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Applying Ligninolytic Fungi on *Eucalyptus grandis* Wood for Pulping Pretreatment or Fractionation

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Abstract

The effects of three different fungal treatments on several technology characteristics of *Eucalyptus grandis* wood, were studied on industrial chips and blocks. The percentage of the substrate mass loss by the fungal treatment, and the relative amount of extractives and lignin were determined. The effective capillarity of wood, pH and total reducing sugars concentration in water-soluble fraction (WSF) were also determined. There was a reduced mass loss of the substrate by the fungal treatment (less than 3%). *Gelatoporia subvermispora* FBCC 313 showed the highest reduction in the Klason lignin content, the highest endoglucanase activity on the WSF as well as the highest ability to increase the effectively capillarity in the radial wood direction. This last effect is interesting since it might facilitate the wood impregnation processes and therefore to reduce the consumption of reagents in industrial treatments.

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Keywords: Biopulping, Effective capillarity, Fermentation in solid state, Bioincising, Impregnation

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1. Introduction

Eucalyptus grandis Hill ex Maiden (Myrtaceae) is one of the most important wood species that is being broadly cultivated in Argentina, Brazil and Uruguay. Economically, it is relevant since its productivity and market are continuously increasing (Kolln, R. 2013). *E. grandis* wood is frequently used for several commercial applications, being the papermaking pulp production the main one.

Although this wood is an excellent source for cellulosic fibers for kraft pulping, several issues could be optimized.

The impregnation by pulping chemical reagents (NaOH and Na_2S) has a significant influence on the final pulp properties (Malkov *et al.* 2001). In this sense, technical and/or productive innovations in the pulping process for obtaining products of better quality, increasing process efficiency, reducing costs and/or chemical reagents are required. Knowledge about new byproducts with potential applicability via fractionation, including environmentally sustainable green and white biotechnology processes, are also required. In order to reach these goals, different approaches are being developed such as the selection and optimization of a proper impregnation procedure and the biological pretreatment of wood prior to impregnation (Scott and Swaney 1998, Ferraz et al. 2000).

The pretreatment of wood chips with white rot fungi can be a promising alternative in timber industry and pulp manufacture due to the selective abilities of degradation triggered by these organisms. It may be addressed in two ways: i) a process known as biopulping, which reduces the content of wood lignin mainly in middle-lamella fraction and secondary wall (Dorado et al. 1999). Particularly, for a mechanical pulping process, the lignin elimination by biopulping facilitates the fiber separation, saves electrical energy and improves strength properties of the final product (Camarero et al. 2007). ii) The process known as bioincising, which improves the wood impregnability supposedly through the selective degradation of pit membranes on wood cells with only negligible changes in the structural components (Thaler et al. 2012, Yildiz et al. 2012). While a long time might elapse until the biopulping treatment is applied at industrial scale, bioincising on wood chips using a short incubation period (up to 6 weeks) has gained importance. Therefore great efforts are devoted to develop technologies that enhance the permeability of refractory wood species by incubation with white rot fungi (Thaler et al. 2012, Yildiz et al. 2012). *Physisporinus vitreus* which is a versatile white rot fungus for engineering value-added wood products (Schwarze and M. Schubert 2011), showed promising results on the impregnability of Norway spruce wood (Thaler et al. 2012).

In pulping processes, the impregnation of wood with the kraft liquor implies penetration of liquids and diffusion of chemical reagents. Once wood is saturated in liquid, the relative ion transport capacity of wood, i. e. Effective Capillarity Cross Section Area (ECCSA) is crucial for impregnation. The effective diffusion coefficient in wood can be expressed as the diffusion coefficient in the liquid medium multiplayer by the ECCSA. It can be said that, if the ECCSA of a wood is increased, the impregnation of this wood will be faster and/or easier. ECCSA can be determined based on the analogy with the relation between the electrical conductivities of the wood and the liquid medium.

The aim of this study was to screen three different white rot fungi for their potential to modify *E. grandis* wood chips, including experimental blocks, in order to quantify the improvement of the ions/solutes transport capacity of wood after a short incubation period (30 days). Two of the tested fungi, *Coriolopsis rigida* (Berk. et Mont.) Murrill LPSC 232 and *Grammothele subargentea* (Speg.) Rajch LPSC 436, are autochthonous isolates from rotten wood of a subtropical area in Northern Argentina (Saparrat *et.al* 2002-a, 2008-a), and the third one is *Gelatoporia subvermispora* (Pilát) Niemelä (1985) FBCC 313, which is a fungus phylogenetically related to *P. vitreus*. To evaluate the fungal treatment effect on the wood properties, the following results were considered: dry mass, soluble and insoluble lignin, extractives content and ash content. Furthermore, other properties to monitor the degree of fungal transformation of wood compared to non-inoculated (control) were evaluated: fungal biomass and pH, reducing sugars, optical density at 465 nm and dry mass of the water-soluble fraction (WSF) of the wood as well as its enzyme activity related to cellulolytic, ligninolytic and xylanolytic systems. Also the effective capillarity on treated wood in radial and tangential directions was analyzed.

2. Experimental

2.1. Wood material

Fresh wood logs from 6-years-old *Eucalyptus grandis* were supplied by INTA (Instituto Argentino de Tecnología Agropecuaria) - Concordia, Argentina. Logs were sawn into disks of about 3.5 cm of thickness and then stored in polyethylene bags at -16 ºC. From the sapwood of disks, using a carpentry saw, blocks of 35 x 35 x 10 mm were obtained by cutting tangential, transverse and approximately radial faces. These blocks were treated and then used for the effective capillarity determination. Figure 1 shows a scheme of the blocks and slices.

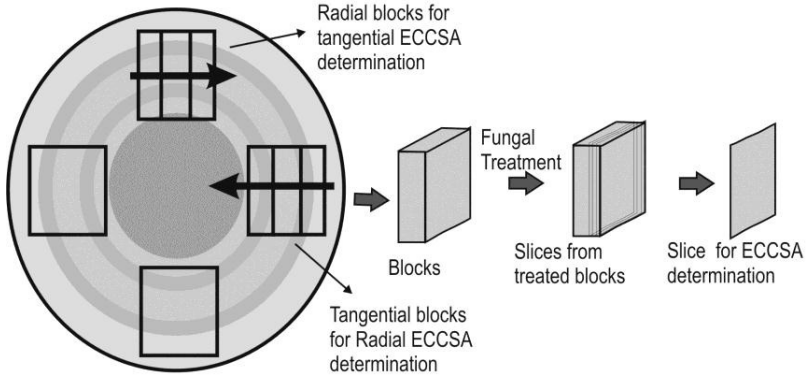

Fig. 1. Blocks for treatment and slices for Effective capillary cross section area determination.

Also, industrial wood chips of 6 years old *Eucalyptus grandis* were provided by UPM Uruguay.

Before inoculation, wood chips and blocks were autoclaved at 121ºC for 20 min immersed in water, and drained in a laminar flow chamber. The wood chips final water content was 2.1 g of water/g wood.

2.2. Fungal isolates and inoculum source

Three ligninolytic fungi with extracellular oxidative activity previously characterized at physiological level were used (Saparrat *et al*. 2002-a, 2002-b, 2004, 2008-a) (table 1). These isolates were inoculated in a liquid medium as reported by Saparrat *et al*. (2002-a) and cultured for 7 days at 150 rpm and 28 ±1.5 °C. Stock cultures were maintained on malt extract agar supplemented with yeast extract (0.4%) and *Populus nigra L.* wood chips at 4 °C (Saparrat *et al.* 2002-a).

Table 1. Fungal isolates used. Culture collection of the Instituto Spegazzini (LPSC) at UNLP, La Plata, Argentina; Fungal Biotechnology Culture Collection (FBCC) at the University of Helsinki's Department of Food and Environmental Sciences, Finland.

Fungal species	Isolate
Coriolopsis rigida (Berk. et Mont.) Murrill	LPSC 232
Gelatoporia subvermispora (Pilát) Niemelä (1985)	FBCC 313 (FP-90031-sp)
Grammothele subargentea (Speg.) Rajch	LPSC 436

2.3. Treatment of Eucalyptus grandis wood under solid-state fermentation (SSF) conditions

The capacity of the three fungi tested to modify industrial chips and experimental blocks of *Eucalyptus grandis* wood was analyzed. Each fungus was inoculated axenically in 2L Erlenmeyer flasks containing sterilized woody

materials under SSF conditions at a humidity level adjusted to 70 %. Uninoculated flasks were used as controls. All the flasks were incubated for 30 days at 27 ±1.5°C. All treatments, including control one, were performed in triplicate.

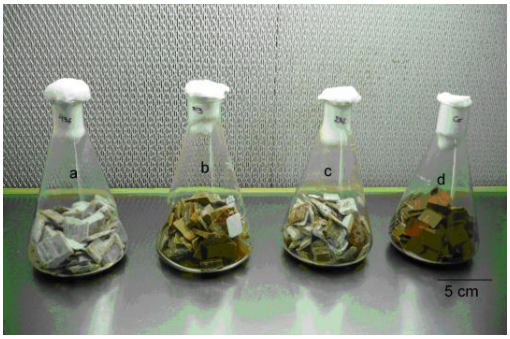

Fig.2. System used for the fungal treatment of *Eucalyptus grandis* wood (a, *G. subargentea* LPSC 436; b, *G. subvermispora* FBCC 313; c, *C. rigida* LPSC 232) and uninoculated control (d).

2.4 Analytical methods and parameters analyzed

- *Dry wood mass*:

It was measured by weighing the flasks content after drying them in an aerated oven at 80 ºC for 36 h. The percentage reduction of inoculated wood in relation to uninoculated one was assessed according to Saparrat *et al.* (2008-a).

- *Fungal biomass and water-soluble fraction (WSF):*

Fungal biomass in treated wood was measured using glucosamine as indicator (Tomaselli Scotti *et al.* 2001). The pH, the concentration of total reducing sugars (Somogyi-Nelson method), the optical density at 465 nm and the dry mass of the WSF were determined according to Dorado et al. (1999) and Saparrat *et al.* (2008-a). Extracellular enzyme activity of cellulolytic, ligninolytic and xylanolytic fungal systems was also determined on the WSF (Saparrat *et al.* 2004, 2002-b, 2008-a y b; Tomaselli Scoti *et al.* 2001).

- *Lipophilic extractives content*:

The amount of acetone-soluble matter in wood was determined according to SCAN-CM 49:03 (2003). About 10 g of wood chips were milled in a Wiley mill (pass 40 mesh) and transferred to an extraction Soxhlet system with acetone. Then, the solvent was evaporated and the residue was weighted.

- *Lignin*:

Acid-insoluble (Klason) and soluble lignin were determined as Sluiter *et al* . (2001). A sample of 3 mg of extractive free wood was place in a tube at 30ºC with 3 ml of 72% sulfuric acid for 60 minutes. Then 84 ml of deionized water was added and the tubes were place in the autoclave for one hour at 121ºC. The sample was filtered and washed with water. The filtrated was used to determine soluble lignin using a spectrophotometer at 205 nm, and the solids were dried and weighted to determine insoluble residue.

- *Ash content*:

Ash content was determined on the acid insoluble residues. They were place on filtering crucibles in the muffle furnace at 575±25ºC for 4 h and weighing the crucibles and ash to the nearest 0,1 mg.

- *Effective capillarity cross section area (ECCSA):*

Slices (350 µm thickness) were obtained from radial and tangential faces of the blocks by a microtome (figure 1). At least 20 slices were obtained from each face. The slices were impregnated with NaCl 0.1N solution. NaCl solution was chosen for ECCSA determination because it is an inert solution, that is, no reaction takes place during ECCSA determination and a constant measured is obtained for each slice. For the determination of the ECCSA, a

laboratory conductivity cell and an especially designed frame were used (Inalbon and Zanuttini 2008). In the procedure, the wood slice is mounted to the frame to keep it flat and equidistant from the electrodes. Both faces of the slice are in contact with the solution. The specific conductivity of the wood is calculated considering slice thickness and electrical resistance in a series circuit with the electrical resistance of the solution existing between electrodes. Wood slices cut in a radial direction allowed the determination of the specific conductivity in the tangential direction and viceversa. In this way, ECCSA was determined for each slice and this value was related to its position considering the original outer face of the blocks. Both face of three blocks were analyzed for each treatment, thus is each capillary profile was determined six times to counteract the wood variability.

2.5 Statistical analysis

Mean and standard deviation were calculated from data obtained for each treatment. Results were analyzed by an oneway ANOVA and means of all variables were contrasted by Tukey's test. Data on enzyme activity were analysed by the Least Significant Difference (LSD) test ($P < 0.05$).

3. Results and Discussion

Eucalyptus grandis wood chips and blocks were inoculated axenically with each of the three fungi tested. Although all of them colonized the wood after 30 days of incubation to different extension compared to an un-inoculated control, covering their surfaces by velvet-like mycelium areas, it was more notorious on wood treated with *G. subargentea* LPSC 436 (figure 2). Several parameters related to the modification of *E. grandis* wood by each fungus, including WSF, are shown in Table 2.

Concomitantly with fungal growth, wood dry mass was reduced, though it was less than 3%, being the fungus *G. subvermispora* FBCC 313 which caused the lower mass loss compared to the others one (Tukey's test, $P < 0.05$). This mass loss is relatively low, which is favorable because it does not influence the overall performance of the process. Ash content was not modified by the treatments.

Table 2. Water soluble fraction (WSF) parameters, mass loss and ash content of wood before (Untreated) and after fungal treatment as well as un-inoculated (control) one.

	WSF mass (mg/100ml)	Optical density of WSF at 465 nm	WSF reducing sugars (mM)	WSF pH	Wood mass loss (%)	Ash content (%)
Untreated	-	-	-	-	-	0.45
Control	422.2±101.8	0.52±0.08	1.07±0.17	4.4	-	0.46
LPSC 232	511.1±38.5	1.38±0.18	0.88±0.28	4.0	1.3±0.4	0.50
FBCC 313	688.9±99.6	1.19±0.05	1.76±0.06	3.5	2.7±0.1	0.60
LPSC 436	499.1±48.3	1.07±0.11	0.98±0.26	4.0	2.1±0.1	0.50

Figure 3 shows that although no endoxylanase and ligninolytic (laccase and manganese peroxidase) enzyme activity was found (data not shown), β-1,4 endoglucanase was detected in WSF of wood treated with the three fungi. In this sense, the highest level of β-1,4 endoglucanase activity was found in the WSF of cultures of *G. subvermispora* FBCC 313 (2.6±1.5 mU/ ml WSF) compared to that when the other two fungi were tested (0.4±0.3 and 0.3±0.4 mU/ ml WSF for ones treated with *C. rigida* LPSC 232 and *G. subargentea* LPSC 436, respectively). *G. subvermispora* FBCC 313 also increased the dry mass and the level of reducing substances from the WSF in relation to a drop in pH (table 2).

Figure 4 shows that the lipophilic extractive content of the wood decreased significantly only when the wood was treated with the fungi LPSC 232 and LPSC 436, which is also attractive since the extractive compounds can affect the paper properties (Prinsen et al. 2012).

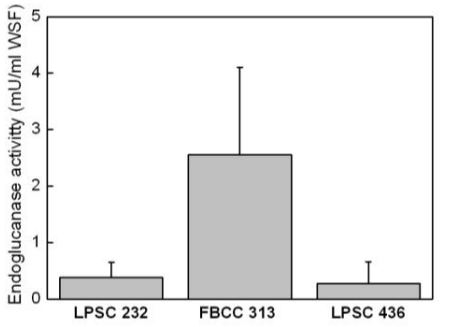

Fig. 3. Endoglucanase activity in WSF from wood treated with different fungi. Values are means of three replicates. Error bars correspond to standard deviation.

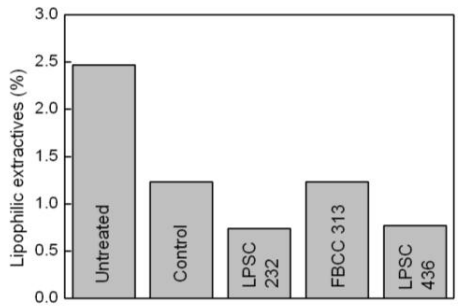

Fig. 4. Lipophilic extractives (%) of treated, untreated and un-inoculated (control) wood.

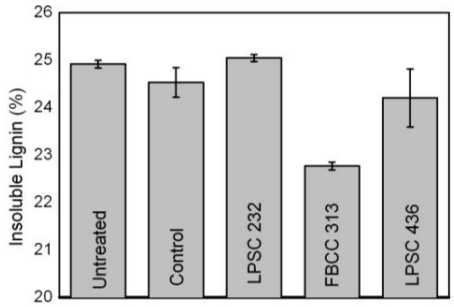

Fig. 5: Insoluble lignin of treated, untreated and un-inoculated (control) wood.

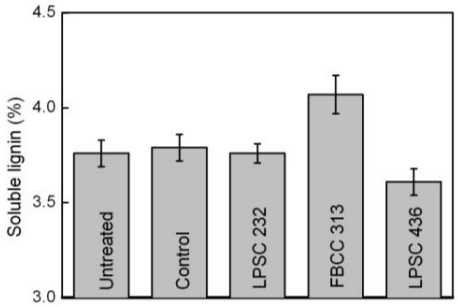

Fig. 6: Soluble lignin of treated, untreated and un-inoculated (control) wood.

Fungal treatment with the isolate FBCC 313 showed also a higher reduction in insoluble lignin (figure 5) compared to untreated wood (Tukey's test, $P < 0.05$), which, at least in part, might be related to the increase in soluble lignin detected (Tukey's test, $P < 0.05$). This suggests that the fungus FBCC 313 has an outstanding ability to attack and solubilize the lignin compared to other two fungi tested. This is relevant since wood carbohydrates are probably preserved such as inferred from data on low wood mass loss, which might also indicate that lignin degradation by FBCC 313 on *E. grandis* was selective in concordance with the available information about the high selectivity of this fungal species to delignify lignocellulose materials (Fernandez-Fueyo *et al*. 2012). Since in fungal-organosolv pulping selective lignin degradation is essential for biopulping (Ferraz *et al*. 2000), the treatment of *E. grandis* wood with the isolates FBCC 313 might facilitate papermaking and pulp production.

Although there are a lot of information about changes in lignin, carbohydrates, ash and lipophilic extractives content of wood substrates by the fungal treatment (Ferraz *et al.* 2001, Hakala *et al.* 2004, Singh *et al.* 2010), knowledge on its effect on wood transport capacity is lacking. Therefore, the ECCSA in the radial and tangential direction of *E. grandis* wood slices corresponding to each treatment was estimated.

Figure 7 shows the ECCSA in the radial direction as a function of the distance from the external face of the wood blocks. It can be observed that all fungal treatment increases the ECCSA in the radial direction, and it is more notorious near the external surface of the samples. FBCC 313 showed the highest effect (ECCSA increased from 0.06 to 0.15 at the interface). However, no changes were found in tangential direction (data not shown).

Since the effective diffusion coefficient of the ions into the wood can be expressed by the diffusion coefficient in water multiplied by the ECCSA (Inalbon *et al.* 20111), the increase found in ECCSA shows that the transport capacity of the wood has been improved. This means that the liquid uptake and ions diffusion might be easier. This is important for wood impregnation with liquor in the pulping process due to the increase in ECCSA would facilitate the uptake of liquor, which might have a positive impact in the impregnation time. Since the main way used by fungi in wood colonizing is longitudinal one (Luna *et al.* 2012), which not analyzed for ECCSA determination in the present study, the increase found in the radial ECCSA might indicate that fungi seems prefer radial direction to tangential one. This might respond to the nutritional requirements of the fungi needed for their establishment in wood and vigorous growth. In parenchymal tissues (radial cells), the availability of carbon sources and other nutrients is greater than in empty axial cells. Previously, Boddy and Rayner (1983) reported the importance of availability of organic nutrients and their distribution as a main factor affecting growth of fungi in wood. In this sense, readily accessible, assimilable substrates for fungal growth such as soluble sugars, lipids, peptides and other primary metabolites, which occur in relatively small amounts (< 10% by dry weight) are located almost exclusively within wood parenchyma (Boddy and Rayner, 1983).

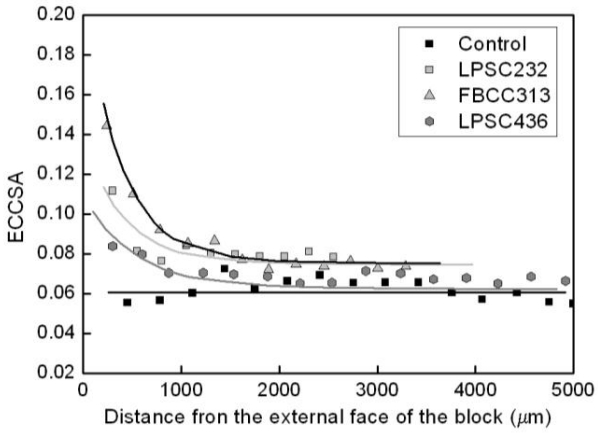

Fig.7: ECCSA in radial direction for different treatments.

4. Conclusions

Treatment of *Eucalyptus grandis* wood with ligninolytic fungi revealed a reduction in the content of lignin and extractives together with a small mass loss of the substrate (less than 3%) according to the development of sustainable technological processes.

A novel assessment for the analysis of the effect of fungal treatment is here applied. This study shows that the transport properties in radial direction of *Eucalyptus grandis* wood are favorably increased by biological treatment (*G. subvermispora*). However, no changes were found in tangential direction, which can be explained by the natural preference of fungus to initially grow in parenchymal tissues as radial cells. The increase in capillarity can be related to a shortening of the time needed to achieve a particular level of impregnation during preservation treatment, pulping or fractionation process. In this sense the fungal action on wood have a potential positive impact in delignification and subsequent impregnation steps of industrial processes.

Biological treatment of wood of *Eucalyptus grandis* using *G. subvermispora* might result promising as a biotechnological tool in environmentally-sound and alternative industrial processes to improve the properties of the starting woody material such as ones related to the pulp and timber industries.

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