerochaete chrysosporium*, *Trametes versicolor* and *Ceriporiopsis subvermispora*. Recently however, there has been a growing interest in studying the lignin-modifying enzymes of a wider array of WRF, not only from the standpoint of comparative biology but also with the prospect of finding better lignin-degrading systems for use in biopulping and other biotechnological applications [16–18]. Fungi from Misiones province (Argentina) usually grow subject to extremely high temperatures and humidity. Therefore, they represent an interesting source in the search of thermo-tolerant enzymes for biotechnological applications. In this work 33 strains of WRF from Misiones, were evaluated for their ability to decolorize Poly R-478. Eight isolates that displayed the fastest decoloration in Poly R-478 medium were screened for their ability to decolorize Azure B, and inoculated on loblolly (*Pinus taeda*) wood chips, to evaluate their potential for biopulping processes. Several relevant hydrolytic enzymes (endoglucanase and endoxylanase) and oxidative enzymes (laccase and Mn-peroxidase) present in the culture extracts were studied. To explore the relationship between the enzyme equipment of each fungus and its corresponding pattern of degradation, wood physical structure and chemical composition were also determined.

2. Materials and methods

2.1. Fungal strains

Pure cultures of 34 white rot fungi strains from the Culture Collection (BAFC) of Universidad de Buenos Aires were used in the present study. Stock cultures were maintained on malt extract (1.2%), agar (2%) slants at 4 °C with periodic transfer.

2.2. Culture conditions

All the strains were inoculated on agar plates (90 mm in diameter, 20 cm^3 medium/Petri dish) containing malt extract (12.7 g dm^{-3}), glucose (10 g dm^{-3}) and agar (20 g dm^{-3}) (MEA) supplemented with Poly R-478 (0.02%) or Azure B (50 μM). Inoculum consisted of a 25-mm^2 surface agar plug from a 7-day-old culture grown on MEA. Uninoculated plates served as controls for abiotic decoloration. The plates were incubated at 28 °C for 21–28 days. Fungal growth was followed by measuring radial extension of the mycelium. Average growth rates (cm day^{-1}) were calculated. A decolorized zone appeared when the fungus degraded the dye. Weekly measurements of the colonies and the decolorized zones (if any) were performed for each strain.

Wood degradation experiments were carried out with 20 g (dry weight) of wood chips (5 cm × 2 cm × 0.5 cm) and 20 cm^3 of aqueous solution of corn steep liquor 1% (C1), corn steep liquor 3% (C3), malt extract 3% (M) or malt extract 5% plus peptone 1.5% (MP), which were sterilized and inoculated with the fungus. Inoculum was grown in 150 cm^3 Erlenmeyer flasks containing 25 cm^3 of liquid medium (shaken at 100 rpm) with the following composition: 1% glucose and 1.3% malt extract. Inoculation was carried out using 5 cm^3 (55% final moisture content of wood chips) of homogenized pellets from 7-day-old shaken cultures. The fungi were incubated at 28 °C for 14 days. After the given period of incubation, wood chips were cut in small fragments (ca. 2.5 cm × 0.4 cm × 0.5 cm), then soluble proteins and sugars were extracted by adding 225 cm^3 of distilled water, stirring for 20 min, followed by filtration and centrifugation. Crude extracts were used for all the determinations of enzyme activities and reducing sugars content. All the steps for crude extraction were performed at room temperature. The supernatants were stored at −20 °C until needed. The experiments were carried out in triplicate parallel cultures.

2.3. Enzyme assays

All enzyme activities were assayed at 30 °C. Laccase activity was determined by oxidation of ABTS [2,2′azinobis(3-ethylbenzthiazoline-6-sulphonate)] (ε_{420} = 36 mM^{-1} cm^{-1}). The reaction mixture contained 0.1 M sodium acetate buffer, pH 3.6 and 5 mM ABTS [19]. MnP activity was determined by oxidation of phenol red (0.01%). The reaction product was measured at 610 nm (ε_{610} = 22 mM^{-1} cm^{-1}). The reaction mixture contained 0.05 M succinate buffer pH 4.8, 0.1 mM $MnSO_4$, 0.1 mM H_2O_2 [20]. Endoglucanase and endoxylanase were determined measuring the reducing sugars produced after hydrolysis of the substrate by the Somogyi–Nelson method [21]. Measurements were made in 0.1 M sodium acetate buffer, pH 4.8, using the following substrates: carboxymethylcellulose (CMC) 0.5% for endoglucanase and xylan from oat spelts 0.2% for endoxylanase. Standard curve with glucose was made to calculate enzyme activities. Enzyme activity has been expressed in International Units (U), as the amount of enzyme needed to release 1 μmol of product in 1 min.

2.4. Analysis of wood composition

Chemical composition of non-treated and bio-treated wood chips was determined. Extractive-free wood samples were prepared according to T204 om-88 "Solvent extractives of wood and pulp" using ethanol–benzene or water as solvent. The solvent extractable material may be considered to consist primarily of resin and fatty acids and their esters, waxes, and unsaponifiable substances. Soluble materials or extractives in wood consist of those components that are soluble in neutral organic solvents. These compounds include waxes, fats, resins, photosterols and non-volatile hydrocarbons, low-molecular-weight carbohydrates, salts, and other water-soluble substances. Klason lignin was determined in accordance with Tappi T-222 om-88, "Acid-insoluble Lignin in Wood and Pulp". To determine acid-insoluble lignin in wood and pulp, the carbohydrates in wood are hydrolyzed and solubilized by sulfuric acid (72%); the acid-insoluble lignin is filtered off, dried, and weighed. Wood contains from about 20 to 30% lignin; removal of which is a main objective of pulping and bleaching processes. Determination of lignin content in wood and pulps provides information for evaluation and application of pulping and bleaching processes respectively. Cellulose content was determined by the Seifert method. The solvolytic method involving treatment of wood meal in a boiling mixture of acetylacetone–dioxane–hydrochloric acid gave the most accurate cellulose values and produced the purest cellulose residues [22]. Hemicellulose content was calculated by difference (100% − (total extractives + lignin + cellulose)). Lignin and cellulose analysis were determined on extractive-free wood. Pore size analysis was conducted by mercury porosimetry in a Poresizer 9320.

2.5. Analysis of NMR spectra

The non-treated and bio-treated with *Pycnoporus sanguineus* BAFC 2126 samples of *P. taeda* wood were analysed by ^{13}C CP-MAS NMR as a fine powder. ^{13}C solid-state NMR spectra were recorded at 100.6 MHz (9.4 T) on a Bruker Avance 400 spectrometer. A 7-mm double bearing Bruker rotor was spun in air at 5.0 kHz. In all experiments the ^{1}H and ^{13}C 90° pulses were ca. 4 μs. The CP-MAS spectra were recorded with a 5 s recycle delay and a 2 ms contact time.

3. Results and discussion

In this work 33 strains of WRF from Misiones province (Argentina) were examined for the decolorization of plates with the lignin model substrate Poly R-478. Their decolorizing abilities were compared with a strain of *Coriolus versicolor* var. *antarcticus*, which proved to be a very efficient dye-degrading microorganism [6]. The capability of Poly R-478 decolorization is indicative of peroxidase activity. Such activity includes the combined activity of H_2O_2-producing oxidases and peroxidases, and is correlated with the ability of WRF to degrade the tricyclic aromatic hydrocarbon anthracene [23]. According to de Koker et al. [24] fungi decolorizing Poly R-478 could be placed in different groups: fungi with strong LiP and MnP activity, fungi with strong MnP activity and fungi with strong laccase activity. On the other hand, LiP activity is responsible for Azure B degradation, therefore plates containing this dye were used to assess this activity [25]. All of the fungal strains tested were able to grow on MEA supplemented with Poly R-478 (Table 1), but 13 of them did not decolorize the dye after 21 days of incubation. We found inter-genera, inter-specific and intra-specific differences in the ratio between the growth halo and the decolorization halo, suggesting physiological differences. When surveying Poly R-478 decolorization abilities of wood-inhabiting fungi, similar results were obtained by Freitag and Morrell [26] and Levin et al. [6]. In most cases, when produced, all the decolorized zones were smaller than the diameters of the corresponding colonies, consistent with the decolorization being a secondary metabolic activity of the older mycelium. Similar results were obtained by Levin et al. [6] when screening WRF for their ability to decolorize various dyes. Consistent with previous findings, in our study *P. sanguineus* and *Peniophora* strains were also included among the most rapid Poly R-478 decolorizing agents [6,26,27]. The eight isolates that displayed the fastest decoloration in Poly R-478 medium were tested for their ability to decolorize Azure B. Growth rates observed in this medium were higher than those observed in cultures supplemented with Poly R-478. All of the strains tested in this second screening step were able to decolorize Azure B, showing similar values of decolorization halo diameter than those observed in Poly R-478 medium.

The selected fungi were inoculated on *P. taeda* wood chips to evaluate their potential for biopulping processes. Growth was assessed visually, and while *P. sanguineus* displayed the fastest growth and a dense mycelial mat covered the chips after 7 days, only scatter hyphae were seen on the chips colonized by the other fungi evaluated after 2 weeks of treatment.

Quantification of extracellular enzymes is limited by the not exhaustive extraction method, therefore a preliminary study was conducted to determine the optimum of extraction time. Wood chips did not render a significantly higher enzyme activity by increasing the extraction time from 20 min to 4 h (data not shown). Extraction time used onwards (20 min) was similar to others reported previously [28,29]. Nevertheless, the extraction procedure may have not been appropriate to guarantee the total recovery of the enzymes adsorbed on the wood substrate. Thus, data reported are not comparable to other works where enzyme secreted in solid-state fermentation were measured.

Hydrolytic (endoglucanase and endoxylanase) and oxidative (MnP and laccase) extracellular enzymes were measured after 14 days (Table 2). Regarding ligninases, the highest laccase activity (5560 mU g^{-1}) was produced by *Trametes villosa* in M supplemented cultures. This fungus produced also a high MnP value (217 mU g^{-1}) similar to that obtained in *P. sanguineus* (332 mU g^{-1}). A negligible laccase activity and no MnP activity were detected in *Trametes elegans* as well as in *Steccherinum* sp. which produce very low titres of MnP and no laccase activity. Taking into account that in MnP determination lactate or other Mn-chelating agents were not included, MnP levels may be underestimated [30]. Maximal hydrolytic enzyme secretion was observed in *Peniophora* sp. and *P. sanguineus*, highest endoglucanase titre (332 mU g^{-1}) was produced by *P. sanguineus* on wood chips supplemented with C3, while *Peniophora* sp. produced the highest endoxylanase activity (660 mU g^{-1}) on MP.

All dry weight loss values ranged from 2 to 4.5%, after 2 weeks of treatment. The biopulping effect can be observed at very low weight losses (<5%) for sulfite pulping process. Biopulping may not require degradation of nonphenolic structures; modification and weakening of lignin rather than bulk removal of lignin appears to be sufficient to give the biopulping effect. Then, good biopulping is observed when the

Table 1
Growth and solid-plate dye decolorization on media containing glucose (10 g dm^{-3}), malt extract (12.7 g dm^{-3}), agar (20 g dm^{-3}), supplemented with either Poly R-478 (0.02%) or Azure B (50 μM)

Strain	Order	Family	Growth in Poly R (cm day^{-1})	Decol. of Poly R[a] (cm day^{-1})	Growth in Azure B (cm day^{-1})	Decolorization of Azure B[b] (cm day^{-1})
Skeletocutis nivea var. *diluta* (BAFC 2347)	Stereales	Grammotheleaceae	0.114	0		
Peniophora sp. (BAFC 633)	Stereales	Peniophoraceae	1.286	0.957	1.286	1.086
Steccherinum sp. (BAFC 1171)	Hericiales	Stecherinaceae	1.286	0.814	1.286	0.614
Phanerochaete sordida (BAFC 2122)	Telephorales	Meruliaceae	1.286	0.571	1.142	Diffuse
Ganoderma applanatum (BAFC 1168)	Ganodermatales	Ganodermataceae	0.450	0.111		
G. applanatum (BAFC 1172)	Ganodermatales	Ganodermataceae	0.450	0.356		
Coriolus pinsitus (BAFC 663)	Poriales	Coriolaceae	0.714	0.271		
C. pinsitus (BAFC 667)	Poriales	Coriolaceae	1.186	0		
Coriolus pavonius (BAFC 752)	Poriales	Coriolaceae	0.643	0.322		
C. pavonius (BAFC 753)	Poriales	Coriolaceae	0.500	0.414		
C. pavonius (BAFC 760)	Poriales	Coriolaceae	0.643	0.536		
Coriolopsis rigida (BAFC 2101)	Poriales	Coriolaceae	1.014	0.686	0.95	0.772
Fomitopsis sp. (BAFC 746)	Poriales	Coriolaceae	0.536	0.273		
Hexagona papyracea (BAFC 1173)	Poriales	Coriolaceae	0.636	0		
Lenzites sp. (BAFC 669)	Poriales	Coriolaceae	0.554	0.143		
Pycnoporus sanguineus (BAFC 2126)	Poriales	Coriolaceae	1.286	0.943	1.143	0.643
Rigidoporus ulmarius (BAFC 1160)	Poriales	Coriolaceae	0.471	0		
Tyromyces caesius (BAFC 2283)	Poriales	Coriolaceae	1.286	0.570		
Trametes elegans (BAFC 2127)	Poriales	Coriolaceae	1.286	0.943	1.286	0.757
Trametes villosa (BAFC 2755)	Poriales	Coriolaceae	1.286	1.000	1.286	0.444
Lentinus afín *villosus* (BAFC 1743)	Polyporales	Lentinaceae	1.171	0		
Schizophyllum commune (BAFC 583)	Polyporales	Schizophyllaceae	1.214	0		
Pleurotus ostreatus (BAFC 215)	Polyporales	Lentinaceae	0.714	0.553		
Pleurotus pulmonarius (BAFC 0076/2002)	Polyporales	Lentinaceae	0.818	0.464		
Polyporus tenuiculus (BAFC 1020)	Polyporales	Polyporaceae	0.364	0		
P. tenuiculus (BAFC 1154)	Polyporales	Polyporaceae	0.657	0		
Polyporus sp. (BAFC 665)	Polyporales	Polyporaceae	1.286	0.182		
Polyporus sp. (BAFC 739)	Polyporales	Polyporaceae	1.071	0		
Polyporus sp. (BAFC 2452)	Polyporales	Polyporaceae	0.686	0		
Phellinus calcitratus (BAFC 2152)	Hymenochaetales	Hymenochaetaceae	0.364	0		
Scytinostroma sp. (BAFC 2740)	Lachnocladiales	Lachnocladiaceae	1.286	0.167		
Eichleriella leveilliana (BAFC 670)	Tremellales	Exidiaceae	0.179	0		
Auricularia polytricha (BAFC 2095)	Tremellales	Exidiaceae	0.545	0		
Coriolus versicolor var. *antarcticus* (BAFC 266)	Poriales	Coriolaceae	1.286	1.286	1.286	0.857

The plates were incubated for 3 weeks at 28 °C.
[a] Ligninolytic activity (Poly R-478 solid-plate decoloration rate (cm day^{-1})). The values are the mean of three replications, S.D. < 5%.
[b] LiP activity (Azure B solid-plate decoloration rate (cm day^{-1})). The values are the mean of three replications, S.D. < 5%.

major cell wall components: lignin, cellulose and hemicellulose are largely intact [31].

Biotreatment of *P. taeda* wood chips with four of the selected fungi (*Steccherinum* sp., *C. versicolor* var. *antarcticus*, *P. sanguineus* and *T. elegans*) resulted in a decrease of Klason lignin content. While incubation with *Steccherinum* reduced Klason lignin content/total wood weight components (%, w/w) from 29 (control) to 26.4 (2.6%), up to 3.1% decrease in lignin content was registered when using *P. sanguineus*, after 2 weeks (Table 3). These results compared favorably with previously findings by Guerra et al., that demonstrated a weight loss of only 3% after 30 days biotreatment of *P. taeda* wood chips with *C. subvermispora* [32] accompanied by a lignin content decrease from 28.2 to 26.1% [33]. *P. sanguineus* was selected for additional studies, taking into account that it caused the highest reduction in loblolly wood lignin content among the fungi evaluated. The extractive fraction, a group of non-structural components of wood that usually contains resin acids, fatty acids, terpenes and phenolic compounds, increased with fungal treatment. Previous studies attributed this raise to biodegradation products [34]. Cellulose content of treated wood with *C. versicolor*, *P. sanguineus* and *T. elegans* was higher than the control, showing thus selective delignification. Lignin removal was also accompanied by hemicellulose degradation in these fungi. In selective delignification hemicelluloses and lignin are preferentially attacked, especially in early stages [35].

Nevertheless, the high levels of hydrolytic and oxidative enzymatic activities detected after 14 days of decay (Table 2) did not correlate with the visualized wood colonization or with the extent of initial wood weight or component losses (i.e. *T. elegans* grew on wood even giving very low titers of oxidative enzymes, but diminished its lignin content in 3%). Similar results were obtained by Ferraz et al. [36], when analyzing wood biodegradation and enzyme production by *C. subvermispora* during solid-state fermentation of *Eucalyptus*

Table 2
Enzyme production (mU g^{-1}) on *P. taeda* wood chips, with different amendments, after 14 days.

Strain	Amendments	Laccase	MnP	*Endo*-glucanase	*Endo*-xylanase
C. versicolor var. *antarcticus* (BAFC 266)	C1	0	20	275	59
	C3	273	14	64	200
	M	30	6	58	160
	MP	65	94	126	230
Peniophora sp. (BAFC 633)	C1	614	46	322	339
	C3	26	0	371	420
	M	0	0	197	440
	MP	35	0	320	660
Steccherinum sp. (BAFC 1171)	C1	0	43	197	115
	C3	0	23	143	209
	M	0	0	110	144
	MP	0	40	90	208
P. sanguineus (BAFC 2126)	C1	72	101	305	162
	C3	0	133	466	283
	M	0	19	181	242
	MP	117	332	72	239
T. elegans (BAFC 2127)	C1	0	0	154	90
	C3	0	0	124	278
	M	19	0	48	193
	MP	0	0	84	253
C. rigida (BAFC 2101)	C1	684	35	79	50
	C3	1540	98	25	50
	M	0	28	39	54
	MP	2140	147	54	66
P. sordida (BAFC 2122)	C1	0	28	89	70
	C3	0	35	95	108
	M	0	98	12	41
	MP	85	112	72	63
T. villosa (BAFC 2755)	C1	513	35	108	63
	C3	1110	98	124	107
	M	5560	217	22	70
	MP	1356	189	159	114

Amendments: C1: corn steep liquor 1%; C3: corn steep liquor 3%; M: malt extract 3%; MP: malt extract 3% plus peptone 1.5%. Data are mean values with S.D. less than 10%.

grandis after 15 days of decay. On the other hand, the capacity of WRF for wood degradation seemed to be correlated with the levels of oxidative activities only after longer biodegradation periods [36]. Lignin was degraded when peroxidase (MnP and/or LiP) activity was detected in fungal cultures, but no correlation in an analytical sense between peroxidase activity and the extent of delignification on spruce wood was found after 2 weeks of treatment [37]. These findings support that also systems which are not related to extracellular activities of oxidative enzymes are important in this early stages of white rot degradation. A direct correlation between the levels of oxidative enzymes and the rate of lignin removal was not expected because the lignin removal expresses the final stage of lignin degradation (mineralization), which starts with the

Table 3
Chemical composition of control (non-inoculated) and 14-days bio-treated wood chips (%, w/w of extractives, Klason lignin, carbohydrates)

Strain	Solvent extractives	Water extractives	Total extractives	Klason lignin	Cellulose	Hemi-cellulose
Steccherinum sp.	2.6	2.1	4.7	26.4	38.1	30.8
C. versicolor var. *antarcticus*	2.2	5.7	7.9	26.1	50.4	15.6
P. sanguineus	2.2	3.3	5.5	25.9	50.9	17.7
Peniophora sp.	1.5	2.9	4.4	28.9	45.9	20.8
T. elegans	2.1	2.5	4.6	26.0	51.0	18.4
C. rigida	1.0	3.0	4.0	28.8	45.0	22.2
P. sordida	1.3	3.9	5.2	28.0	44.5	22.3
T. villosa	1.6	1.7	3.3	28.9	43.9	23.8
Control	1.7	1.1	2.8	29.0	44.4	23.8

Lignin, cellulose and hemicellulose were determined on extractive free wood chips. Fungi were cultured on *P. taeda* wood chips supplemented with CSL 1%. Data are mean values with S.D. less than 5%.

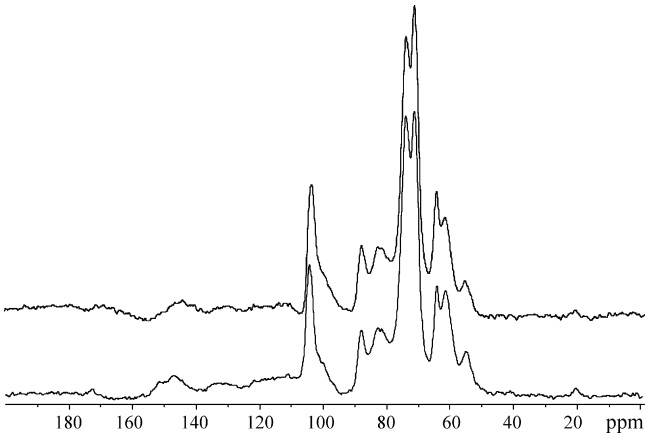

Fig. 1. ^{13}C CP-MAS NMR spectra of sound *Pinus taeda* wood (bottom spectrum) and wood bio-treated with *Pycnoporus sanguineus* (top spectrum). 151–152 ppm: C-3/C-5 in syringyl (S) units, C-3 in 5-5′ and 4-O-5′ units, C-4 in structures with Cα=O and vinyl moieties; 144 ppm: C-4 in phenolic structures; 60 ppm: β-*O*-4 structures (Cγ); 55.6 ppm: carbon resonance in OCH_3.

depolymerization steps. However it has previously been shown that the amount of aryl-ether linkages of lignin decreases and lignin is extensively fragmented at the early stages of cultivation before being mineralized by *C. subvermispora* [38]. These modifications of the lignin structure by the fungus may be beneficial for the downstream pulping processes. In addition, a selective removal of lignin apparently correlates with beneficial biomechanical pulping performance [39]. In our work, *P. taeda* wood suffered notable structural changes of lignin and hemicelluloses during treatment with *P. sanguineus* as revealed from ^{13}C CP-MAS NMR spectra (Fig. 1). Thus, a group of signals at 151–152 ppm, assigned to C-3/C-5 in syringyl (S) units, C-3 in 5-5′ and 4-O-5′ units and C-4 in structures with Cα=O and vinyl moieties [1], decreased remarkably in the spectrum of biotreated wood. Simultaneously, the resonance at 144 ppm (C-4 in phenolic structures) [1] increased remarkably showing clearly the cleavage of lignin interunit linkages. The depolymerization of lignin may include also the cleavage of β-*O*-4 structures since a shoulder at around 60 ppm, assigned to Cγ in those structures, decreased notably after wood biotreatment. Previously, degradation of β-*O*-4 structures in lignin of *P. taeda* wood during biotreatment with white rot fungus *C. subvermispora* was suggested based on wet chemistry and NMR analyses [40]. Additionally, the decrease of resonance at 55.6 ppm (carbon resonance in OCH_3) in the spectrum of biotreated wood, when compared to starting woody material, indicated the lignin demethoxylation. The biotreatment of *P. taeda* wood with *P. sanguineus* affected also the hemicelluloses, which was evidenced from the significant decrease of resonance at around 21 ppm assigned to methyl carbon in acetyl groups, belonging mostly to galactoglucomannan [41]. The degradation of the last one was also confirmed by the decrease of resonance at 61.8 ppm (C-6 in hexoses of galactoglucomannan) in the biotreated wood.

Fig. 2 shows pore size distribution after the incubation during 14 days with *P. sanguineus*. Pore size increased in treated wood chips in the range 10 and 100 μm compared to non-treated wood chips. The more remarkable increase was observed around pore size 100 μm, which reveals a high amount of such pore size, resulting from fungal degradation. Average pore size values of treated and non-treated wood chips were 0.3025 and 0.2377 μm, respectively; on the other hand, the porosity was 83% for treated wood chips while in the control was 68%. The resulting effect is a more open structure which enhances chemical impregnation. A previous report showed an increase in the porosity of the secondary wall during incipient stages of decay [42]. Pore size in *Pinus resinosa* wood fibers was also enlarged after treatment with *Phlebiopsis gigantea* [43].

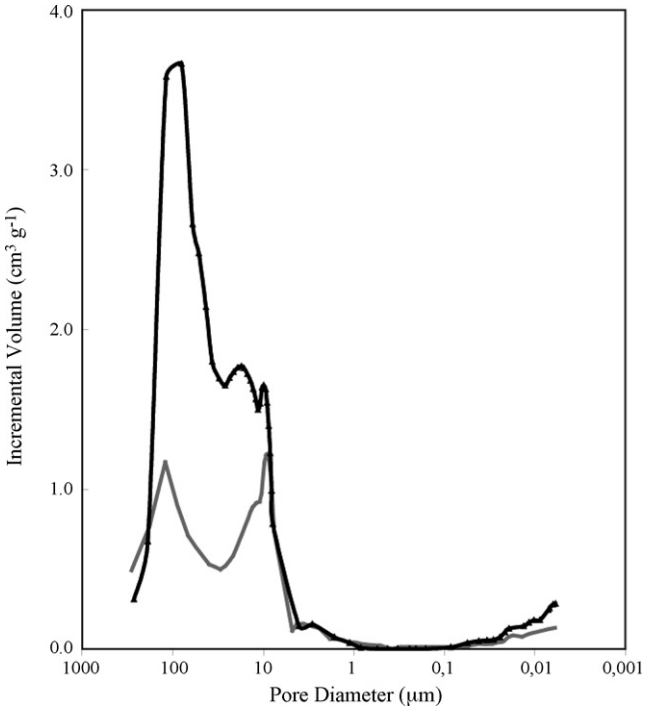

Fig. 2. Pore size distribution of *P. taeda* wood after 2-week treatment with *P. sanguineus*. Control, lower curve; bio-treated wood, upper curve.

4. Conclusions

Understanding the mechanism of wood decay under biopulping simulated conditions could be useful for improving this technological procedure. Results from this work allowed the identification of a new strain with potential for loblolly wood biopulping. *P. sanguineus* was able to reduce total Klason lignin content in 11% in 14 days of treatment, but also *P. taeda* wood suffered notable structural changes of lignin and hemicelluloses during the treatment, as revealed from ^{13}C CP-MAS NMR spectra. Similarly, porosity of treated wood increased 15%. In addition to the chemical modification caused by selective delignification, physical changes, as the increase in pore size, affects directly the penetration of cooking chemicals. Both combined effects increase reaction's rate, leading to shorter cooking times or the possibility of decreasing chemical charge reducing in consequence the environmental impact of the process [31].

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