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BIODEGRADATION OF *EUCALYPTUS GLOBULUS* AND *E. NITENS* BY THE WHITE-ROT FUNGUS *CERIPORIOPSIS SUBVERMISPORA* AND LIGNIN CHARACTERIZATION BY THIOACIDOLYSIS AND CuO OXIDATION

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ABSTRACT

Wood chips of *Eucalyptus globulus* and *E. nitens* were biodegraded by the white-rot fungus *Ceriporiopsis subvermispora* for periods from 15 to 60 days. Two analytical techniques, oxidation with copper(II) oxide (CuO) and thioacidolysis were used for *in situ* characterization of lignin, and determination of changes in the amount of syringyl (S) and guaiacyl (G) units, frequency of *β*-O-4 linkages and S/G ratio. Wood weight losses after degradation varied from 1 to 10 %. A preferential degradation pattern of lignin over cellulose was observed with lignin losses ranging from 5 to 26 % for both *Eucalyptus* species. Thioacidolysis analysis showed a reduction in the amount of S units of 42 % and 24 % for *E. globulus* and *E. nitens* lignin, respectively. No variation in the amount of G units in the residual lignin of decayed wood chips was observed. The S/G ratio for *E. globulus* and *E. nitens* determined by oxidation with CuO was 3.9 and 3.1, respectively. Results showed that during the degradation of *Eucalyptus* spp. wood by *C. subvermispora* there were a preferential degradation of syringyl structures and non-condensed aryl-ether bonds of lignin.

KEYWORDS: *Eucalyptus globulus*, *Eucalyptus nitens, Ceriporiopsis subvermispora,* biodegradation, lignin, thioacidolysis.

INTRODUCTION

Wood decay fungi play an important role in the carbon cycle by degrading lignocellulosic biomass, as well as, other organic components. There are three main types of decay caused by fungi: soft rot, brown rot and white rot (Eriksson et al. 1990, Martínez et al. 2005). White-rot fungi (WRF) can present two types of decay: the simultaneous and the lignin-selective. In the simultaneous type carbohydrates and lignin are almost uniformly degraded at the same time and at a similar rate during all decay stages (Eriksson et al. 1990). The lignin-degrading WRF of the lignin-selective type present a preferential attack of lignin in the initial period of wood decay, degradation and mineralizing the macromolecule, leaving cellulose relatively intact (Eriksson et al. 1990, Martínez et al. 2005). In order to degrade lignin these fungi produce extracellular oxidative enzymes such as laccase (Lac), lignin peroxidase (LiP), manganese peroxidase (MnP) and also low molecular mass compounds that mediate the action of these enzymes (Kirk and Cullen 1998, Hofrichter 2000). Several works have been published on the use of WRF and its enzymes in biotechnological applications in the pulp and paper industry, textile effluent treatment, dyes discoloration, bioethanol production and others (Pointing 2001, Eichlerová et al. 2005, Reinprecht et al. 2007, Muñoz et al. 2007, Ferraz et al. 2008, Mendonça et al. 2008).

Several WRF have been evaluated in its ability to degrade selectively lignin for the biopulping process, being *Ceriporiopsis subvermispora* the most suitable for this purpose in both mechanical and chemical pulping processes (Akhtar et al. 1998, Mendonça et al. 2002, Ferraz et al. 2008, Mendonça et al. 2008). Together with high lignin and low carbohydrates decay during wood colonization, the WRF also cause modifications in the lignin structure that could not be assessed only by the determination of weight and component losses. Modifications in the structure of residual lignin are also important to follow and understand the mechanism of wood decay by WRF. Guerra et al. (2002) using in situ analysis by oxidation with CuO and derivatization followed by reductive cleavage (DFRC) techniques demonstrated that *C. subvermispora* heavily degraded the lignin of *Pinus taeda* in the first stages of wood degradation even when low lignin losses are detected. With the aim to contribute with the study of wood decay and determine modification in the lignin structure caused by WRF, in this work we evaluated the degradation of wood chips from *Eucalyptus globulus* and *E. nitens* by the biopulping fungus *C. subvermispora*. Modifications in lignin structure were determined by two in situ analytical techniques: thioacidolysis and oxidation with copper (II) oxide (CuO). These techniques have the advantage that can be used directly on wood with no need to isolation of lignin, giving results that were more representative of the changes in the macromolecule structure.

MATERIAL AND METHODS

Wood degradation

The white-rot fungus *Ceriporiopsis subvermispora* (Pilát) Gilb. & Ryvarden was from the culture collection of the "Laboratorio de Recursos Renovables, Centro de Biotecnología", Universidad de Concepcion, Chile. The strain CS-1 was kindly donated by Dr. A. Ferraz (EEL/USP, Sao Paulo, Brazil) and maintained at 4°C on solid medium contained 2 % malt extract, 0.5 % soy peptone and 2 % agar. Wood chips from 10-12 years old *Eucalyptus globulus* and *E. nitens* were obtained in a local pulp mill (Bío-Bío Province, Chile). The wood chips with an average size of 2.5 x 2.0 x 0.3 cm were air-dried until 10 % moisture and stored in dry conditions. Before fungus inoculation, wood chips were immersed in water for a 16 h period. Residual water

was drained and 50 g (dry basis) of moist wood chips (55 % moisture) were sterilized in 1-L bioreactors at 121°C for 30 min.

Sterilized liquid culture medium (200 mL) composed by 2 % (w/v) malt extract and 0.5 % (w/v) soybean peptone was inoculated with 20 discs (4 mm in diameter) of *C. subvermispora* pre-cultured solid medium. These liquid cultures were incubated under stationary conditions for 10 days at 27°C. The grown mycelium mat was filtered and washed with 300 mL of sterilized water. Washed mycelium obtained from several cultures was blended in laboratory blender with sterilized water in 3 cycles of 15 s. The mycelium suspension was used to inoculate the sterilized wood chips with a volume of suspension corresponding to 500 mg fungal mycelium per kg dry wood. The inoculated wood chips were incubated at 27°C and 55 % of relative air humidity in an acclimatized room and maintained stationary for 15, 30, 45 and 60 days. Bioreactors with sterilized but non-inoculated wood chips were also prepared for use as control samples. Three bioreactors were prepared for each period of time evaluated. After each biodegradation period, bioreactors were opened and the wood chips washed with abundant water to remove the superficial mycelium. Wood chips were air-dried and the exact moisture determined for calculation of wood mass loss due to biodegradation.

Wood characterization

Milled wood samples (40/60 mesh) were extracted with ethanol/toluene according to TAPPI standard method 204 cm-97. Wood and pulp samples were characterized for total lignin, glucan and xylan according to methodology described by Mendonça et al. (2008). In a test tube were weighed 300 mg of sample and added 3 mL of 72 % (w/w) H_2SO_4. The hydrolysis was carried out in a water bath at 30°C for 1 h with a glass-rod shaking every 10 min. Later, the acid was diluted to 4 % (w/w) with 79 mL of distilled water and the mixture transferred to a 250-mL Erlenmeyer flask and autoclaved for 1 h at 121°C (post-hydrolysis). The residual material was cooled and filtered through a porous glass filter number 3 (Schott Duran, Germany). Solids were dried to constant weight at 105°C and determined as insoluble lignin. Soluble lignin was determined by measuring the absorbance of the solution at 205 nm. The concentration of monomeric sugars in the soluble fraction was determined by HPLC (Merck Hitachi, Japan) in an Aminex HPX-87H (Biorad, USA) column at 45°C, eluted at 0.6 mL.min^{-1} with 5 mM H_2SO_4 and using a refractive index detector (LabChrom L-7490, Merck Hitachi, Japan). The cellulose amount in wood was expressed as glucans (sum of anhydroglucose + anhydrocellobiose + hydroxymethylfurfural) and the hemicellulose was expressed as xylans (sum of anhydroxylose + uronic acids + anhydroarabinose + acetyl groups + furfural). The standard deviation from the average values was lower than 3 %. Component losses were calculated in basis of wood composition from undecayed and decayed wood chips. Due to the different pattern of degradation of some wood components (some were decayed more extensively than others and with different rates), the amount of the less decayed components increased in the decayed wood as compared with the undecayed wood chips.

Lignin characterization

Thioacidolysis was performed on 20 mg of extracted milled wood in 10 mL of reagent according to the method published by Rolando et al. (1992). The reagent was prepared by introducing 2.5 mL of BF_3 etherate (Aldrich) and 10 mL of ethanethiol EtSH (Aldrich) into a 100 mL flask and adjusting the final volume to 100 mL with dioxane. The reagent (15 mL) and 1 mL of a solution of GC internal standard (tetracosane in CH_2Cl_2, 0.6 mg.mL^{-1}) were added to the sample in a glass tube closed with a Teflon-lined screw cap. Thioacidolysis was performed at 100°C (in an oil bath) for 4 h. The cooled reaction mixture was diluted with 30 mL of water and

the pH adjusted to 3.0-4.0 with aqueous 0.4 M $NaHCO_3$ followed by extraction with 3 x 30 mL CH_2Cl_2. The combined organic extracts were dried over Na_2SO_4, and the solvent was evaporated under reduced pressure at 40°C. The final residue was dissolved in 1 mL of CH_2Cl_2 and analyzed by GC-FID (Clarus 600, Perkin Elmer, USA) after silylation in a DB-5 column (Agilent J&W, Australia). Program temperature was the same described by Lu and Ralph (1997). The amount of syringyl and guayacyl units (mmoles per g of lignin) bonded by *β*-O-4 linkages and also the frequency of *β*-O-4 linkages in the lignin were obtained with this analysis.

The determination of syringyl and guaiacyl units in the lignin of decayed wood by oxidation with copper(II) oxide was carried out following the methodology described by Chen (1992). In a 80 mL stainless steel reactor it was added 400 mg of sample (milled and extracted wood), 2 g CuO, 15 mL NaOH 2 M and 100 mg $Fe(NH_4)_2(SO_4)_2.6H_2O$. Nitrogen was bubbled inside the reactor which was tightly closed and immersed in an oil bath for 3 h at 170°. After the reaction, it was added to the reactor 10 mL NaOH 1 M and 10 mL of distilled water. The mixture was acidified to pH 1 with HCl. The content of the reactor was transferred to a centrifuge tube and centrifuged at 2500 rpm for 10 min. The liquid fraction was collected, transferred to a separation funnel and extracted three times with 50 mL of diethyl ether. The organic fraction was further evaporated (40°C) at reduced pressure (450 mbar). The solid residue was dissolved in 1 mL pyridine and 0.5 mL of sample was silylated with 0.5 mL N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). Derivatization products were quantified by GC/FID using the conditions published elsewhere (Chen 1992). The amount of syringyl units was the sum of syringaldehyde and syringic acid; and the amount of guaicyl units was the sum of vanilin and vanillic acid. Degree of condensation (DC) represented the amount of lignin fraction that was not bonded by aryl-ether linkages and was calculated as the subtraction of the percentage of *β*-O-4 linkages from 100 % (DC = 100 % - % of *β*-O-4).

RESULTS AND DISCUSSION

Chemical composition of undecayed wood chips of *Eucalyptus* spp. shows that *E. globulus* presents slight higher amount of glucans, lower amount of lignin and extractives than *E. nitens*, and similar amount of xylans (Tab. 1). Values found for chemical composition were according to expect for *E. globulus* hardwood.

Tab. 1: Chemical composition of undecayed Eucalyptus globulus and E. nitens.

	E. globulus	***E. nitens***
Glucans (%)	51.3±0.4	48.2±0.4
Xylans (%)	20.7±0.2	19.4±0.4
Lignin (%)	26.9±0.4	27.8±0.9
Extractives (%)	1.0±0.1	2.8±0.1

With the chemical composition of each species and the weight losses determined during the different degradation time, it was calculated the component losses during the time-course of the decay assays (15 to 60 days). Degradation of *E. globulus* wood chips by *C. subvermispora* led to an extensive decrease in lignin and xylans amount (Fig. 1) and, as a result, the amount of glucans (representing the cellulose fraction in wood) increases slightly. These results corroborate a typical behavior of this fungus that is the preferential decay of lignin in the initial steps of

wood degradation (Guerra et al. 2002, Mendonça et al. 2002, 2008). Specifically, in the decay of *E. globulus*, weight loss varied from 1.6 % to 6 %, while lignin and xylan losses were from 6-25 % and 11-15 %, respectively (Fig. 1A). The amount of glucans in wood increased (6-8 %) in the first 30 days of decay, indicating that the fungus did not significantly mineralize this component. However, after the 30 days of incubation with *C. subvermispora*, glucans started also to be consumed by the fungus and its amount in *E. globulus* wood decreased 10 % at 60 days of decay (Fig. 1A). When decaying *E. nitens*, *C. subvermispora* causes similar degradation in lignin with losses from 5 to 26 %, but with lower degradation of xylans and glucans than observed for *E. globulus* (Fig. 1B). In this case, the selectivity of the fungus starts to decrease after 60 days of decay. Results obtained were according to the previously published for both wood species (Mardones et al. 2006, Mendonça et al. 2008).

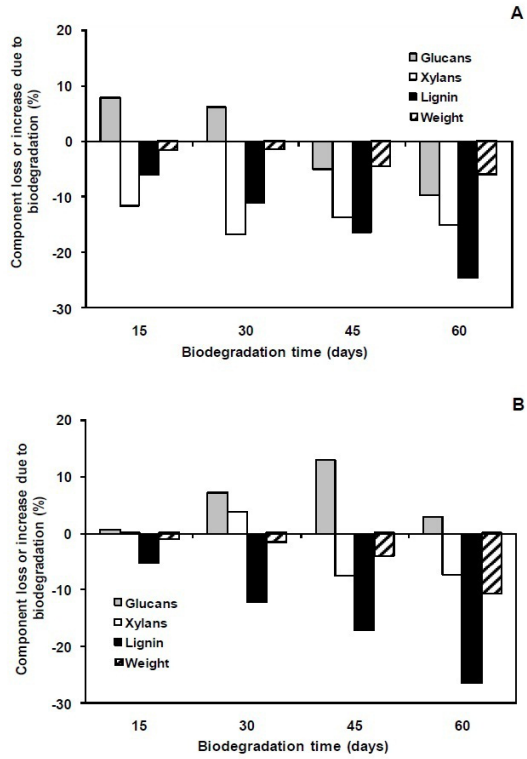

Fig. 1: Weight and components variation during the biodegradation of (A) E. globulus and (B) E. nitens by C. subvermispora.

Component losses reported here only represented the fraction of the wood that was mineralized by the fungus to CO_2 and H_2O and water soluble fragments, but do not indicate the extended of degradation of the residual biopolymers in wood. Guerra et al. (2002, 2003) showed that for *Pinus taeda* decayed by *C. subvermispora*, the DFRC monomers originating from aryl-ether linkages decreased drastically at the initial stages of degradation (27 % and

53 % after 15 and 30 days, respectively) while the wood weight and lignin losses reached only 2.3 % and 3 %, and 9.6 % and 10.9 %, respectively, for the same degradation periods. Authors also showed that cellulose, which is poorly mineralized by the fungus, presented a diminution in its polymerization degree as determined by GPC analysis. This result indicated that an extensive depolymerization of lignin occurs before its mineralization becomes significant. To determine the structural modifications in *E. globulus* lignin of the decayed wood, two analytical techniques for in situ characterization of lignin were used: thioacidolysis and oxidation with CuO. With the first technique it is possible to quantify the amount of syringyl and guaiacyl units bonded by aryl-ether *β*-O-4 linkages, as well as, the frequency of these linkages and the condensation degree of lignin (carbon-carbon bonds); with the second technique it is possible to determine all the syringyl and guaiacyl units in the macromolecule and estimate the syringyl/guaiacyl (S/G) ratio (Rolando et al. 1992, Chen 1992). The syringyl/guaiacyl ratio (S/G ratio) and the aryl-ether bonds are the main structures in lignin that could be modified by the white-rot fungi during wood decay as showed previously (Srebotnik et al. 1997, Guerra et al. 2002).

Lignin from hardwoods is composed mainly by syringyl (S) and guaiacyl (G) units linked by ether and carbon-carbon (C-C) bonds, being the aryl-ether *β*-O-4 the most frequent (Ralph et al. 2004). For both *E. globulus* and *E. nitens* the initial amount of S or G units bonded by *β*-O-4 linkages were rather similar (1560 μmol.g^{-1} lignin and 380 μmol.g^{-1} lignin, respectively). During wood decay, S units content decreased with the increase of incubation time and reduction of lignin amount in wood. This decrease is more accentuated in lignin of *E. globulus* than *E. nitens* (Fig. 2).

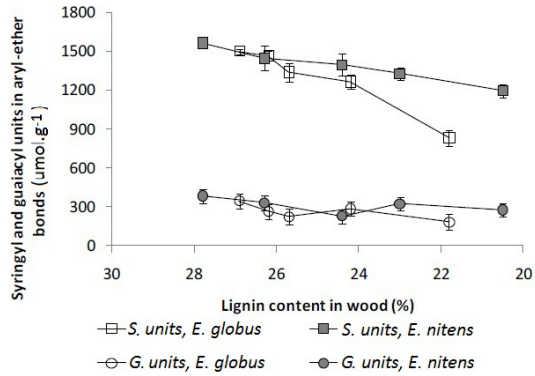

Fig. 2: Amount of syringyl and guaiacyl units in aryl-ether (β-O-4) bonds in the lignin of E. globulus and E. nitens decayed by C. subversmispora.

S units decreased from 1550 to 830 μmol.g^{-1} lignin in *E. globulus* and from 1560 to 1200 μmol.g^{-1} in *E. nitens*. The amount of G units in lignins of decayed wood also presented a decrease in its amount from approximately 360 to 200 μmol.g^{-1} lignin. S units (mainly bonded by aryl-ether linkages) were more susceptible to degradation by *C. subvermispora* than G units that were present in highly condensed structures. The decrease in the amount of S units in the lignin of *E. globulus* was followed by a reduction in the amount of *β*-O-4 bonds (Fig. 3A). For both *E. globulus* and *E. nitens* the amount of *β*-O-4 linkages is close to 40 % and was reduced to 27 % (*E. globulus*) and 32 % (*E. nitens*). As a consequence, there was an increase in the condensed

structures due to the accumulation of non-decayed G units in the lignin (Fig. 3B). The oxidative system of *C. subvermispora* probably lacks of enough oxidation potential to degrade the G units bonded bycarbon-carbon linkages. The condensation degree represents the fraction of lignin that is bond by C-C structures and is inversely proportional to the amount of aryl-ether structures.

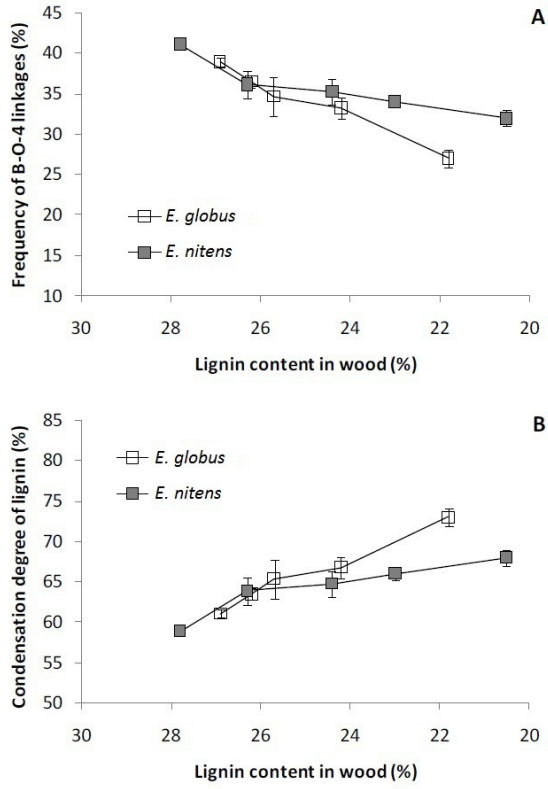

Fig. 3: (A) Frequency of β-O-4 linkages and (B) condensation degree of lignin from E. globulus and E. nitens decayed by C. subvermispora.

The determination of the syringyl/guaiacyl ratio (S/G) was obtained by the in situ oxidation of lignin with CuO in alkaline medium. The main products of this reaction are vanillin and vanillic acid from G units, and syringaldehyde and syringic acid from S units. The S/G of *E. globulus* genus varied widely according to the species and some of values reported in the literature were 5.9 for *E. globulus*, 2.8 for *E. grandis* and 2.2 for *E. urograndis* (Pinto et al. 2005).

In this work, the values found for the undecayed wood of *E. globulus* and *E. nitens* were 3.9 and 3.1, respectively (Fig. 4). Results showed that during wood degradation by *C. subvermispora* the S/G ratio decreases with the increase in lignin removal (less lignin in wood).

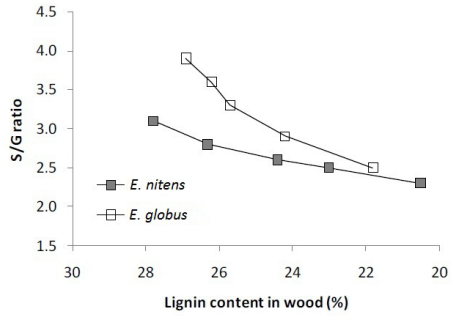

Fig. 4: Syringyl/guaiacyl (S/G) ratio in E. globulus and E. nitens wood chips decayed by C. subvermispora.

The decrease in the S/G ratio is also related with the decrease in the S units in the lignin with the apparently unchanged amount of G units as observed before. This confirms that S units are preferentially decayed by the oxidative complex of the fungus and more condensed structures remained in wood. At this point fungal degradation mechanism could change toward increasing the carbohydrates degradation as showed by Guerra et al. (2003). The different behavior could be due to different features of wood composition or anatomy (type of extractives, metals content, cell morphology and vessel frequency, for instance) that could affect both homogeneity of wood colonization and oxidative enzymes production during decay.

CONCLUSIONS

Results obtained allow concluding that for *E. globulus* and *E. nitens* there is similar mineralization rates of lignin during decay with the selective-type white-rot fungus *C. subvermispora*, but a different pattern in the modifications of the structure of the residual lignin. The structural analysis of lignin by thioacidolysis showed that syringyl units were extensively decayed by the fungus with losses up to 50 %. The reduction of S units was followed by a decrease in β-O-4 linkages.

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