Fungal screening for biological removal of extractives from *Eucalyptus globulus* wood

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Abstract: Wood extractives cause production troubles during pulp and paper manufacture. The potentiality of fungal strains for biotechnological removal of extractives from *Eucalyptus globulus* Labill. wood is evaluated here. First, a survey of fungi present in eucalypt woodlands in western Spain was carried out, and 90 species, including fungi strictly associated with eucalypt trees, were collected. Then, a total of 33 basidiomycetes, 21 ascomycetes, and 19 conidial fungi (including some strains from culture collections) were compared in terms of their capacity to decrease the acetone extract of eucalypt wood. High extractive removal (50–70% of initial content) was obtained with *Ophiostoma*, *Mollisia*, and *Pleurotus* species, as well as with *Funalia trogii* (Berk. in Trog) Bond. & Singer, *Melanotus hepatochrous* (Berk.) Singer, and *Paecilomyces* sp. Microscopic observation of the degraded wood revealed a correlation between extractive degradation and removal of spherical deposits in wood rays. Moreover, when extractive biodegradation was analyzed by gas chromatography – mass spectrometry it was found that some of the basidiomycetes were able to remove both free and esterified sitosterol (75–100% degradation by *Poria subvermispura* Pilát, *Phlebia radiata* Fr., *F. trogii*, and *Bjerkandera adusta* (Willd.) P. Karsten), which has been identified as a major constituent of pitch deposits in eucalypt pulps, whereas the action of ascomycetes was mainly limited to hydrolysis of the sitosterol esters.

Key words: fungi, *Eucalyptus globulus*, extractives, sitosterol, fungal diversity, paper manufacture.

Résumé : Les composés extractibles du bois semblent largement impliqués dans la formation de poix lors de la production de pâte et papier. L'efficacité de différentes souches de champignons pour éliminer les matières extractibles a été étudiée pour l'*Eucalyptus globulus* Labill. Une étude préliminaire dans les plantations d'eucalyptus de l'Ouest de l'Espagne a permis de récolter et d'identifier 90 espèces, dont quelques champignons strictement associés à l'eucalyptus. La biodégradation d'extraits à l'acétone a été évaluée pour des souches isolées par les auteurs, ou obtenus à partir de collections (33 basidiomycètes, 21 ascomycètes et 19 champignons imparfaits). Des effets significatifs ont été observés pour certaines espèces d'*Ophiostoma*, *Mollisia* et *Pleurotus*, et pour *Funalia trogii* (Berk. in Trog) Bond. & Singer, *Melanotus hepatochrous* (Berk.) Singer et *Paecilomyces* sp., avec une réduction pouvant atteindre 50–70 % de la teneur initiale en composés extractibles. Lors de l'observation microscopique du bois bio-dégradé, il a été constaté une élimination des nodules présents dans les rayons. Un suivi en chromatographie en phase gazeuse – spectrométrie de masses a montré que quelques basidiomycètes pouvaient éliminer le sitostérol libre et esterifié, principal constituant de la poix dans les pâtes d'eucalyptus. Si les *Poria subvermispura* Pilát, *Phlebia radiata* Fr., *F. trogii* et *Bjerkandera adusta* (Willd.) P. Karsten sont capables de dégrader 75–100% de la teneur initiale de ces composés, l'action des ascomycètes se limite à l'hydrolyse des esters du sitostérol.

Mots clés : champignons, *Eucalyptus globulus*, composés extractibles, sitostérol, diversité fongique, industrie papetière.

Introduction

Wood extractives include a large number of compounds, such as resin acids, fatty acids, glycerides, steroids, and polyphenols, that are soluble in organic solvents. Extractives cause different production problems during paper pulp manufacture related to pulp discoloration and brightness reversion, wastewater toxicity, and pitch deposition (28). These problems are important during alkaline pulping (kraft pro-

cess) of eucalypt wood when Cl_2 -free sequences are used to bleach the pulp. Although numerous studies exist on extractive composition in several softwoods and hardwoods (18, 27, 35), only limited information was available on its composition in eucalypt wood (8, 37), which is extensively used for paper pulp manufacture in Spain, Brazil, and other countries. Recently, extractives from *Eucalyptus globulus* Labill. have been characterized by different analytical techniques, and the main compounds responsible for pitch deposition

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during ECF (elementary chlorine free) and TCF (totally chlorine free) kraft pulping of eucalypt wood were identified (9, 10, 24).

The use of organisms and their enzymes offers the possibility to introduce environmentally friendly procedures in the pulp and paper industry (13). Fungi are efficient degraders of wood constituents including not only polymer fractions (plant polysaccharides and lignin) (14) but also low molecular weight compounds in the extractive fraction (30). The biological removal of extractives is being investigated by treating wood with fungi or pulp with enzyme preparations (6, 17, 20). This includes industrial treatment of softwood with a white strain of *Ophiostoma piliferum* (commercialized by Clariant as Cartapip™) (17). However, no successful control of pitch during kraft pulping of eucalypt wood has been reported with this or other fungi.

Most of the studies on fungi growing on *Eucalyptus* species in Australia and other countries concern pathogenic fungi (22, 33). However, fungal diversity in eucalypt forests has been investigated also, including general mycoflora (34, 36), mycorrhizal associations with *Eucalyptus* species (7) and endophytic fungi (3, 21). The aim of this study is to identify the most suitable fungi to remove extractives from *E. globulus* wood to control problems caused by these compounds during kraft pulping of this type of wood. To identify the most promising strains, a preliminary survey of fungal diversity in eucalypt woodlands in Spain was conducted (including fungi from eucalypt piles in a paper mill). Then, a screening of a total of 68 fungal species for removal of extractives from *E. globulus* wood was carried out, and degradation of the main lipophilic constituents of the extractive fraction was analyzed by gas chromatography – mass spectrometry (GC–MS).

Materials and methods

Fungal species

Different fungi were collected and identified from *E. globulus* and *Eucalyptus camaldulensis* Dehn. woodlands in Spain (see Table 1). They were obtained from wood logs from the ENCE (Empresa Nacional de Celulosas) mill in Pontevedra (Spain), and from dead wood, bark, plant debris, and soil of plantations of *E. globulus* and *E. camaldulensis* in the western (Cáceres) and northwestern Spain (Pontevedra). Samples from fruiting bodies and decayed wood were plated on malt extract (2%) agar or yeast extract (0.5%) – malt extract (2%) agar containing streptomycin (50 mg/L) and oxytetracycline hydrochloride (50 mg/L), and incubated for 15–20 days at 20°C under natural daylight (“direct plating”). Other isolates were obtained by “surface sterilization.” For this, wood blocks were washed, surface sterilized by immersion in 70% ethanol (1 min), 3% sodium hypochlorite (3 min), and 70% ethanol (1 min) and plated on the above media. Benomyl (3 mg/L) was added to the agar media for isolation of basidiomycetes.

Additional fungi were obtained from Clariant, *O. piliferum* white strains commercialized as Cartapip™ 58 and 97, and from the Centraalbureau voor Schimmelcultures (CBS; Baarn, the Netherlands), the Institute Jaime Ferran de Microbiologie (IJFM; Centro Investigaciones Biológicas (CIB), Madrid), and Merck Sharp-Dome (MSD) culture collections. These included strains isolated from eucalypt such as *Bjerkandera adusta* (Willd.) P. Karsten CBS 230.93, *Chaetospermum chaetosporum* (Pat.) A.L. Smith & Ramsbottom CBS 306.75, *Exophiala jeanselmei* (Langeron) McGinnis & Padhye CBS 642.82, *Monocillium tenue* W. Gams

CBS 743.83, *Mycosphaerella parkii* Crous, Wingfield, Ferreira & Alfenas CBS 516.93, *Penicillium megalosporum* Orpurt & Fennel CBS 324.83, *Pestalotiopsis crassiuscula* Steyaert CBS 264.37, and *Phialophora mustea* Neergaard CBS 643.82. Other fungal strains were isolated from other woody and herbaceous plants such as *Ceratocystis allantospora* Griffin CBS 185.86, *Ceratocystis crassivaginata* Griffin CBS 402.77, *Ceratocystis deltoideospora* Olchow. & Reid CBS 187.86, *Ceratocystis tenella* Davidson CBS 190.86, *Dichomitus squalens* (P. Karsten) Reid MSD SM3-92, *Fumalia trogii* (Berk. in Trog) Bond. & Singer IJFM A615, *Ganoderma australe* (Fr.) Pat. IJFM A130, *Gietrichium candidum* Link ex Pers. CBS 820.71, *Ophiostoma ainoae* Solheim CBS 205.83, *Ophiostoma huntii* (Robinson-Jeffrey) de Hoog & Scheffer CBS 399.77, *Ophiostoma megalobrunneum* (Davidson & Toole) de Hoog & Scheffer CBS 560.83, *Ophiostoma microsporum* (Davidson) von Arx CBS 412.77, *Ophiostoma olivaceum* Mathiesen CBS 502.86, *Ophiostoma piceae* (Munch) H. & P. Sydow IJFM A237, *Ophiostoma piliferum* CBS 158.74, *Ophiostoma rostrocoronatum* (Davidson & Erlyn) de Hoog & Scheffer CBS 434.77, *Ophiostoma valdivianum* (Butin) Rulamort CBS 454.83, *Phanerochaete chrysosporium* Burds. VKM-F1767 (= CBS 184.83), *Phellinus torulosus* MSD SM3-92, *Phlebia chrysocrea* (Berk. & Curt. apud Berk.) Burds. apud Lombard IJFM A469, *Phlebia radiata* Fr. CBS 184.83, *Pleurotus eryngii* (DC.) Quél. IJFM A169 (= CBS 613.91), *Pleurotus ostreatus* (Jacq.) Quél. CBS411.71, *Pleurotus pulmonarius* (Fr.) Quél CBS 507.85 and *Poria* (synonym: *Ceriporiopsis*) *subvernisporea* Pilát CBS 347.63. Finally, several *Fusarium* strains with high lipase activity were also assayed including *Fusarium decemcellulare* Brick IJFM A686 and several *F. oxysporum* strains (IJFM A682, IJFM A685, and IJFM A686).

Screening for fungal degradation of the extractive fraction

The 73 fungal strains assayed for extractive removal from eucalypt wood (see Figs. 1–3) were selected from those isolated from eucalypt wood in Spain (according to growth and culture characteristics) or obtained from fungal culture collections as described above. Wood treatment was carried out in triplicate, using 100-mL flasks with 2 g of small chips (1–2 × 10–20 mm) of *E. globulus* wood (industrial chips including heartwood and sapwood from 14-year-old trees, provided by ENCE, Pontevedra Spain) and 6 mL of water, which were sterilized (at 120°C for 20 min), inoculated with two plugs of mycelium from cultures on malt extract agar, and incubated at 26°C. Non-inoculated sterilized wood was incubated under the same conditions as a control. After 40 days, the wood was dried at 60°C in an aerated oven for 12 h, weighed, and Soxhlet extracted with acetone for 6 h; then, the extracts were dried for weight estimation and GC–MS analysis. The Klason lignin content was determined according to Tappi 222 om-88 (38).

Microscopy

Samples of sound wood and wood treated with fungi were processed for light microscopy. They were fixed with 3% glutaraldehyde, dehydrated in an ethanol–butanol–water series and embedded in paraffin. Thin sections (12 µm) were stained with 1% safranin (in 96% ethanol) and 0.5% Astra blue (in 2% tartaric acid) (1). The control wood was stained also with butanol-HCl for leucoanthocyanin and ellagitannins localization (27). Microscopy observations and pictures were made with an Olympus BX50 using bright field.

Chromatographic analysis of extractives

Gas chromatography (GC) analyses of chloroform-soluble compounds (extractive lipophilic fraction) were performed on a Hewlett Packard HP 5890 gas chromatograph equipped with a flame ionization detector (FID), using a high temperature, poly-

Table 1. List of fungi identified in Spanish eucalypt woodlands.**Ascomycota**

Arachnopeziza aurelia (Pers.) Fuckel
Coniochaeta tetraspora Cain
Nectria coccinea (Pers.) Fr.
Mollisia discolor (Mont.) Phill.*
Mollisia melaleuca (Fr.) Sacc.*
Mollisia sp.*
Orbilbia coccinea (Sommerf.) Fr.

Basidiomycota

Bjerkandera adusta (Willd.) P.Karsten*
Byssomerulius corium (Pers.) Parm.*
Cerocorticium notabile (H. Jackson) Jül. & Stalp.
Clitocybe ericetorum Bull.
Clitocybe paropsis (Fr.) Sacc.
Collybia dryophila (Bull.) Kumm.
Coniophora arida var. *suffocata* (Peck) Ginns
Coniophora puteana (Sch.) P.Karsten*
Coprinus plicatilis (Curt.) Fr.
Crepidotus epibryus (Fr.) Quéf.
Crepidotus variabilis (Pers.) Gray*
Crucibulum laeve (Huds. ex Relh.) Kambly
Cyathus olla (Batsch) Pers.*
Dacrymyces capitata Schw.*
Daedalea quercina L.
Descolea rheophylla (Bertault & Malençon) Malençon
Exidia glandulosa (Bull.) Fr.
Exidia truncata Fr.
Flammulaster carpophiloides (Kunner) Watling
Galzinia incrustans (Hönel & Litsch.) Parm.
Hyphoderma eucalypti Dueñas & Tellería
Hyphoderma medioburiense (Burt) Donk*
Hyphoderma praetermissum (P. Karsten) J. Eriksson & Strid
Hyphodontia aspera (Fr.) J. Eriksson
Hypholoma fasciculare (Huds.) Kumm.
Hypholoma subericaeum (Fr.) Kühn.
Hypochnicium caucasicum Parm.*
Laccaria bicolor (Maire) P.D. Orton
Laccaria fraterna (Cooke & Mass.) Pegl.
Lopharia spadicea (Fr.) Boidin
Macrolepiota procera (Scop.) Singer
Marasmius anomalus Lasch in Rabh.
Melanotus hepatochrous (Berk.) Singer*
Mycena acicula (Sch.) Kumm.
Mycena aetites (Fr.) Quéf.
Mycena alba (Bres.) Kühn.
Mycena flavoalba (Fr.) Quéf.
Mycena leucogala Cooke
Mycena meliigena (Berk. et Cooke) Sacc.
Mycena sp.
Omphalotus olearius (DC.) Singer*
Panellus stypticus (Bull.) P. Karsten
Peniophora cinerea (Pers.) Cooke*
Peniophora incarnata (Pers.) P. Karsten*
Peniophora limitata (Chaill.) Cooke*
Peniophora meridionalis Boidin*
Peniophora nuda (Fr.) Bresad.*
Phanerochaete avellanea (Bresad.) J. Eriksson*
Phanerochaete sordida (P. Karsten) J. Eriksson & Ryv.

Table 1. (concluded).**Basidiomycota**

Phanerochaete velutina (DC.) P. Karsten
Phellinus torulosus (Pers.) H. Bourdot & Galzin
Phellinus sp.
Pholiota highlandensis (Peck.) Smith & Hesl.
Pluteus cervinus (Sch.) Kumm.
Polyporus arcularius Batsch.*
Pseudoclyocybe cyathiformis (Bull.) Singer
Psilocybe argentina (Speg.) Singer
Pulcherricium caeruleum (Schrader) Parm.*
Ripartites tricholoma (A. & S.) P. Karsten
Schizophora paradoxa (Schrader) Donk
Schizophyllum commune Fr.*
Stecherrium ochraceum (Pers.) Gray
Stereum hirsutum (Willd.) Gray*
Trechispora vaga (Fr.) Libertá
Trametes versicolor (L.) Pilát*
Tremella foliacea Pers.
Tremella mesenterica Retz.*
Trichaptum bifforme (Fr. in Kl.) Ryv.
Tubaria conspersa (Pers.) Fayod
Tubaria furfuracea (Pers.) Gill.
Tubaria hiemalis Romagn. ex Bon
Tubaria romagnesianae Arnolds
Tubulicrinis calotrix (Pat.) Donk

Conidial fungi

Alternaria alternata (Fr.) Keissler
Alternaria sp.*
Arthrinium phaeospermum (Corda) M.B. Ellis*
Colletotrichum sp.*
Epicoccum nigrum Link*
Fusarium oxysporum Schldt.*
Kirramyces eucalypti (Cooke & Massee) J. Walker, B. Sutton, & I. Pascoe*
Paecilomyces sp.*
Periconia igniaria Mason & M.B. Ellis*
Phoma sp.

*Species included in subsequent screening for wood extractive degradation (Figs. 1–3).

imide coated fused silica 5 m × 0.25 mm DB5-HT (0.1 µm film thickness, J&W Scientific) capillary column. The injector and the detector temperatures were set at 300 and 350°C respectively, and the oven was programmed from 100 (1 min) to 350°C (3 min) at 15°C/min. Samples were injected in the splitless mode and helium was used as the carrier gas. As previously described (24), a mixture of sitosterol and cholesteryl oleate (Sigma), with a concentration range between 0.1 and 1.0 µg/mL, was used to obtain a calibration curve for quantitation of free and esterified sterols (the correlation coefficients being higher than 0.99). The different compounds were quantified by peak area using the corresponding calibration curves.

The GC–MS analyses to identify lipophilic compounds were performed on a Varian Star 3400 gas chromatograph with an ion trap detector (ITD, Varian Saturn 2000) using a 15 m × 0.25 mm DB-5HT (0.1 µm film thickness, J&W Scientific) capillary column. Helium was used as the carrier gas, and samples were injected with an autoinjector (Varian 8200) directly onto the column using a programmable injector. The temperature of the injector during the injection was 120°C, and 0.1 min after the injection was programmed to 380°C (200°C/min) and maintained at this temper-

Fig. 1. Extractive removal (% initial content) and weight loss (%) after eucalypt wood treatment with 21 ascomycetes (in all cases the standard deviations for extractive removal and weight loss were less than 13 and 16% of the mean values, respectively).

ature for 10 min. The oven was programmed from 120 (1 min) to 380°C (5 min) at 10°C/min, and the temperatures of the ITD and transfer line were set at 200 and 300°C, respectively. Compounds were identified by mass spectra comparison with those in the Wiley and Nist libraries, by mass fragmentography and the different standards.

Results and discussion

Survey of fungi in Spanish eucalypt woodlands

A large screening of fungi was carried out to evaluate their ability to degrade extractives from *E. globulus* wood, to optimize biotechnological control of production problems caused by these compounds. This was based on previous isolation of fungal species growing in eucalypt plantations, which provided a first approach to fungal diversity of eucalypt woodlands in Spain. A total of 90 fungal taxa were collected and identified from wood, bark, plant debris, and soil in plantations of *E. globulus* and *E. camaldulensis* and from logs of *E. globulus* in a pulp mill (Table 1). From a taxonomical and ecological point of view, most of the spe-

cies recorded in this study are not specific to eucalypt species, since they have been reported on different conifers and deciduous trees. However, at least five of the taxa identified have been described in close association with different *Eucalyptus* species as mycorrhizal or saprophytic fungi and should initially have an Australasian distribution, being introduced into Spain with the eucalypt trees. Among them, *Laccaria fraterna* is one of the most common ectomycorrhizal species of Australian eucalypts, and it has been spread throughout the world with eucalypt plantations (39) including Spain, where it has been cited as *Laccaria lateritia* (31). In contrast, the second ectomycorrhizal species, *Descolea rheophylla*, has been cited only in Europe, including Spain (16). Since it is strictly associated with eucalypts, this species probably originates from Australia where a related species (*Descolea maculata*) has been reported. The three saprophytic fungi were the basidiomycetes, *Melanotus hepatochrous* and *Hyphoderma eucalypti*, and the conidial fungus, *Kirramyces eucalypti*. The first species is widespread in Australia associated with eucalypts but also with some arboreal ferns and Podocarpaceae and has been cited in Spain

Fig. 2. Extractive removal (% initial content) and weight loss (%) after eucalypt wood treatment with 35 basidiomycetes (in all cases the standard deviations for extractive removal and weight loss were less than 16 and 14% of the mean values, respectively).

(15). In fact, *Melanotus hepatochrous* was one of the most frequent species found on eucalypt piles. *Hyphoderma eucalypti* was originally described in Spain (12), and it is still to be reported in Australia. Finally, *K. eucalypti* is a very common phytopathogenic species on *E. globulus* and related species (36). This fungus has been cited in Italy (36), but the present work includes the first report in Spain.

Fungal removal of extractives from eucalypt wood

Among all the fungi identified from *E. globulus* and *E. camaldulensis*, the 34 species marked in Table 1 were selected for evaluation of their ability to remove eucalypt ex-

tractives from wood taking into account their growth and propagation characteristics. To complete the screening work, additional strains isolated from eucalypt and other plants were obtained from culture collections (see Materials and methods). Thus, a total of 73 strains, corresponding to 68 fungal species, were tested for extractive removal from *E. globulus* wood under solid-state fermentation conditions. The differences in the extent of wood extractive removal and weight loss produced by the different strains are illustrated in Figs. 1–3. Some of the fungi strongly reduced the content of extractives in wood by up to 50–70% of the initial amount. We also found that several basidiomycetes (includ-

Fig. 3. Extractive removal (% initial content) and weight loss (%) after eucalypt wood treatment with 19 conidial fungi (in all cases the standard deviations for extractive removal and weight loss were less than 12 and 16% of the mean values, respectively).

ing *Coniophora puteana* and *Poria subvermispora*), ascomycetes (including *Nectria coccinea*), and conidial fungi produced an increase in the extractive fraction (over twofold extractive content with respect to control wood in the above species). In some cases this increase could be due to lipid biosynthesis (40), but in the case of basidiomycetes it is most probably due to the release of lignin-derived products, as discussed below.

The most important reduction of extractives in eucalypt wood was obtained with the basidiomycetes *Funalia trogii*, *Melanotus hepatochrous*, and several species of *Pleurotus*, the ascomycetes *Mollisia melaleuca* and several species of *Ophiostoma* and the conidial fungus *Paecilomyces* sp. The basidiomycetes generally caused higher wood degradation, which corresponds to their ability to extensively degrade lignified materials. However, the above species produced low weight losses below 5% (a prerequisite for wood pretreatment in paper pulp manufacture) excepting *Melanotus hepatochrous*. In addition to this species, which was excluded from subsequent studies because of its limited viability in culture, the basidiomycetes *Byssomerulius corium* and *Peniophora incarnata*, isolated from eucalypt wood, also

caused high weight losses (around 25%). In the case of ascomycetes and conidial fungi, their growth on wood is limited to the utilization of easily degradable components (such as water-soluble and extractive fractions) and did not result in high losses of weight. White strains of *O. piliferum* (Cartapip™) (17) can reduce the extractive content of pine chips (triglycerides, fatty acids, and resin acids) and control growth of undesired organisms improving chemical pulping efficiency (2, 6). Moreover, the ability of several basidiomycetes to remove extractives from *Pinus sylvestris* L. wood (29) and decrease extractive toxicity has also been reported (11).

Micromorphological aspects of eucalypt wood degradation

Fungal growth was not very apparent during wood treatment but some wood discoloration was caused by several fungi. This includes a brighter color than the control in wood treated with *Trametes versicolor* and *Bjerkandera adusta* and darker color in the case of *Melanotus hepatochrous* and *Periconia igniaria*. Moreover, some micromorphological aspects of wood could be related to changes in the total con-

Fig. 4. Micromorphological aspects of eucalypt wood treatment with fungi (B, C, E, and F) and the corresponding controls (A and D). Spherical pitch deposits in wood rays (A), which are partially degraded by *Ganoderma australe* (B) or totally removed by *Ophiostoma piliferum* (C). The presence of leucoanthocyanins and ellagitannins is evidenced by specific staining in A. Control eucalypt wood (D), selective degradation pattern caused by *Poria subvermispora* (E), and simultaneous degradation pattern caused by *Crepidotus variabilis* (F). Safranin staining of lignin is visible in D. Sections (which are radial in Figs. 4A and 4C, tangential in Fig. 4B, and transverse in Figs. 4D and 4F) were stained with butanol-HCl (A) or with safranin – Astra blue (B–F). Scale bar = 15 μ m.

tent of extractives, such as the presence of spherical deposits localized in rays. These deposits contain some leucoanthocyanins and ellagitannins (Fig. 4A) as revealed by specific bright red staining (26), but they are also visible in sections stained with safranin – Astra blue. The microscopy study of treated wood revealed that some of the fungi decreasing the extractive content of wood were able to remove deposits from rays, compared with the control wood or with wood treated with fungi that did not remove extractives. Partial

degradation of the spherical deposits was observed in some cases, as illustrated for the wood treated with *Ganoderma australe* (Fig. 4B). Moreover, a total removal of ray deposits was found in wood samples treated with different fungi including *O. piliferum*, *Paecilomyces* sp., *B. adusta*, and *Phanerochaete chrysosporium* (Fig. 4C).

It was possible to follow the fungal removal of lignin from wood tissues using safranin – Astra blue staining (1) and identify different micromorphological degradation pat-

Fig. 5. Chromatographic analysis of the different lipophilic compounds found in eucalypt wood prior to (A) and after fungal treatment with *Bjerkandera adusta* (B) and *Poria subvermispora* (C). The same sample volume for the chloroform soluble fraction obtained from the same amount of wood was injected in each case, and the relative chromatographic responses are shown.

terns, which are of interest for application of these fungi in paper pulp manufacture (5, 32) (Figs. 4D–4F). The pattern caused by *Poria subvermispora* (Fig. 4E) and *B. adusta* corresponds to preferential lignin degradation (selective degradation pattern), characterized by specific attack of the middle lamella causing separation of fibers. This micromorphological pattern agrees with lignin degradation estimated by the Klason method (up to 31% of the initial content in the case of *Poria subvermispora*) and low weight loss. A similar degradation pattern was reported in wood degraded by *Poria subvermispora* (4), a fungus studied for biopulping and extractive removal from *Pinus taeda* L. and *Picea abies* (L.) Karst. wood (19). However, extensive alteration of wood tissues due to fungal attack to both lignin and cellulose was observed in wood treated with other fungi, such as *Crepidotus variabilis* (Fig. 4F), *Melanotus hepatochrous* or *Phanerochaete chrysosporium* causing a simultaneous deg-

radation pattern, characterized by destruction of wood fibers and higher weight losses.

Gas chromatography analysis of lipophilic compounds

Wood lipophilic compounds are responsible for the formation of pitch deposits in paper pulp manufacture (28). The removal of these compounds after fungal treatment of eucalypt wood was investigated by GC–MS using high-temperature capillary columns. Representative chromatograms of the lipophilic extractive fraction from *E. globulus* wood treated with fungi and the control wood (the latter showing the same pattern of lipophilic compounds during the whole incubation period) are shown in Fig. 5. Free and esterified sitosterol, free fatty acids (palmitic, linoleic, oleic, and stearic acids), squalene, steroid hydrocarbons, steroid ketones, and small amount of triglycerides were identified as the main constituent of this fraction. Small amounts of free

Table 2. Degradation of lignin and free and esterified sterols by selected fungi.

Species name and strain number	Degradation (% initial content)		
	Lignin	Sterol esters	Free sterols
<i>Ophiostoma valdivianum</i> CBS 454.83	0	45	−55*
<i>Ophiostoma piliferum</i> Cartapip™ 97	0	68	−23
<i>Mollisia</i> sp. IJFM A711	0	25	−13
<i>Paecilomyces</i> sp. IJFM A710	0	38	23
<i>Pleurotus eryngii</i> IJFM A169	0	37	14
<i>Pleurotus pulmonarius</i> CBS 507.85	0	76	39
<i>Funalia trogii</i> IJFM A615	2	89	89
<i>Phanerochaete chrysosporium</i> CBS 481.73	4	79	73
<i>Bjerkandera adusta</i> CBS 230.93	13	76	77
<i>Crepidotus variabilis</i> IJFM A675	18	89	82
<i>Phlebia radiata</i> CBS 184.83	21	100	96
<i>Poria subvermispora</i> CBS347.63	31	100	98

Note: Lignin was analyzed by the Klason method and sterols (sitosterol and other minor sterols) by gas chromatography (in all cases the standard deviations for sterol and lignin degradation were, respectively, less than 15 and 7% of the mean values shown).
*The total content of free sterols (mainly sitosterol) was increased by some of the species.

and esterified sterols (mainly stigmastanol and fucosterol) were detected as shoulders of the peaks of sitosterol and sitosterol esters, respectively. Strong degradation of most lipophilic compounds in wood was produced by some of the fungi assayed, such as *Poria subvermispora* and *B. adusta* (Figs. 5B and 5C).

Among the different constituents of the lipophilic extractive fraction, free and esterified sitosterol has been identified as the main compound responsible for deposit formation in Cl₂-free kraft pulps of eucalypt wood (9). Table 2 presents sitosterol and sitosterol esters degradation by several representative fungi after GC–MS analysis of lipophilic extractives from wood treated with fungi and control. Degradation of these compounds was produced even by some fungi, such as *Poria subvermispora*, that did not decrease the total extractive content of wood. Since this and other white-rot fungi are able to degrade eucalypt lignin (Table 2), an increase in the extractive content can be produced by the release of low molecular weight, lignin-derived products. Additional evidence for this has been obtained by thermochemolysis analysis of lignin-derived products in wood treated with fungi (25). As shown in Table 2, significant reduction of sitosterol ester content in eucalypt wood was obtained with both ascomycetous and basidiomycetous fungi, although the highest removal corresponded to the latter species. However, a strong removal of free sitosterol was obtained only after wood treatment with some of basidiomycetes. In contrast, the ascomycetes assayed (including the *O. piliferum* and *O. valdivianum* strains) increased the free sitosterol content in pulp, preventing its utilization for pitch control in paper pulp manufacture from eucalypt wood. This could be related to the sterol esterase activity described recently in *O. piceae* (23) releasing sitosterol from sitosterol esters, and the lack of enzymes degrading the sitosterol molecule. The results obtained suggests that strains from *Poria subvermispora*, *Phlebia radiata*, *F. trogii*, *B. adusta*, *Crepidotus variabilis*, and *Pleurotus* spp. are the most promising for biological control of pitch deposition in *E. globulus* pulp, since they strongly removed both free and esterified sitosterol (although the latter fungus showed reduced growth rates after long maintenance in culture). An additional advantage of these

white-rot basidiomycetes is the degradation of eucalypt lignin during wood treatment (up to 30% of the initial content), which could result in lower alkali consumption during kraft pulping and (or) lower lignin content in crude pulp.

The results obtained show biodegradation of total or specific compounds in eucalypt extractives, by treating wood with selected fungal strains, as a suitable approach to control the different production problems caused by extractives during pulp and paper manufacture. Further investigation is currently in progress with the most promising strains to optimize the effectiveness of fungal treatments (in terms of maximizing removal of problematic compounds and limiting cellulose degradation) in the course of larger scale experiments.

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