

Fragmentation of Lignin from Organosolv Black Liquor by White Rot Fungi

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The mycelial growth ability of 13 white rot fungi were separately evaluated in kraft, organosolv, and soda black liquor agar-plates. The fungus able to best grow and decolorize black liquor agar-plates was grown in organosolv black liquor to investigate whether it reduced organosolv lignin molar mass. The fungus *Bjerkandera adusta* showed fair mycelial growth and decolorization ability in 10% black liquor-agar plates. To obtain low-molecular weight (MW) lignin, *B. adusta* was cultivated in 150-mL Erlenmeyer flasks containing 10% black liquor and maintained in a shaking culture for 15 days. Lignin was recovered from each Erlenmeyer flask by acid precipitation and was analysed by size exclusion chromatography (SEC) and Fourier transform infrared (FTIR) spectroscopy. The lowest MW of lignin from black liquor was observed on the 11th and 12th days, at 1461 and 1790 kDa, respectively, with the polydispersity close to 1.0, indicating that the molecules were similar in size. Fourier transform infrared spectra bands showed modification of the lignin structure during 9 days, with new bands appearing after five days of lignin biodegradation.

Keywords: Lignin; White-rot fungi; Black liquor; Decolorization; Molecular weight

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INTRODUCTION

Worldwide, forestry industries have significant holdings in the economy of many countries. In 2010, the United States, Canada, and Brazil led the ranking of countries producing pulp from wood, while China, the United States, and Japan were the largest paper producers (FAO 2012). However, in the pulp and paper industry, waste generation grows concomitantly with the increase in industrial production because most of the industries do not have adequate technology that allows for the reuse of industrial waste (James *et al.* 2012).

Since the 1950s the kraft pulping process has been dominant in the world because this process produces pulp with the best strength properties. Many years later, in 1993, a new process called organosolv pulping has started to be used. Nowadays the organosolv process is still in its developing stage, and much time should pass until it replaces kraft pulping (Muurinen 2000). For both processes, black liquor is the primary waste generated after the production of pulp, and its composition varies with the type, age, and section of

the wood used, as well as the technology employed in the production of the pulp. The bulk of the organic solids content in black liquor consists of lignin, while the remainder is composed of phenolic resins, fatty acids, color, tannins, terpenes, alcohols, phenols, and chloroform along with lignin-derived chromophores and chlorolignin (Chandra and Abhishek 2001; Chandra *et al.* 2011; ; Pokhrel and Viraraghavan 2004).

The dark colour of black liquor together with its recalcitrance to biodegradation makes it a waste product of environmental importance (Pokhrel and Viraraghavan 2004; Da-Re and Papinutti 2011). Often the industry burns the black liquor as a low-value energy source (Gouveia *et al.* 2012) and the vast majority of pulp and paper industries in the world do not have a suitable alternative for the black liquor (Sena-Martins *et al.* 2008). The use of black liquor to obtain energy can be further justified based on avoidance of environmental contamination. For example, the use of black liquor in the United States has become eligible in politics, technology research, and a wide range of tax issues, including tax planning and accounting for income taxes. Thus, the paper industry has the opportunity to use the black liquor fuel certified as eligible for the credit AFM (Alternative Fuels Mixture, enacted by the Tax Technical Corrections Act of 2007) (Kern 2012). Another alternative of black liquor is its gasification, which can provide electricity and steam used by the pulping plant. In the case of a biorefinery, this by-product can be tailored to produce high-value liquid fuels or chemicals (NETL 2014). Although black liquor has economic importance to industries, aromatic compounds as phenols derived from lignin fragmentation can be used as chemical products with high added value (Peng *et al.* 2014).

Around 90% of the lignin content in black liquor can be recovered through chemical processes (Pokhrel and Viraraghavan 2004; Da-Re and Papinutti 2011), but only a small fraction between 1 to 2% of isolated lignin is used to create a wide range of specialty products (Lora and Glasser 2002). Commercial lignins are available as by-products of the pulping processes, with kraft lignin and lignosulfonates being the major derivatives (Raquezza *et al.* 2010). The characterisation of lignin is a particularly difficult task due to its macromolecular structure, whose monomeric units do not repeat on a regular basis, unlike cellulose and other natural polymers. The heterogeneity of lignin is caused by variations in polymer composition, size, and types of crosslinking functional groups (Toledano *et al.* 2010). Lignin has highly reactive sites in its structure that can be modified through a selection of physical and/or enzymatic reactions. For this purpose, the enzymatic modification of lignin has been studied as a promising way to obtain new products (Sanchez 2009).

In nature, the only organisms able to mineralise lignin are the white rot fungi (Blanchette 2000), which produce non-specific oxidative enzymes that reduce dissolved oxygen to peroxide (Chandra and Abhishek 2001; Raj *et al.* 2007; Ahmad *et al.* 2010). The key enzymes involved in this process are laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), and peroxidases with a wide range of specificity (Lewis and Yamamoto 1990; Rabinovich *et al.* 2007). Several studies have recently been conducted in searching of low molecular weight compounds (LMWC), like phenols, generated by lignin bioconversion from black liquor (Lara *et al.* 2003; Xiong *et al.* 2007; Sanchez 2009; Arun and Eyini 2011).

The most frequently discussed monomeric degradation products from lignin are vanillin and gallic acid (Ribbons 1987). Fractions composed of a mixture by monomers, dimers, and trimers can be obtained through depolymerization of lignin (Beauchet *et al.* 2012).

Monomers such as catechols have a wide array of application in pesticide and pharmaceutical synthesis (Lavoie *et al.* 2011) or with antioxidants properties (Ergües *et al.* 2014). Products derived from lignin conversion as catechols and vanillin can be potentially commercialized at market prices such as \$1.5/lb and \$5.9/lb, respectively (Bozell *et al.* 2007).

Nowadays, petroleum-based phenol is widely used to produce phenolic resins and adhesives. However, lignin is also a great source of bio-phenols, making it very attractive to many industries. They can be produced through the cleavage of ether and C-C linkages during pyrolysis of lignin, *i.e.* by a thermal decomposition process in absence of oxygen (Bu *et al.* 2014). Antioxidants from organosolv lignin have been demonstrated antiradical activity (Garcia *et al.* 2010)

The aim of this study was to obtain low molecular weight lignin through the cultivation of *Bjerkandera adusta* in organosolv black liquor. The experiment was carried out in order to develop an alternative form of lignin reuse by changing or shrinking its molecular structure.

EXPERIMENTAL

Background

Because of the large numbers of wood-decay fungi able to degrade lignin, 13 basidiomycetes species were initially cultivated *via* three methods, generating black liquor agar plates in two concentrations.

Parameters evaluated by these assays included the type of black liquor that provided the best fungal growth and the fungus that grew the fastest in a high concentration of black liquor. After these tests, one fungus was used for lignin biodegradation by cultivation it in 10% black liquor.

Materials

Black liquor samples

Soda black liquor was generated from wheat straw pulping. Kraft and organosolv black liquors were generated by hardwood pulping and donated by AV Nackawic Company, Inc. (Nackawic, New Brunswick, Canada).

Fungi

Ten isolates of fungi were purchased from the University of Alberta Microfungus Collection and Herbarium, University of Alberta, Alberta, Canada (UAMH); the names and respective codes of these fungi were as follows: *Pleurotus ostreatus*, three isolates (UAMH 7963, UAMH 7964, and UAMH 7969); *Bjerkandera adusta* (UAMH 8258); *Ganoderma applanatum* (UAMH 8168); *Ganoderma lucidum* (UAMH 8026); *Phanerochaete chrysosporium* (UAMH 3642); *Trametes versicolor* (UAMH 8272); *Coriolopsis gallica* (UAMH 8260); and *Sporotrichum pruinosum* (UAMH 4521). In addition, three isolates were obtained from the Fungal Collection, Faculty of Forestry, University of Toronto, ON, Canada: *Piptoporus betulinus* (UTPH 0308); *Postia placenta* (R698); and *Trametes versicolor* (SR013). The fungal growing cultures were maintained on a commercial potato dextrose agar (PDA) (BD Biosciences, Canada) at 26 °C.

Methods

Lignin recovery

Preliminary analysis of lignin from soda, kraft, and organosolv black liquor was performed by acid precipitation with 3 M sulfuric acid, according to the procedures of Abacherli and Doppenberg (2001). After acid precipitation, the lignins were placed in Petri dishes and oven dried at 60 °C for 24 h.

Non-modified lignin

For fungi cultivation, it was necessary to sterilise the black liquor by autoclaving. However, after the heating process, the final pH of black liquor changed. Table 1 shows the pH value of kraft and organosolv black liquor after autoclaving at 120 °C. An increase in pH was observed in both black liquors for all tested pH. It is well known that most fungi can grow in substrates with pH values between 5 and 6. For this reason, the culture medium was pre-set at a pH of 5.0 for carrying out the biodegradation study of black liquor.

Table 1. pH Changes in Organosolv and Kraft Black Liquor Before and After Autoclaving at 120 °C

Kraft		Organosolv	
Before	After	Before	After
3.6	3.9	3.2	3.5
5.0	5.4	5.0	5.6
6.0	7.8	6.0	7.8
6.0*	8.7*	6.0*	8.7*

*Samples with 10 g.L⁻¹ of glucose

The solids contents and quantity of lignin recovered from black liquor after acid precipitation varied depending on the type of black liquor (Table 2). Kraft and organosolv black liquor provided a high quantity of lignin compared to the soda black liquor.

Table 2. Solids Contents and Quantity of Lignin Recovered from Soda, Kraft, and Organosolv Black Liquor

Black Liquor	Solids Contents ^a (g. L ⁻¹)	Lignin ^b (g.L ⁻¹)	pH
Soda	123.22	5.16	5.0
Kraft	170.84	26.2	5.0
Organosolv	150.01	21.2	5.7

^asolids contents obtained by drying black liquor at room temperature
^bquantity of lignin obtained after acid precipitation with sulphuric acid solution

Lignin analyses

The non-modified lignin from soda, kraft, and organosolv black liquor was analysed by nuclear magnetic resonance (¹H-NMR) to evaluate their molecular structures. Fungus-modified lignin from organosolv black liquor was analysed by size exclusion chromatography (SEC) and Fourier transform infrared spectroscopy (FTIR). The SEC was performed to understand the variations in the biodegraded lignin's

molecular weight throughout the 15-day period of fungus growth in organosolv black liquor. The FTIR analysis was performed to obtain information about changes in the biodegraded lignin's molecular structure throughout nine days of biodegradation.

Nuclear magnetic resonance was carried out using a Varian Unity Plus 500 MHz spectrometer (USA) housed in the Department of Chemistry, University of Toronto. The instrument was operated in the quadrature mode, with typical $^1\text{H-NMR}$ data points recorded with an acquisition time of 4.0 s, number of scans of 126, and relaxation time of 1.0 s at room temperature. During the measurements, the lignin was dissolved in dimethylsulfoxide-*d*₆ (DMSO-*d*₆) as the standard solvent.

The FTIR analyses of biodegraded lignin (from the 2nd to the 9th days of fungal growth) were carried out using a Bruker spectrophotometer, Model Vertex 70 (Germany). The samples were pulverised in a quartz mortar and collected in the range of 4000 to 500 cm^{-1} . The data were collected in ATR mode using the spectrometer with a resolution of 4 cm^{-1} .

The SEC analysis of the fragmented lignin was followed according to the standard ASTM 5296-11 (2011) and Manesh (2012). Lignin samples were analysed by a Dionex DX-600 HPSEC system equipped with an UV detector (Dionex Corp.; USA) and PSS MCX column (1000 Å, 300 × 8 mm). The eluting solvent was 100 mM NaOH, used in the mobile phase at a rate of 1 mL/min. The amount of sample placed in each tube was 2.5 mL. The molecular weight obtained by the retention time was calibrated with polystyrene standards 1K, 2K, 4K, and 6 K. The reproducibility of analyses was checked in duplicate.

Fungi growth assays

(a) Black liquor agar-plates

Three types of black liquor, *i.e.*, soda, kraft, and organosolv black liquors, were used for preparing agar plates to assay mycelial growth. Each agar media type was prepared with 5% and 10% black liquor diluted in distilled water. After dilution in water, the pH was adjusted to 5.0 and 12 g/L of agar (Difco Laboratories, USA) was added to each black liquor.

Nutrients were added only to the organosolv black liquor agar plates according to Barratt *et al.* (1965). The following chemicals (Sigma-Aldrich, USA) were used to prepare 1 L of agar: sodium nitrate (6 g), potassium chloride (0.52 g), magnesium sulphate (0.52 g), potassium dihydrogen phosphate (1.52 g), iron sulphate (0.01 g), zinc chloride (0.01 g), and glucose (10 g). For this media, control treatments were considered to be those without added nutrients. After diluting the chemicals in 5% and 10% black liquor, the pH was adjusted to 5.0 with 3 M sulfuric acid solution. The sterilisation was performed by autoclaving at 121 °C for 15 min.

The factorial experimental design for black liquor agar media was the 13 fungal species × the 2 concentrations of black liquor × 3 the media types, with four replications. All fungi inoculated in the three types of media were incubated at 26 °C. After 8 days of grow, the rate of the mycelial growth was measured.

(b) Sawdust

In this assay, fungi were cultivated in *Fagus* sp. sawdust in order to know the capacity of each fungus to colonize wood. The major limitation to microbial growth is imposed by the presence of lignin (Dix and Webster 1995). So, it was considered that wood degradation capacity of fungi is related mainly to the production of lignolytic

enzymes. If any fungus can grow on wood with black liquor it is a major producer of lignolytic enzymes. This type of assay was chosen because it is a quick and simple way to observe this ability between various species, which can be potentially used to degrade a rich-lignin substrate.

The sawdust was prepared from branches of *Fagus* sp., collected on campus near the Faculty of Forestry, University of the Toronto. The branches were first cut into discs 5 cm in diameter with a Delta X5 bandsaw (USA). After that, the discs were cut into smaller pieces with the bandsaw, dried in an oven for 72 h at 60 °C, and finally transformed in sawdust in a Thomas Model 4 Wiley® Mill (Thomas Scientific; USA).

To determine the capacity of fungal growth in wood containing organosolv black liquor, an assay was performed using the isolates (UAMH) 7963, 7964, 7969, 8258, 8026, 8168, 8272, 4521, 8260, 3642, and SR013, and UTPH 0308 with the following treatments: (a) *Beech* sp. sawdust and water (control treatment); (b) *Fagus* sp. sawdust, 20% black liquor, and 10 g/L glucose; and (c) *Fagus* sp. sawdust and 20% black liquor. Each 150 mL Erlenmeyer flask received 3 g of *Fagus* sp. sawdust and 3 mL of distilled water (final pH 7.0) or 3 mL of black liquor (final pH 5.0) in order to obtain 60% humidity in the flask.

Five PDA disks (5 mm) containing mycelia were deposited on the sawdust surface, followed by incubation in the dark at 26 °C for 14 days. The experimental design was 12 fungal species x 3 treatments x 5 disks, in duplicate. Biomass generation or mass losses were evaluated by drying the sawdust in an oven at 60 °C until a constant weight was reached.

Biodegradation of black liquor and lignin analyses

The purpose of this assay was to cultivate *B. adusta* in 10% organosolv black liquor with nutrients (Barrat *et al.* 1953) in order to obtain low molecular weight lignin. After preliminary tests, organosolv black liquor was chosen, as it provided fair mycelial growth and allowed for easy recovery of lignin. The white rot fungus *B. adusta* (UAMH 8258) was chosen due to its great ability to grow and decolorize black liquor agar plates.

A solution containing 10% organosolv black liquor with nutrients at pH 5.0 was used to cultivate *B. adusta* in a shaking culture for 15 days at 26 °C. Each 150-mL Erlenmeyer flask received 100 mL of 10% organosolv black liquor that was sterilised by autoclaving at 120 °C for 15 min.

Liquid substrate is a non-common environment to grow wood rot fungi. For this assay, sawdust was used to reinforce fungal growth and to support its development in a strange substrate. The fungus inoculum was prepared by mixing 15 g *Beech* sp. sawdust, 3 g wheat germ, 7.5 mL organosolv black liquor, and 17.5 mL of distilled water (to allow for 60% humidity). One gram of this mix was placed in a 15 mL glass flask, autoclaved (120 °C, 15 min), inoculated with a colonised PDA disc by *B. adusta*, and maintained at 26 °C during the 15 days. After that, the contents from each 15 mL flask were transferred to the Erlenmeyer flask containing 10% organosolv black liquor and kept under a shaking culture (150 rpm) at 26 °C and a photoperiod of 12 h during the 15 days of treatment. Sampling was obtained from the 2nd day until the 15th day, in duplicate.

On each evaluation day, two flasks containing the black liquor were filtered with n° 1 Whatman paper and placed in a 50 mL centrifuge tube. The pH was set to 2.5 and was maintained in a resting state overnight at 25 °C. The tubes were then centrifuged at 5000 rpm for 15 min at 15 °C. The supernatant was discarded, the solid lignin was dried in an oven at 60 °C for 24 h, and then milled in a quartz mortar and pestle.

For SEC analysis, 0.005 g/L of lignin was solubilised in 3 M NaOH solution (pH 11). The molecular weight of the lignin was determined according to the method described previously in the lignin analysis section.

RESULTS AND DISCUSSION

Mycelial Growth in Sawdust

Although all fungal species grew on *Fagus* sp. sawdust, mass loss was not observed in all treatments (Fig. 1). Besides *C. gallica* (UAMH 8260; Fig. 1l), all fungi caused mass loss in *Fagus* sp. sawdust and water. The greatest wood consumers were *T. versicolor* (SR013; Fig. 1f) and *P. ostreatus* (UAMH 7969, Fig. 1e), causing mass losses of around 44 and 29%, respectively. Increases were observed in some of the treatments with 20% black liquor in the range of 2.19% (UAMH 8168, Fig. 1k) to 8.31% (UAMH 0308, Fig. 1j). The range of weight increase in the treatment with black liquor and glucose varied between 1.03% (UAMH 8272; Fig. 1a) and 9.81% (UAMH 0308; Fig. 1j). Glucose is a nutrient easily metabolised by fungi and serves as a primary source of carbon, although the rate of carbohydrate polymer hydrolysis can limit fungi growth and affect growth indices such as production of protein (Boyle 1998).

Only the basidiomycetes *G. lucidum* (UAMH 8026; Fig.1b), *P. ostreatus* (UAMH 7963; Fig. 1c), and *T. versicolor* (UAMH 8272; Fig. 1a) caused mass losses in the sawdust in the treatment with 20% organosolv black liquor. The range of mass losses varied from 1.23% to 5.03% after 15 days of wood biodegradation. In this case, the presence of lignocellulose-containing substrates led to the production of ligninolytic enzymes, indicating that some compounds, such as extra lignin, low molecular weight aromatic compounds, and dissolved carbohydrates, were present in the black liquor and induced secretion of the lignocellulolytic enzymes, consequently causing mass losses (Kapich *et al.* 2004).

The presence of glucose repressed wood biodegradation for all isolates during the 15 days of mycelial growth. Wu *et al.* (2005) observed that lignin degradation by *P. ostreatus* was faster with 1 g/L than 10 g/L glucose concentration. According to these authors, mass loss of *Fagus* wood can occur only after glucose consumption reached a minimum level that should be after 15 days of fungus growth. In this condition, laccase can start to be secreted (Galhaup *et al.* 2002). Oxidation of glucose allows for the production of hydrogen peroxide, which is necessary for peroxidase activity, another important class of enzymes responsible for the breakdown of biomass (Elisashvili and Kachlishvili 2009). All fungi need metals during their metabolism, reproduction, and differentiation. Essential and non-essential metals can interact with fungal cells and be accumulated by physico-chemical mechanisms. High concentration of inorganic components can negatively influence the mycelial development of some fungi species, which will vary depending on the organism (Gadd 1992). Further studies with concentration of metals in black liquor under mycelial growth should be carried out. In the case of *P. chrysosporium*, growth is possible under a high concentration of Pb, but cellulolytic enzymes can be inhibited (Huang *et al.* 2010). On other side, fungal organisms are able to keep metals outside the cell by the extracellularly active melanin (Fogarty and Tobin 1996).

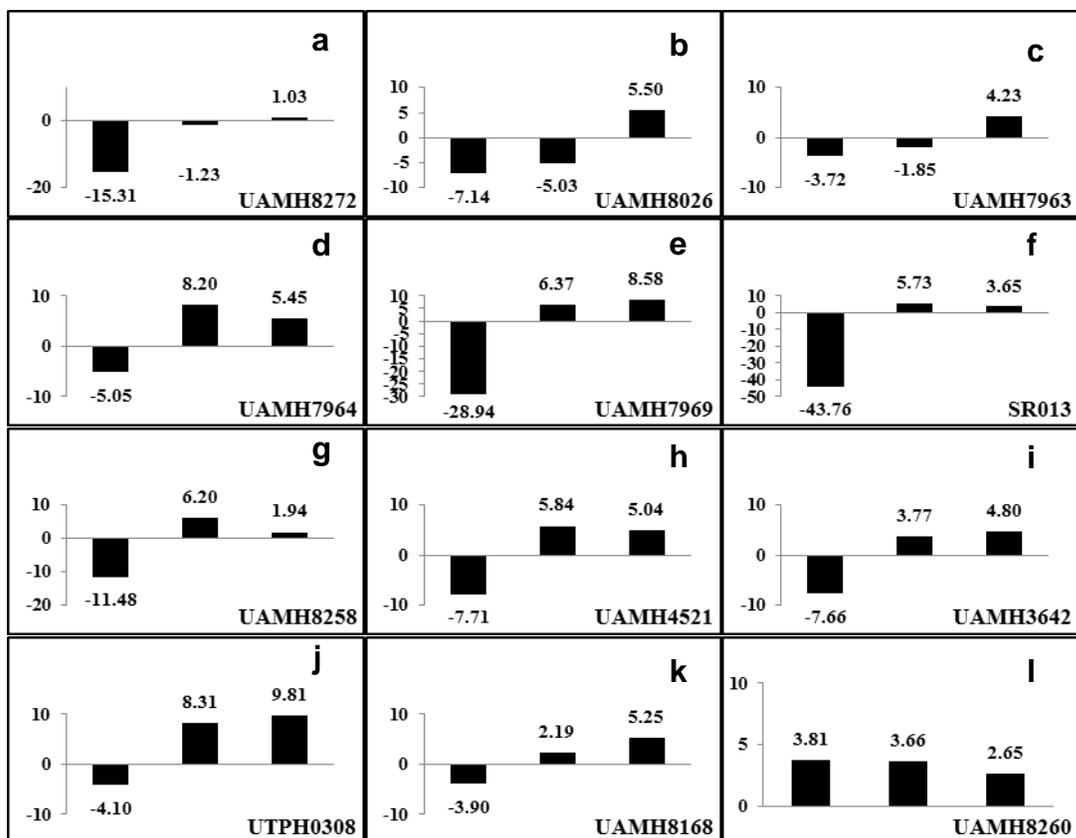


Fig. 1. Mass losses and generation of biomass of 12 fungi isolates cultivated in *Fagus* sp. sawdust with water or organosolv black liquor added: The first column indicates the treatment with sawdust and water; the second column indicates sawdust and 20% organosolv black liquor; the third column indicates sawdust, 20% organosolv black liquor, and 10 g/L glucose. Values are respective percentage of mass loss (negative) or increase of the mass (positive).

Mycelial Growth on Black Liquor Agar Plates

Figure 2 shows the mycelial growth of selected white rot fungi on three different black liquor agar plates. The rate of growth varied with the type and concentration of black liquor in the agar plates. Not all fungi tested could grow in the black liquor agar plates at a 10% concentration. Some isolates produced a halo around the mycelial growing zone. Removal of the colour indicates the degradation of chromophores and lignin residing in the black liquor.

Organosolv black liquor agar plates (OAM) were found to be the best substrate for fungi growth (Fig. 2a). From all eight fungi isolates tested, only the *T. versicolor* (SR013) did not grow on OAM without nutrients. On the contrary, *P. ostreatus* (UAMH 7963) grew better on control treatment, that is 10% OAM with no nutrients added. All fungi produced a halo in the 5% OAM. Halo-producing fungi at 10% OAM with nutrients were *G. lucidum* (UAMH 8260), *P. chrysosporium* (UAMH 3642), *B. adusta* (UAMH 8258), and *P. ostreatus* (UAMH 7963). Only *G. lucidum* (UAMH 8026) produced halos at 5 and 10% in control treatments (without nutrients) *B. adusta* showed the greatest ability to grow and decolorize in 5 and 10% organosolv black liquor agar media. Therefore, this fungus was chosen for further study of the biodegradation of black liquor aiming to obtain enzyme-fragmented lignin.

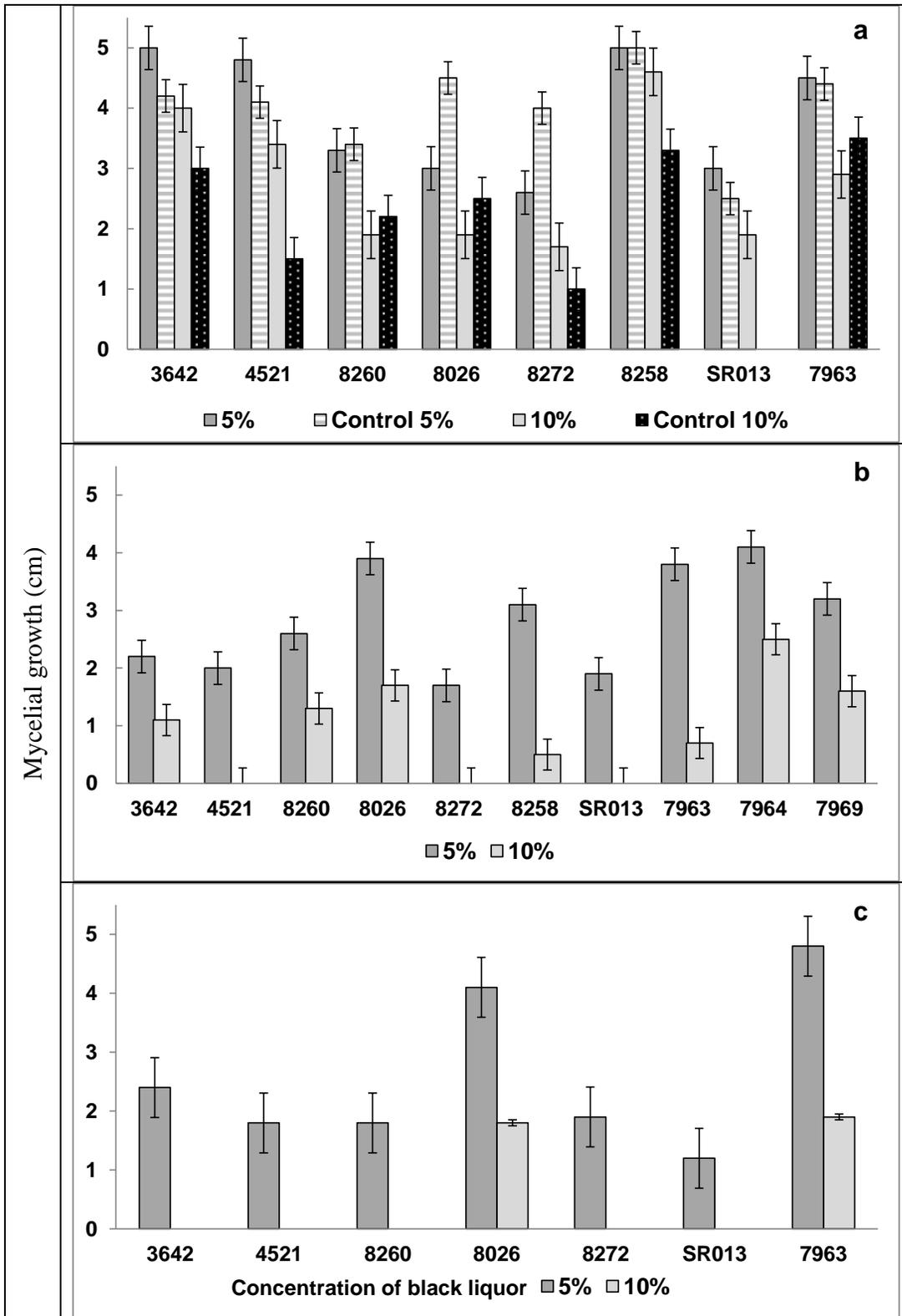


Fig. 2. Mycelial growth (cm in diameter) of different fungal isolates on (a) organosolv, (b) kraft, and (c) soda black liquor agar after eight days of incubation at 26 °C. Data presented as the mean ± standard deviation (SD)

All tested fungi grew in kraft black liquor agar plates (KAM) with *P. ostreatus* (UAMH 7963 and UAMH 7964) and *G. lucidum* (UAMH 8026), growing the fastest on the 5% black liquor agar plates (Fig. 2b). The fungi *S. pruinosum* (UAMH 4521) and *T. versicolor* (UAMH 8272 and SR013) did not grow on the 10% kraft black liquor agar plates (Fig. 2b). Halo-producing fungi in 5% KAM were (UAMH) 3642, 8260, 4521, 7963, 7996, 8272, and 8026, and the last isolate produced slight mycelium. The isolate UAMH 8258 was the only that produced halo at 5 and 10%.

Soda black liquor agar plates (SAM) provided mycelial growth for seven isolates out of the 10 species used in this assay (Fig. 2c). The fungi *G. lucidum* (UAMH 8026) and *P. ostreatus* (UAMH 7963) were the only fungi that grew in 10% black liquor without nutrients, that also it caused a lighter halo around the colonies. The halo-producing fungus at 5% SAM was *G. lucidum* (UAMH 8026).

G. lucidum (UAMH 8026) and *T. versicolor* (UAMH 8272) showed little mycelial growth when cultivated on OAM without nutrients (NaNO_3 , KH_2PO_4 , MgSO_4 , KCl , FeSO_4 , ZnCl_4 , and glucose). For *B. adusta* (UAMH 8258) and *P. ostreatus* (UAMH 7963), addition of nutrients did not affect the growth rate (Fig. 2a). In these assays, the mycelial growth of some fungi can be disturbed by increasing the black liquor concentration, as it is a very heterogeneous waste composed of low concentrations of nutrients such as nitrogen, and by high concentrations of chlorine, potassium, sulfur, sodium, and carbon (Cardoso *et al.* 2009).

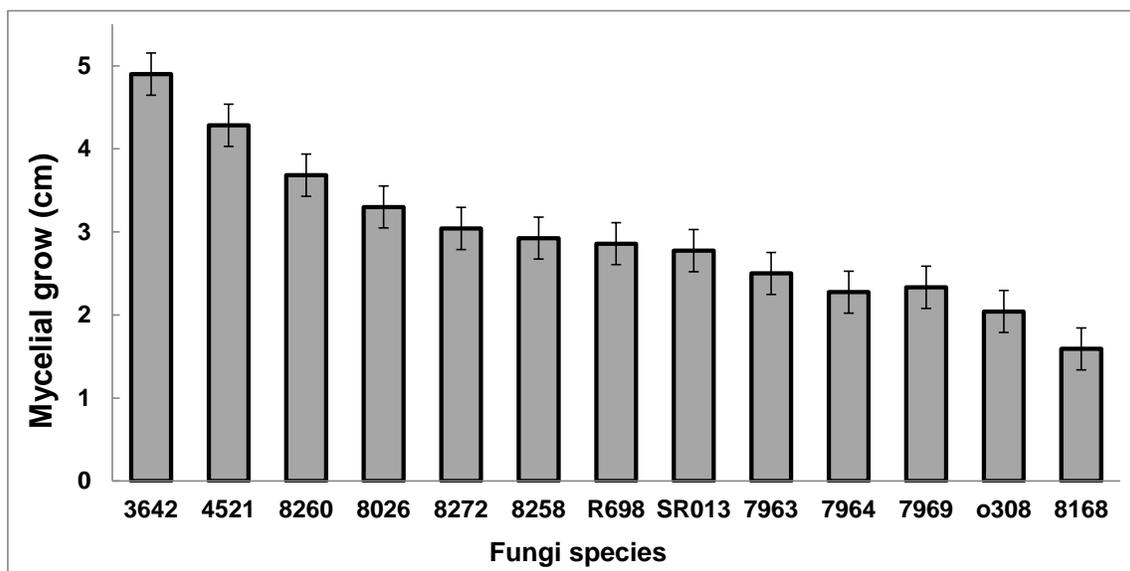


Fig. 3. Mycelial growth (cm) of 13 species of fungi in minimal medium after eight days of incubation at 26 °C. Data presented as the mean \pm SD

Figure 3 shows the different mycelial grow behaviours of 13 fungi species in the minimal medium (Barrat *et al.* 1965) containing nutrients commonly used for fungal growth, that were the same chemicals used to prepare black liquor solid media with nutrients. Faster growing fungi include *P. chrysosporium*, *S. pruinosum*, and *C. gallica*, (UAMH 3642, 4521, and 8260, respectively) while the three *Pleurotus ostreatus* isolates (UAMH 7963, 7963, and 7969), *P. betunilus*, and two *G. applanatum* isolates (UAMH 0308 and 8168) were the slowest growers.

The mycelial growth of UAMH 8258 and 7963 on minimal media was slower when compared with OAM at 5 and 10% concentration. It is clear these fungi can quickly consume organosolv black liquor components, without being nutrient-dependent. On KAM, the same behaviour was observed with these fungi as well with UAMH 8026, 7964 and 7969. Only the isolate UAMH 7963 grew faster on SAM than on minimal media. These results showed the behaviour under mycelial growth velocity with and without chemicals (nutrients).

In some cases, additional chemicals disturbed mycelial growth velocity, as observed with *Pleurotus* isolate UAMH 7963 in all black liquor media tested. In general, at low concentrations of black liquor, some fungi can grow faster than others on minimal media. However, with increasing of black liquor concentration, mycelial growth velocity decreases and additional chemicals as nutrients do not help them. All isolates of *Pleurotus ostreatus* and *B. adusta* have high potential to be applied in biotechnology.

Da-Re and Papinutti (2011) also reported similar results for fungi, including some basidiomycetes. However, the authors observed mycelial growth and decolorization of black liquor agar by removing salts from black liquor through dialysis. In addition to salts, various inorganic compounds present in the extractives can also be deleterious to some fungal species and are a natural barrier against fungal attack on standing wood (Kirker *et al.* 2013).

Analysis of Non-Modified Lignin

Proton NMR spectroscopy is one of the most valuable analytical tools for elucidating the molecular structures of the cell wall, including lignin (Fukushima and Hatfield 2003).

The results of the $^1\text{H-NMR}$ analyses clearly demonstrate a difference in the distribution of the values of protons between soda lignin with kraft lignin and organosolv lignin (Figs. 4a, 4b, and 4c). According to the groups formed by hydrogen assignment of the signals described by Saliba *et al.* (2001), $^1\text{H-NMR}$ analysis of soda lignin (wheat straw lignin) showed several peaks in the region of 5 ($\delta \sim 2.50$ to 5.18), that are not present in the kraft and organosolv lignin. The presence of a vinyl aromatic was also observed in the region of 2 ($\delta \sim 6.28$ to 8.0), similar to the results obtained by Singh *et al.* (2011). Organosolv and kraft lignin showed two peaks in the regions 2 ($\delta \sim 6.28$ to 8.0), 4 ($\delta \sim 5.18$ to 5.74), 6 ($\delta \sim 2.1$ to 2.5), 7 ($\delta \sim 1.58$ to 2.1), and 8 ($\delta \sim 0.38$ to 1.53).

In relation to hydrogen signals in region 5 ($\delta \sim 2.5$ to 5.18), organosolv lignin had more peaks compared to kraft lignin. According to She *et al.* (2010), signals in this region are assigned to two aromatic protons in syringyl and guaiacyl propane structures. Intensive signals detected between 6.7 and 6.8 ppm suggest that the lignin fraction contains more syringyl than guaiacyl units, which is characteristic of hardwoods (Saliba *et al.* 2001).

Analysis of Fungus-Modified Lignin

The changing of the colour of the organosolv black liquor after fungal growth can be seen in Fig. 5. The ability of *B. adusta* to decolorize a dark waste such as black liquor makes it a promising microorganism for biotechnological purposes as well as in environmental studies.

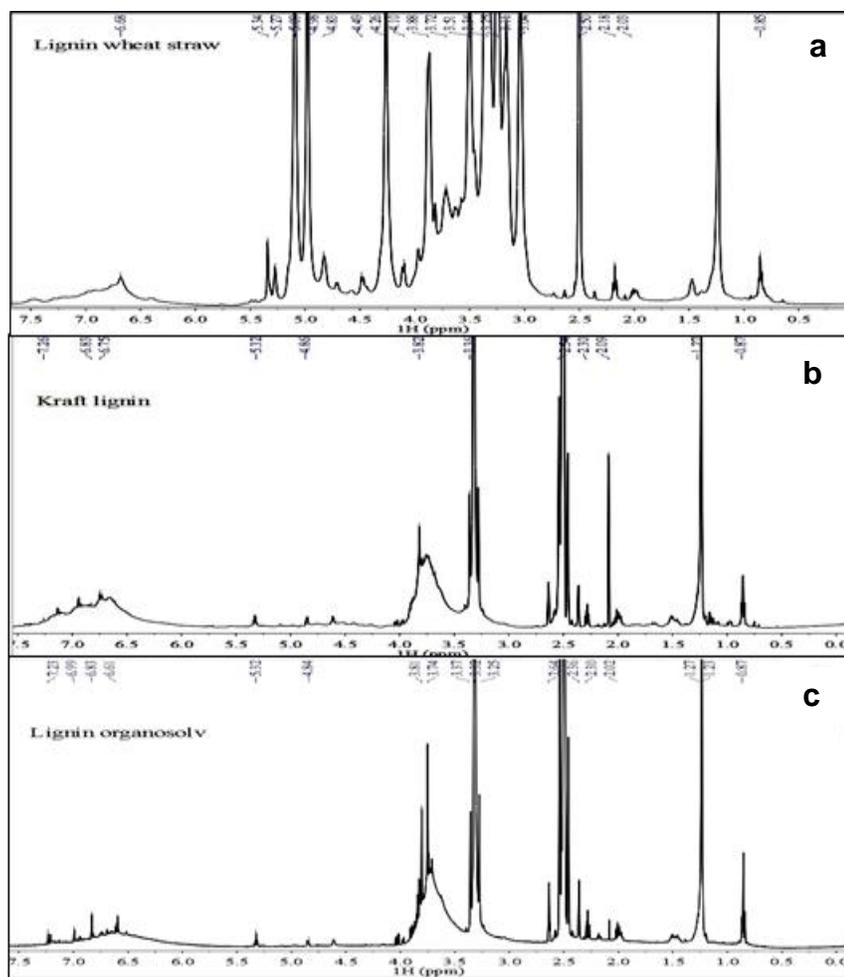


Fig. 4. ¹H-NMR analyses of lignins obtained from (a) soda (wheat straw), (b) kraft, and (c) organosolv black liquors

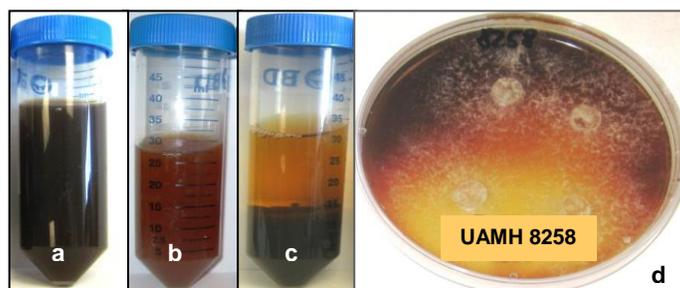


Fig. 5. (a) 10% non-biodegraded organosolv black liquor (control), (b) 10% organosolv black liquor biodegraded by *B. adusta* after 10 days, (c) 10 days-biodegraded 10% black liquor after acid precipitation (the dark color in the tube c is insoluble lignin), and (d) decolorization area of 10% organosolv black liquor media, inoculated with *B. adusta*.

The FTIR analyses of organosolv lignin were performed on samples obtained from the 2nd through the 9th day of culture (Fig. 6). The FTIR spectrum of lignin obtained from organosolv black liquor biodegraded by *B. adusta* showed significant variations between 3500 and 500 cm^{-1} (Figs. 6 and 7). With the continued fungal growth in the black liquor, some peaks in the lignin molecule almost disappeared or showed only a

weak peak; this was also seen in the lignin control samples obtained from the 2nd and 5th days (Fig. 6). Compared to commercial and organosolv lignin, the spectra of control samples had peaks with weak absorbance in the regions between 1300 and 1400 cm⁻¹.

Band absorption between 1330 and 1320 correspond to C=O stretching of the syringyl units (Kline *et al.* 2010), and bands between 1380 and 1370 cm⁻¹ correspond to C-H deformation in cellulose and hemicellulose (Zhao *et al.* 2008). Control samples (2nd and 5th days; Fig. 6) also showed a weak absorption band between 1700 and 1800 cm⁻¹. The absorption band at 1703 cm⁻¹ is attributed to the carbonyl moieties of aldehyde/ketone groups. Lacking the spectral band in this region suggests the chemical modification of most carbonyl groups under high pH conditions (Kline *et al.* 2010).

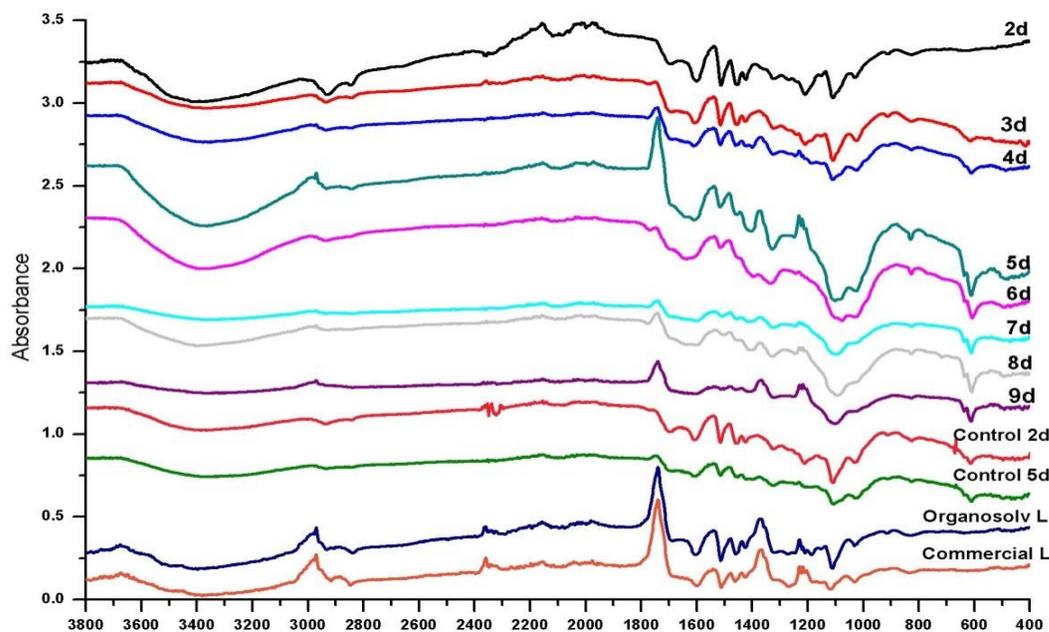


Fig. 6. FTIR spectra of lignin samples obtained by acid precipitation from organosolv black liquor biodegraded by *Bjerkandera adusta* for 9 days (samples named 2d to 9d). Control lignin samples from 2d and 5d were obtained from autoclaved black liquor non-inoculated with fungus. The organosolv lignin sample was obtained from the non-autoclaved black liquor.

The last region of the control samples lacked as compared to the organosolv lignin sample was located between 2800 and 3000 cm⁻¹, and is related to C-H stretching in methyl and methylene groups (Faix 1991). Lignin obtained from the 5th through 9th days and lignin control samples from the 2nd and 5th days showed a double peak in the region between 601 and 630 cm⁻¹. This new absorption band became apparent after the autoclaving process. There is no reference in the literature regarding these bands in this absorption region.

Commercial and organosolv lignin has a double peak in the region between 1210 and 1234 cm⁻¹, while these bands disappeared on autoclaved control lignin (Fig. 6). Bands in this region are assigned to C-C, C-O, and C=O stretching (Xiong *et al.* 2007) of the guaiacyl unit (Kline *et al.* 2010). These bands reappeared strongly at the 5th, 8th, and 9th days of fungal growth in black liquor. According to Rodrigues *et al.* (1998), a band at 1239 cm⁻¹ in *Eucalyptus globulus* wood corresponds to O-H in plane in polysaccharides.

The autoclaving process caused some modifications to the molecular structure of the lignin sample. However, compared to non-autoclaved black liquor lignin, some structures reappeared during fungus growth as well with incubation time. Xiong *et al.* (2007) also observed that lignin suffered modifications to its structure after fungus treatment, in this case, through detection of a new band at 1665 cm^{-1} , which can be assigned to a carbonyl bond.

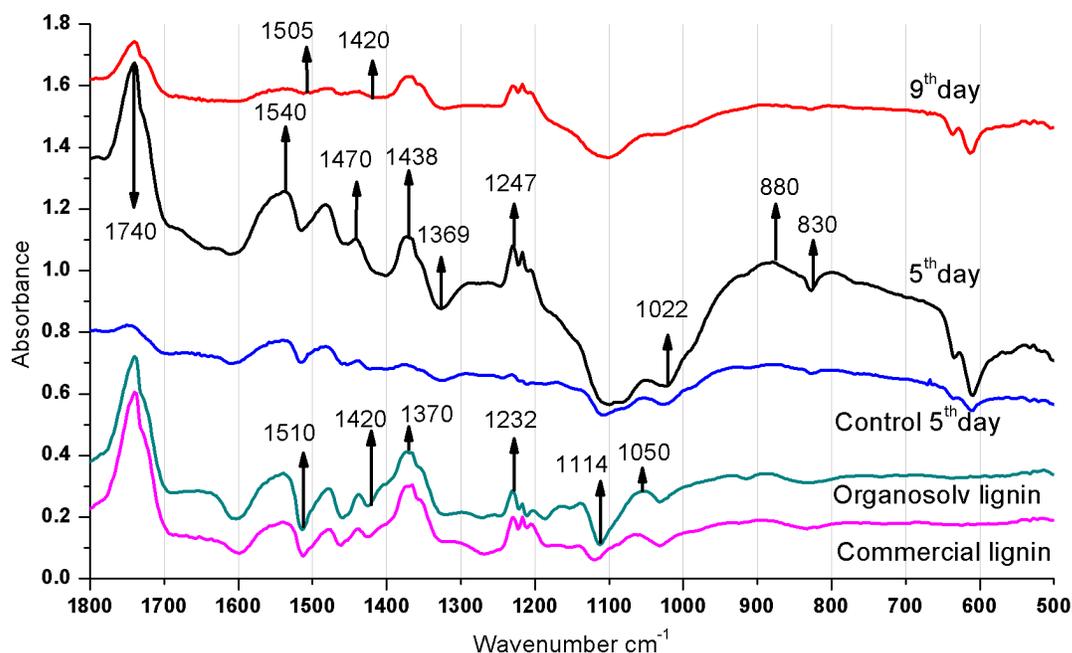


Fig. 7. FTIR spectra of lignin obtained from biodegraded-organosolv black liquor at 5th and 9th day by the fungus *Bjerkandera adusta*. Control lignin from the 5th day was obtained from autoclaved black liquor non-inoculated with the fungus. The organosolv lignin sample was obtained from non-autoclaved black liquor.

Compared to organosolv and commercial lignin, the distinct peak in the lignin control sample, 5d, that changed with the autoclaving process, is assigned to the region of 1740 cm^{-1} (Figs. 6 and 7). This peak can be seen in spectra, but it is very weak in the lignin control samples 2d and 5d. This is related to ketone/aldehyde C=O stretching in unconjugated ketone, carbonyl, and in ester groups, frequently of carbohydrate origin (Faix 1991), especially xylans (Zhao *et al.* 2008) or free esters of hemicellulose (Kubo and Kadla 2005).

Lignins obtained from the 2nd and 3rd days show a similar spectral profile, except for the additional bands at 2160 , 2219 , and 1969 cm^{-1} (Fig. 6). Compared to organosolv lignin, these samples lost a peak located at 2973 cm^{-1} , which can be attributed to C-H stretching in a methyl, methylene, or methane group (Xiong *et al.* 2007).

There are new peaks in the 600 cm^{-1} region only present in the lignin from the 5th day (Fig. 7). Thus, this structure also appeared after the autoclaving process (Control 5th day). The spectra of lignin obtained from the 5th day began to have a similar structure to that of organosolv lignin, though with some peaks missing after the autoclaving process, mainly the peak at 1740 cm^{-1} . On this sample day it is possible to observe a broad and

convex band around 3700 to 3000 cm^{-1} , and a peak at 2160 cm^{-1} that is also stronger in the 2nd day lignin sample (Fig. 6).

The 5th day lignin sample showed a peak at 1330 cm^{-1} , decreasing until the 8th day, which is not present in organosolv and commercial lignin. The absorption band between 1325 and 1330 cm^{-1} corresponds to the syringyl ring plus condensed guaiacyl ring (Faix 1991). The sample from the 5th day shows a deeper band in the region between 1220 and 900 cm^{-1} (Fig. 7). The absorption band between 1221 and 1230 cm^{-1} corresponds to C-C, C-O, and C=O stretching (Faix 1991).

In general, FTIR spectra showed modifications in lignin structure that occurred after fungal treatment, mainly at the 5th and 9th days. The most changes occurred in bands between 1700 to 1100 cm^{-1} that correspond to C-C and C-O bonds and the presence of a new peak at 880 cm^{-1} region. The spectra of lignin obtained at 9th showed absence of aromatic rings at regions 1600, 1505, and 1420 cm^{-1} , which implies a corresponding breakdown of this structures by the fungal treatment. This technique is very useful to complement lignin characterization process, even though the sensitivity of the method was not high enough to detect slightly changes in lignin (Mattinen *et al.* 2008).

Table 3. Signal Assignments and Relative Intensities in FTIR Spectra Band of Lignin Biodegraded by *Bjerkandera adusta* During 15 Days of Shaking Incubation at 26 °C.

Band (cm^{-1})	Assignment
2800 to 3000	C-H stretching
1703 to 1718	Unconjugated C=O stretching
1604 to 1596	C-C stretch vibration in aromatic skeletal
1660 to 1640	Aromatic ring vibration and C=O stretching
1595 and 1505	Aromatic skeletal vibrations in lignin
1513 to 1507	C-C stretch vibration in aromatic skeletal
1458 to 1456	C-H deformation in methyl and methylene groups
1375	C-H deformations on polysaccharides
1260	C-O stretch vibration in guaiacyl
1215 to 1208	C-C, C-O, and C=O stretch
1127 to -1109	Aromatic C-H deformation in syringyl ring
1047 to 1031	Aromatic C-H in plane deformation
897	C-H deformation
Table adapted from Gouveia <i>et al.</i> (2012)	

SEC Analyses

The molecular weight of lignin is an important measurement parameter for raw materials in the manufacturing of biocomposites. From size exclusion chromatography analysis it was observed that there was lignin modification, fragmentation, and/or repolymerisation during the 15 days of treatment (Table 4). From the 2nd to 5th days, the average molecular weight (MW) of the lignin (MWL) appeared to be constant. At the 6th day, there was likely some repolymerisation of lignin.

Table 4. Molecular Weight Average of Lignins Obtained from Organosolv Black Liquor Biodegraded or not by *Bjerkandera adusta* for 15 days as Determined by Size Exclusion Chromatography.

Samples	Mn ^a	MW ^b	PDI ^c	Mp ^d	pH
Lignin ^e	953	3122	3.3	2297	5.6
Lignin ^f	1308	2150	1.6	2162	6.0
2d ^g	1470	2217	1.5	2097	5.9
3d	1517	2236	1.5	2123	5.8
4d	1602	2122	1.3	2093	5.8
5d	1502	2655	1.8	2645	5.5
6d	2305	5185	2.3	4753	5.5
7d	1607	3061	1.9	2975	5.6
8d	1819	3902	2.1	3761	5.5
9d	2063	3384	1.7	3531	5.6
11d	1309	1461	1.1	1986	4.8
12d	1629	1790	1.1	2101	4.8
13d	1622	3067	1.9	2777	4.8
14d	1323	2373	1.8	2179	5.0
15d	1286	1997	1.6	2059	4.7

^aMn: number average molecular weight
^bMW: weight average molecular weight
^cPDI: Polydispersity
^dMp: Peak molecular weight
^eLignin from non-autoclaved black liquor
^fLignin from autoclaved black liquor, recovered after 1 d
^gLignin samples recovered after 2 d to 15 d of biodegradation

From the 7th to 9th days, there was a decreasing MW, but it was still higher than in the first days. A lower MW was obtained on the 11th and 12th days as well as lower polydispersity, which means that the sizes of the lignin molecules were uniform. Lignin samples obtained from the 13th to 15th days suffered decreases in MW. The molecular weight of lignin increased when re-polymerisation occurs during further incubation. Some of these differences can occur by interaction between lignin and its compounds with laccase, an oxidative enzyme often produced by basidiomycetes.

Radicals generated during the reaction between laccase and lignin can create different mesomeric forms that may couple in many possible ways, forming new linkages in only a few hours (Gouveia *et al.* 2012). Increasing in the MW on biodegraded-lignin sample observed at 6th day can be indicative of the presence of new linkages caused during fungus growth. The molecular weight of lignin can suffer increase or decrease in the MW after exposition to laccase, mainly after 30 min of reaction under controlled conditions (Rocha *et al.* 2014) or even after 24 h (Mattinen *et al.* 2008). In general, with *B. adusta* growth in the organosolv black liquor, the pH also decreases, being more acidic on the 11th and 12th days and with lower MW.

Black liquor is a hazardous waste if improperly discarded, and its degradation by fungi has been the subject of many studies. In addition to the fungi evaluated in this study, *Phanerochaete sordida*, *Pycnoporus sanguineus*, and *Trametes elegans* show great ability to remove the colour of kraft black liquor malt agar plates (Da-Re and Papinutti 2011; Sanchez 2009). The genera *Bacillus* and *Streptomyces* also have shown great

capacity to degrade lignin by generating low molecular weight compounds and by removing the colour of black liquor (Chandra *et al.* 2011; Raj *et al.* 2007; Ahmad *et al.* 2010).

Black liquor is known for its unpleasant smell, especially when the pH is lowered with sulphuric acid, and for its dark colour. After three days of fungal growth, these characteristics began to change. The colour became brownish, and a weak sweet smell was detected, probably due to vanillic acid production. Transformation of lignin-based aromatic acids or its compounds by microorganisms in vanillic acid has been investigated by several authors (Chen *et al.* 1983; Ander *et al.* 1984; Zamzuri *et al.* 2013), showing that fragmentation of lignin can generate a flavouring compound. With advancing degradation, these characteristics were more easily detected.

CONCLUSIONS

1. Biologically modified lignin was obtained by the cultivation of the white-rot fungus *Bjerkandera adusta* in organosolv black liquor.
2. The lowest MW of lignin recovered from organosolv black liquor after cultivation with *B. adusta* was observed on the 11th and 12th days, at 1461 and 1790 KDa, respectively, and a polydispersity index close to 1.0, indicating molecular size uniformity.
3. The FTIR spectra bands showed modification of the lignin structure after 9 days, with new bands appearing after five days of lignin biodegradation.
4. Organosolv black liquor provided a great mycelial growth rate to all fungi tested. Adopting the best conditions of fungus growth in a hazardous waste is essential to obtaining success in the biological modification of lignin.
5. Monitoring measures of colour changes, pH, and smell are rapid methods to determine the degree of modification of lignin structure and molecular weight.

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