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EFFECT OF ALBINO OPHIOSTOMA STRAINS ON *EUCALYPTUS NITENS* EXTRACTIVES

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ABSTRACT

Wood extractives promote pitch formation during pulp and paper manufacturing. To date, this problem has been controlled by extended storage of the chips and/or chemical additives. Biotreatment of the wood prior to pulping provides an alternative that not only decreases the negative impact of the extractives but may also improve the kraft pulping efficiency. This initiative seeks to verify the quantity and chemical composition of Eucalyptus nitens wood extractives following biotreatment with three albino fungi species *(Ophiostoma floccosum, Ophiostoma piceae* and *Ophiostoma piliferum)*. *Eucalyptus nitens* wood chips were sprayed with spore suspensions of *Ophiostoma piliferum, Ophiostoma piceae* and *Ophiostoma floccosum* albino strains (1×10^8 spore concentration). After 7 and 21 days of fungal treatment, the extractive content was determined via Soxhlet extraction with an 80:20% n-hexane:ethyl acetate solvent mixture. The *Ophiostoma floccosum* F1A94, *Ophiostoma piliferum* F2D8 and *Ophiostoma piceae* F2A68 strains proved to be most capable of bioreduction with reductions of 35.1%, 33.2% and 29.3%, respectively. The chemical composition of the extract was analyzed via gas chromatography coupled with mass spectrometry, which demonstrated that most of the tested strains could reduce the β-sitosterol content.

Keywords: Albino fungi, *Eucalyptus nitens,* extractives, pitch, *Ophiostoma*, sitosterol.

INTRODUCTION

Wood extractives are organic compounds soluble in organic solvents and include polyphenols, terpenes, fats, waxes, complex polysaccharides and nitrogenized compounds, of which a fraction are saponified (fatty acids and sterol esters) and the rest (hydrocarbons, sterols, diverse alcohols and aldehydes) are unsaponifiable (Browning 1975, Rowe and Conner 1979, Fengel and Wegener 1984). The lipophilic extractives, which consist of fats, fatty acids, steryl esters, sterols, terpenoids and waxes (Fengel and Wegener 1989, Martínez-Íñigo *et al*. 2000, Gutiérrez *et al.* 2006), significantly contribute to pitch formation (Fengel and Wegener 1989, Burnes *et al.* 2000, Mouyal 2005, Sitholé *et al.* 2010). Pitch deposits result in low-quality pulp products, cause pulping equipment to wear out prematurely, can block and stop pulping operations, require costly chemical additives and can lead to economic losses (Fischer *et al.* 1996, Gutiérrez *et al.* 1999a, Soto 2001, Gutiérrez *et al.* 2001, Maltha *et al.* 2011, Pepijin *et al.* 2012). Pitch can be controlled both by the storage time of the pulpwood materials and by the addition of chemicals to the pulp suspension (Burnes *et al.* 2000, Soto 2001). Albino fungi strains from the Ophiostoma genus have been proposed as a biological treatment for wood deresination (Blanchette *et al.* 1992, Farrell *et al.* 2000, Martínez *et al.* 1995, Gutiérrez *et al.* 2001, del Río *et al.* 2001, Calero *et al.* 2004, Herrera *et al.* 2008).

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Blanchette *et al.* (1992) and Fisher *et al.* (1994) inoculated *Pinus taeda* and *Picea abies* wood chips with the colorless *piliferum* (Cartapip 58) strain and found a 30% decrease in the total resin content after two weeks of biological treatment. Similarly, Chen *et al.* (1994) examined the effect of Cartapip on *Pinus contorta* and poplar wood chips and found a 55% and 70–80% decrease, respectively, in their triglycerides after 2 weeks of fungal treatment. In another study, Burgos (2006) found that a mixed *O. piliferum, O. piceae* and *O. floccosum* spore suspension reduced the total extractive quantity of radiata pine woodchips by 40%. Ophiostoma albino fungi have also been used to biologically treat *Eucalyptus* species. Gutiérrez *et al.* (1999b) found that *O. valdivianum* and *O. piliferum* strains could decrease the total extractives content by 70% after 40 days of fungal treatment. In another study, Calero *et al.* (2004) found that biotreatment with *O. piceae* could reduce sterol esters by 70%.

Because biological treatments can significantly reduce the quantity of wood extractives, we conclude native Chilean albino *Ophiostoma* strains can be developed and their efficacy verified for reducing the extractive content in *E. nitens* wood, which is an increasingly important wood species for Chilean forestry.

Thus, the objectives of this study were a) to evaluate the effect of biological treatment on the extractive content of *E. nitens* wood, particularly its lipophilic fraction, using 30 albino strains of the *Ophiostoma* genus after 7 and 21 days of fungal treatment and b) to determine the effect of the three best albino strains, *O. piliferum, O. piceae* and *O. floccosum*, on the chemical composition of the *E. nitens* extractive lipid component over the same treatment period.

MATERIALS AND METHODS

Ten twelve-year-old *Eucalyptus nitens* (Deane and Maiden) Maiden trees were felled and three 2.4-m logs were cut from each tree. The logs were cut to 30, 50 and 80% of their commercial height, which was defined as the total stem length measured from the bottom to the point at which the diameter was 12 cm.

The 30 sampled logs were shipped to industrial facilities in CMPC Planta Santa Fe, Nacimiento (Chile). Logs from each tree were debarked and chipped, and the chips were classified and bagged at the industrial facility. The bags were identified and shipped to the Laboratorio de Biodeterioro at the Universidad del Bío-Bío. Chips from each tree were mixed in a 0.3-m^3 drum mixer for 3 minutes, and a representative sample was obtained based on the total weight. The sample chips were mixed again for 3 minutes, and 10% of the chips by weight were removed, bagged in sacks, placed in boxes and transported to the Compañía Chilena de Esterilización S.A., a facility located near Santiago, for sterilization using a 15-kGy dose of gamma irradiation.

Inoculum preparation and chip biotreatment

Isolated albino strains of *Ophiostoma floccosum, O. piceae* and *O. piliferum* were used for this study, and 10 isolates of each species were obtained from the Laboratorio de Biodeterioro culture collection at the Universidad del Bío-Bío (Table 1). Each albino isolate was cultured and aseptically transferred to several Petri dishes containing malt extract agar (MEA, 1.5% Difco malt extract and 2.0% agar) amended with antibiotics (0,025% streptomycin and 0,025% chloramphenicol). The petri dishes were rinsed with sterile water after two weeks of culturing, and the obtained spore suspensions were poured into 250-ml flasks containing 50 ml of liquid media (15 g of malt extract per 1000 ml of distilled water) (Held *et al.* 2003). The inoculum spore concentration, measured using a Neubauer hemocytometer, was approximately 1×10^5 spores/ml. The cultures were centrifuged at 3,600 rpm for 10 minutes after growing in a shaker at room temperature (20–25°C) for 5 days. The supernatant was removed and a treatment solution was prepared for each isolate by resuspending the concentrate in distilled water to a final concentration of 1×10^8 spore/ml. One-hundred twenty bags containing 2,7 kg *E. nitens* chips, 60 bags per treatment period and four per isolate, were inoculated using 10 ml of the prepared spore suspension. The control bags, eight per treatment period, were sprayed with 10 ml of sterile water.

Table 1. Strains selected for the *Eucalyptus nitens* chip biotreatment.

Nº	*O. piceae* strain code	Nº	*O. floccosum* strain code	Nº	*O. piliferum* strain code
1	PcF2A68	11	FlF1A94	21	PlF2D8
2	PcF1A23	12	FlF1A55	22	PlF1A7
3	PcF1A32	13	FlF1A9	23	PlF2C60
4	PcF2A14	14	FlF1A65	24	PlF1A1
5	PcF2A70	15	FlF1A2	25	PlF2B82
6	PcF1A39	16	FlF1A7	26	PlF2C57
7	PcF2A9	17	FlF1A8	27	PlF2D6
8	PcF2A3	18	FlF1A5	28	PlF1A4
9	PcF2A35	19	FlF1A18	29	PlF2D88
10	PcF2A29	20	FlF1A11	30	PlF2B68

Determining the reduction in wood extractive

After the desired fungal treatment time, the wood chips were placed on racks and dried in a 6-m^3 gas kiln. The dry and wet bulb temperatures were 30 and 20°C, respectively. The air velocity was adjusted to obtain a final moisture content of 12% after 24 hours. The dried wood chips from each treatment were ground in a Retsch SM 2000 mill according to the ASTM D1105-96 standard. The acceptable fractions, those between the 40 and 60 mesh sieves, were weighed, and 100 g was poured through paper filters.

The *E. nitens* wood samples were Soxhlet extracted for 4 hours using an n-hexane:ethyl acetate (80:20 (v/v)) solvent mixture. The extract was weighed using gravimetric analysis after evaporating the solvent mixture to dryness in a rotary evaporator.

Determining the percent extractives, bioreduction factor and extractive chemical composition

The extract was dried in a Buchi rotatory evaporator and weighed. A two-sample *t*-test was applied to each control and fungus-treated chip using a significance level of 5% (Table 2). The extractive chemical compositions were analyzed via gas chromatography coupled with mass spectrometry (GC-MS) in the Laboratorio de Productos Naturales of the Facultad de Ciencias Naturales at the Universidad de Concepción.

The extract samples were seeded in a silica gel stationary phase using a mixture of n-hexane: ethyl acetate (70:30) v/v as the mobile phase. Sulfuric acid was sprayed at 30%, and the silica gel plate was heated to observe the analytes.

Table 2. Extractive content of *E. nitens* chips after 7 and 21 days of biotreatment.

Nº	Strain	Extractives Content 7 days (g)	Standard Deviation 7 days	Factor Bioreduction 7 days	Extractives Content 21 days (g)	Standard Deviation 21 days	Factor Bioreduction 21 days
1	PcF2A68	0,22	0,02	16,62*	0,21	0,01	29,27*
2	PcF1A23	0,28	0,03	-5,69	0,21	0,01	28,90*
3	PcF1A32	0,32	0,09	-21,41	0,21	0,03	28,15*
4	PcF2A14	0,21	0,02	22,34*	0,21	0,02	27,95*
5	PcF2A70	0,46	0,66	7,71	0,22	0,01	27,31*
6	PcF1A39	0,22	0,01	16,57*	0,23	0,01	23,72*
7	PcF2A9	0,26	0,02	1,83	0,23	0,03	22,31*
8	PcF2A3	0,27	0,04	-1,08	0,27	0,04	8,85
9	PcF2A35	0,31	0,04	-17,43	0,27	0,05	8,2
10	PcF2A29	0,22	0,01	17,78*	0,31	0,03	-5,2
11	FlF1A94	0,27	0,02	-2,23	0,19	0,02	35,08*
12	FlF1A55	0,24	0,03	11,45	0,20	0,03	33,49*
13	FlF1A9	0,26	0,07	2,51	0,21	0,01	30,21*
14	FlF1A65	0,22	0,02	18,99	0,21	0,02	29,57*
15	FlF1A2	0,33	0,04	-23,41	0,21	0,03	28,76*
16	FlF1A7	0,30	0,04	-11,42	0,22	0,01	25,55*
17	FlF1A8	0,21	0,02	19,86	0,23	0,02	21,98*
18	FlF1A5	0,28	0,04	-5,28	0,23	0,04	21,19*
19	FlF1A18	0,36	0,04	-34,24	0,32	0,09	-7,26
20	FlF1A11	0,23	0,07	13,1	0,44	0,58	-47,03
21	PlF2D8	0,28	0,03	-5,06	0,20	0,02	33,23*
22	PlF1A7	0,21	0,02	19,84	0,20	0,01	30,99*
23	PlF2C60	0,25	0,01	7,71	0,21	0,02	29,47*
24	PlF1A1	0,39	0,02	-46,51	0,21	0,00	28,08*
25	PlF2B82	0,37	0,30	-39,49	0,21	0,02	27,85*
26	PlF2C57	0,23	0,01	13,19	0,23	0,01	23,87*
27	PlF2D6	0,29	0,13	-7,82	0,23	0,03	21,22*
28	PlF1A4	0,21	0,02	21,67*	0,24	0,05	17,88
29	PlF2D88	0,21	0,01	21,65*	0,24	0,02	17,34*
30	PlF2B68	0,23	0,01	13,33	0,43	0,58	-43,78
31	Control	0,27	0,04	-	0,30	0,05	-

* significant difference with respect to the control for 7 and 21 days.

Sample preparation for gas and gas-mass chromatography

Several 50-ml glass balls were marked and weighed before adding 1000 µl of the extract. The extract was then dried in a rotatory evaporator, left to cool and weighed again to determine the dry extract weight. The initial volume of the extract samples reconstituted with ethyl acetate was calculated according to equation (1):

$$C_i \times V_i = C_f \times V_f \quad (1)$$

in which
C_i = initial concentration in mg
V_i = initial volume in ml
C_f = final concentration = 50 mg
V_f = final volume = 1 ml

The initial volume calculated using equation (1) was added to the dry sample. The reconstituted samples, were stored in a 1,5-ml vial for chromatographic analysis and left uncovered in the chamber until completely dry.

Sample methylation and chromatographic analyses

The dry extract samples were treated with 50 µl of diazomethane. The methylated samples were placed in a double boiler at 50°C for 15 minutes and left uncovered for 5 minutes under an extractor to evaporate the solvent. The sample was reconstituted to a final concentration of 50 mg/ml using 1000 µl of ethyl acetate. The methylated samples were analyzed in a gas chromatograph using a flame ionization detector (FID) (Agilent technologies 6890N; maximum temperature 325°C, 10,09 psi, nitrogen mobile phase, 30 m × 320 µm × 0,25

µm column). From the GC analyzed samples, two representatives of the *O. floccosum* strain, three of the *O. piceae,* three of the *O. piliferum* and the control samples were selected for culturing for 7 and 21 days. In all cases, 50-mg samples were analyzed in a gas-mass chromatograph (Hewlett Packard 5890 Series II, HP5-MS column) with a mass detector (model 5972 series, 50–550 amu sweep range, 70-eV electro-impact ionization, 280°C temperature).

RESULTS AND DISCUSSION

Table 2 provides the reduction in extractives for the *E. nitens* wood chips biotreated with albino fungi (genus *Ophiostoma.*) for 7 and 21 days then extracted using an n-hexane:ethyl acetate solvent mixture. After 7 days of fungal growth, nine of the 30 strains caused a statistically significant reduction in the extractives relative to the untreated wood chips. Increasing the fungal treatment to 21 days yielded 23 out of 30 biological strains adequate for treatment and increased the extractive reduction from 30% to 70%. The number of *O. piceae* strains that significantly reduced the extractives relative to the control increased from four to seven when the biotreatment was extended from 7 to 21 days. The maximum extractive reduction for both treatments was achieved with PcF1(WxPc6A)32, while the PcF2A29 strain caused the least reduction relative to the control. For the *O. floccosum* species, the number of strains capable of extractive bioreduction increased from 2 to 8 from 7 to 21 days. The strain with the maximum extractive reduction was FlF1(1ªxA)2 for both biotreatment durations.

For the 7-day biotreatment, *O. piceae* yielded the most significant difference relative to the control, which indicates this fungus species colonized and consumed the *E. nitens* extractives faster than the other species; however, *O. piceae* did not perform best in the 21-day biotreatment because both the *O. floccosum*- and *O. piliferum-treated* samples differed more significantly from the controls. The performance of these three species did not allow us to draw conclusions regarding their bioreduction effectiveness.

The biotreated *E. nitens* extracts analyzed via GC exhibited different degradation patterns for the pitch-forming compounds. The 7-day chromatograms demonstrated no correlation between the strains with the highest extractive reduction and the lowest peak retention times. However, some strains with high extractive reductions yielded chromatograms with many retention peaks that could result from secondary metabolites. Neither of the 21-day chromatograms demonstrated a clear trend. Some strains greatly reduced the extractive concentration and presented numerous peaks similar to those of the control chromatogram, while others had increased final extractive contents and chromatograms with few peaks that differed significantly from the control chromatogram.

Extracts for the selected strains were analyzed via GC-MS (Figure 1). An analysis of the biotreatment time revealed the sharpest decline in lipophilic compounds after 7 days of biotreatment with most strains yielding an extractive reduction and most compounds, such as free sterols and fatty acids, decreasing with respect to the control. However, the free sterol content of a few fungi increased, and the breakdown resulted in variable efficiencies. The chromatographic profiles of the lipophilic extract after the 7-day biotreatment differed significantly from those of the control because most of the strains showed fewer peaks than the control.

The 21-day biotreated samples contained more free sterols and fatty acids than the control. The chromatographic profiles of the lipophilic extracts after 21 days demonstrated a limited decrease in the identified sitosterol peaks but a strong reduction in the remaining compounds relative to the control.

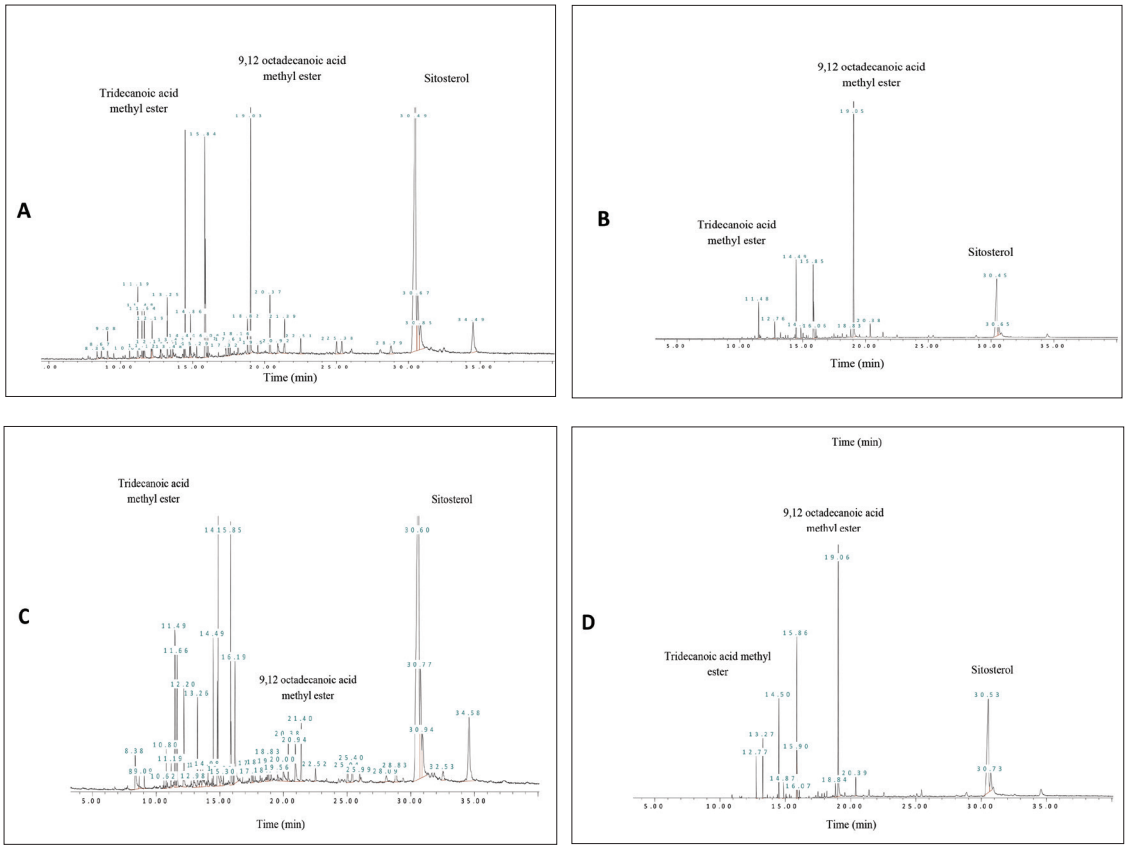

Figure 1. GC/MS chromatogram of A) *E. nitens* wood chip extractives without biotreatment after 7 days of exposure. (B) *E. nitens* extractives of chips biotreated with the *O. piceae* PcF2A3 strain after 7 days of exposure (C) *E. nitens* wood chip extractives without biotreatment after 21 days of exposure. (D) *E. nitens* extractives of chips biotreated with the *O. piceae* PcF2A3 strain after 21 days of exposure.

The extractive contents were found to depend on the biotreatment. Some fungi decreased the free sterols while increasing the secondary metabolite content. A similar pattern was observed in the biotreated radiata pinewood (Burgos 2006). The pinewood treated with *O. piliferum* (Clariant Cartapip strain) exhibited reduced pitch during mechanical wood pulp processing. However, this strain was less efficient on eucalyptus because *O. piliferum* breaks down free sterols (Calero *et al.* 1999).

Tables 3 and 4 list all identified compounds. The lipid compounds detected in large quantities in *E. nitens* were sitosterol, tridecanoic acid methyl ester, hexadecanoic acid methyl ester, tetracosanoic acid methyl ester, heneicosanoic acid methyl ester, and 9-12-octadecadienoic acid methyl ester. Sitosterol and stigmastenol were the primary compounds in the extract.

Table 3. Wood extractives composition determined via GC/MS as an area percentage for 7-day biotreated and control *E. nitens* wood chip extracts.

Time	Compound	Control %	FlF1A55 %	FlF1A2 %	PcF2A70 %	PcF2A3 %	PcF1A23 %	PlF2C60 %
11,19	2,6-dimethoxybenzoquinone	1,809						
12,19	4-hydroxy-3,5-dimethoxybenzaldehyde	1,124						
13,25	4-hydroxy-3,5dimethoxybenzoic acid	1,301					1,434	
14,48	Tridecanoic acid methyl ester	5,403	6,343	17,336	5,176	6,848	6,411	2,516
14,86	Dibutyl phthalate	1,057					2,494	
15,84	9,12 octadecadienoic acid methyl ester	7,654	7,83	14,067	6,683	10,906	7,684	4,194
19,03	Benzene-1,2-dicarboxylic acid	6,242				41,783		
20,37	Tetracosanoic acid methyl ester	1,985					1,952	
30,49	Sitosterol	44,497	49,06	31,659	32,629	27,96	31,571	43,958
30,85	Fucosterol	3,09			3,74			
34,49	Stigmast-4-en-3-one	3,826					2,845	4,593
	Unidentified	22,012	26,477	2,097	18,518	6,436	22,405	25,093

Table 4. Wood extractive composition determined via GC/MS as an area percentage for 21-day biotreated and control *E. nitens* wood chip extracts.

Time	Compound	Control %	FlF1A55 %	FlF1A2 %	PcF2A70 %	PcF2A3 %	PcF1A23 %	PlF2C60 %
9,10	3-hydroxy-4-methoxybenzaldehyde	0,234						
11,49	Diethyl phthalate	2,207					3,908	
12,20	4-hydroxy-3,5-dimethoxybenzaldehyde	2,023						
13,26	4-hydroxy-3,5-dimethoxybenzoic acid	1,446	4,714	2,916	3,368	3,882	1,941	1,509
14,49	Tridecanoic acid methyl ester	1,858	2,781		7,75	5,761	5,249	3,12
14,87	Dibutyl phthalate	6,246						
15,85	9,12 octadecadienoic acid methyl ester	4,652	6,282	4,429	11,479	10,171	10,279	6,704
16,19	9,12-octadecadienoic acid (Z,Z)	4,372						
30,60	Sitosterol	40,993	49,869	46,1	51,513	35,17	44,995	46,15
30,77	Stigmastan-3-ol (3 beta, 5 alpha)	6,512	9,081	9,174				7,15
34,58	Stigmast-4-en 3-one	5,862	3,826	5,004	4,28		4,093	4,26
	Unidentified	23,64	15,658	28,243	19,162	11,787	27,381	25,743

According to Gutiérrez *et al.* (2001), several *Ascomicetes* fungi included in Cartapip were inefficient at controlling *Eucalyptus* sp. pulp pitch and decreased the sterol ester content without affecting the free sterol quantity (sitosterols). Martínez *et al.* (1995) and Dorado *et al.* (2001) demonstrated that certain fungi of the genus *Ophiostoma* efficiently broke down all lipophilic components responsible for pitch deposition in *Eucalyptus* wood, whereas other species, including *O. piliferum,* decreased the sterol ester content while increasing the free sitosterol content. The reduction in sterol ester levels was related to the activity of the esterase enzyme produced by these fungi. These findings coincide with this study because the major lipids present were sitosterols.

Furthermore, Calero (2004) found that *O. piceae* sterol esterase hydrolyzed sterol esters, but increased the free sterol content, which agreed with this study in which the free sterol content of the biotreated samples increased relative to the control. This enzyme was found in several fungi studied for biocontrol over pitch (Leone and Breuil 1999, Calero *et al.* 1999). No sterol esters or triglycerides were detected in this study. A complete identification of the sterol esters via GC-MS was impossible (Lusby *et al.* 1984, Evershed *et al.* 1989). In recent years, attempts to characterize *Eucalyptus* wood extractives have indicated that triglycerides form a small fraction of *E. globulus* wood extractives, which primarily contain the sterol and sterol ester compounds responsible for kraft pulp pitch deposits (del Río *et al.* 1998, Gutiérrez *et al.* 1999a).

According to Gutiérrez *et al.* (2001), basidiomycetes provide an alternative for reducing lipid content that is resistant to chlorine dioxide whitening and responsible for the pitch that decreased the sitosterols and sitosterol esters 60 and 70%, respectively. The author suggested combining both methods to remove the extractives and lignin from the white-rot fungi-treated wood biopulp. Moreover, the fungi pretreatment significantly decreased the effluent toxicity due to the biological removal of certain extractives.

CONCLUSIONS

The strains with the best bioreduction were PcF2A14, FlF1A8 and PlF1A4 after 7 days of biotreatment and PcF2A68, Fl F1 A94 and Pl F2D8 after 21 days of biotreatment. The lipophilic extract fraction in the gas chromatographic analysis revealed that the best strains were PcF2A3 and FlF1A2 for the 7-day biotreatment and PlF1A4 for the 21-day biotreatment.

Pretreating *E. nitens* with 30 strains of fungi from the genus *Ophiostoma* for 7 and 21 days reduced the free sterol and fatty acid content of the lipophilic extracts. The primary lipophilic compounds found in the *E. nitens* extracts were free sterols, fatty acids and ketone steroids.

The best strains for industrial pretreatment to control pitch should be selected based on the optimum elimination of lipophilic extractives and the quality of the paper produced.

These results demonstrate, on the laboratory scale, a promising biological treatment for reducing and/or controlling pitch during the production of pulp and paper from *E. nitens*.

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