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Biodegradability of Extractives in Sapwood and Heartwood from Scots Pine by Sapstain and White Rot Fungi

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Keywords

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White rot
Biodegradation
Fungal inhibition

Summary

The fungal degradation of lipophilic extractives in sapwood and heartwood from Scots pine (*Pinus sylvestris*) was studied. In sapwood, the white rot fungi, *Bjerkandera* sp. and *Funalia trogii*, removed higher amounts of extractives than the sapstain strains, *Ophiostoma ainoae* and *Ceratocystis allantospora*. Triglycerides, long chain fatty acids, steryl esters and waxes in pine sapwood were almost completely degraded by all the fungi. Sterols and resin acids were also extensively degraded by the white rot strains; however, these components were not or only poorly removed by the sapstain fungi. The removal of total extractives by all the fungal strains was higher in sapwood as compared to heartwood. The highly concentrated extractive fraction in pine heartwood mainly consists of resin acids. As observed in sapwood, sapstain were also poorly effective in the degradation of the resin acids present in heartwood. The fungal degradation of heartwood extractives was not only limited by the degradative ability of the various test microorganisms, but also by the inhibitory effect exerted by the extractive fraction. The white rot fungus *F. trogii* was particularly inhibited on heartwood. *Bjerkandera* sp. showed a higher tolerance to toxic extractives and was the most efficient fungus in degrading extractive constituents in both Scots pine heartwood and sapwood. Therefore, *Bjerkandera* sp. strain BOS55 should be considered as a potential agent for pitch control in pulp and paper manufacture.

Introduction

Wood extractives cause pitch problems in pulp and paper manufacture. Extractives are low-molecular weight lipophilic components in wood consisting mainly of triglycerides, waxes, steryl esters, sterols, free long chain fatty acids and resin acids (Fengel and Wegener 1989a). The deposits of wood extractives associated with fibers, inorganic salts and additives, technically referred to as pitch, disrupt the runnability of paper-making machinery and reduce strength and brightness of pulp (Allen 1980).

Fungal removal of extractives from wood prior to pulping is a promising technology to control pitch formation. Research on the potential use of wood-inhabiting fungi for reducing the levels of extractives in wood chips has mainly focused on sapstain fungi that spontaneously infect freshly cut logs. An albino isolate of *Ophiostoma piliferum* (Cartapip™) is nowadays commercially available for pulpwood depitching (Blanchette *et al*. 1992; Farrell *et al.* 1993; Messner 1998). The main sources of nutrition for *O. piliferum* and other staining fungi are the readily available carbohydrates (sugars and starch) as well as the lipophilic wood extractives (Gao *et al*. 1994; Brush *et al*. 1994). Recently, the white rot fungi *Phlebiopsis gigantea*, *Ceriporiopsis subvermispora* and *Phanerochaete chrysosporium*, have also been reported to reduce the extractive content in wood chips (Behrendt and Blanchette 1997; Fischer *et al*. 1994).

Biotechnological processes based on the use of white rot fungi could be very advantageous compared to existing biological methods for pitch reduction. In addition to the benefits directly resulting from the removal of wood extractives, white rot fungi could accomplish other significant benefits. Wood chip pretreatment with white rot fungi capable of selective lignin degradation, a process known as biopulping, enables substantial savings in energy required for refining mechanical pulps (Messner 1998). Biopulping also improves various paper strength properties (Akhtar *et al.* 1993; Fischer *et al*. 1994). Unlike white rot fungi, ascomycetes such as the commercial pitch control fungus *O. piliferum* are generally not able to attack the woody cell wall (Eaton and Hale 1993a; Zabel and Morrell 1992a). Therefore, such fungal treatments are not expected to reduce refining energy requirements. Another limitation of sapstain fungi is their poor ability to colonize the heartwood of most softwood species (Chen *et al.* 1994; Eaton and Hale 1993; Zheng *et al*. 1995). The total amount of extractives in heartwood is generally much higher than in sapwood. Therefore, the low susceptibility of heartwood to biological deterioration by sapstain fungi will limit the effectiveness of wood depitching.

The ability of white-rot fungi to degrade all major components of the woody cell wall, including lignin, cellulose and hemicellulose, is well characterized (Blanchette 1995). In contrast, information regarding the decomposition

of non-structural components in the woody tissue by white rot fungi is scarce (Fischer *et al*. 1996; Rayner and Boddy 1988). Improved knowledge of the fungal degradation of wood extractives can facilitate the development of new biological approaches for controlling pitch problems in pulp and paper manufacture.

In the present study, sapstain and white rot fungi were compared for their ability to degrade extractive constituents in Scots pine sapwood and heartwood. The extent of extractive removal by the different fungal strains was related to the possible inhibitory effect exerted by the extractive constituents present in sapwood and heartwood.

Materials and Methods

Microorganisms

The strain *Bjerkandera* sp. BOS55 was obtained from the Division of Industrial Microbiology, Department of Food Science, Wageningen Agricultural University, The Netherlands. *Funalia trogii* strain A 615 was a gift from the Center of Biological Research (CIB-CSIC) in Madrid, Spain. *Ophiostoma ainoae* (CBS 205.83) and *Ceratocystis allantospora* (CBS 185.86) were supplied by the Centraalbureau voor Schimmelcultures (CBS) in Baarn, The Netherlands.

Wood

Fresh logs of Scots pine (*Pinus sylvestris* L.*)* were supplied by the pulp and paper mill Parenco Newsprint B.V. (Renkum, The Netherlands). Sapwood and heartwood were separated, ground and sieved. Wood meal (18–40 mesh) was collected and stored at –20 °C. Extractive-free samples from sapwood and heartwood were prepared by Soxhlet extraction with acetone for 6 hours. The content of acetone extractives in sapwood and heartwood was 3.1 % and 5.1 % oven dry (o.d.) weight, respectively.

Solid state fermentation

Solid state fermentation experiments were carried out in 250 ml serum flasks containing 4 g (o.d.) of wood meal. Extractive-free sapwood and heartwood samples were used as a reference in the experiments investigating the fungal inhibition by extractives. Distilled water was added to adjust the wood moisture to 50 % (on wet wood basis). Subsequently, all flasks were sterilized by autoclaving at 120 °C during 20 min. Each flask was inoculated with three plugs (6 mm-diameter) from the edge of the mycelium grown on malt agar plates (10 g glucose, 3.5 g malt extract and 15 g agar per liter). Non-inoculated controls were run in parallel. All experiments were conducted in triplicate. The incubation conditions were 27 °C and 70 % relative air humidity during 6 weeks.

Analytical techniques

Total weight loss was estimated from the change in the dry weight of milled wood (103 °C, overnight). Fungal mediated losses in wood weight were corrected by abiotic weight losses determined in incubated sterile controls.

The total extractive content in the fungal treated and sterile wood samples (approx. 3.5 g o.d. weight) was determined by Soxhlet extraction with distilled acetone for 6 h. Extractions were performed in triplicate following drying of the wood samples at 60 °C. The extract was concentrated and evaporated to dryness by rotatory evaporation at 45 °C and then the extract was determined gravimetrically. The degradation of total extractives attained by fungal pretreatment was corrected by abiotic losses determined in incubated sterile controls. Abiotic losses of total wood extractives under the test conditions used in this study did not exceed 5 % of the initial extractive content.

The components of the acetone extracts were separated and quantified by gas chromatography using a modification of the analytical procedures described by Örså and Holmbom (1994) and Sithole *et al*. (1992). A gas chromatograph Hewlett Packard HP 5890 equipped with a split/splitless injector and a flame ionization detector (FID) was used. The high temperature capillary column was a DB5-HT (5 m-length × 0.25 mm-diameter, 0.1 mm film thickness, J&W Scientific). Samples (1 ml) were injected in the splitless mode. Helium was used as the carrier gas. The selected temperature program was as follows: started at 100 °C, held for 1 min at this temperature, heated to 350 °C at a rate of 15 °C min^{-1} and held for 3 min. The temperatures of the injector and the FID detector were set at 300 °C and 350 °C, respectively. This analytical procedure enabled direct determination of the main classes of wood extractive constituents, *i.e*., free long chain fatty acids, resin acids (diterpene carboxylic acids), sterols, steryl esters (long chain fatty acid esters), waxes (esters of long chain fatty acids and long chain fatty alcohols) and triglycerides (triacylglycerols). Compound identifications were based on the comparison of retention times of mixtures of standards detected by FID. Fatty acids were determined relative to the palmitic acid standard, resin acids relative to dehydroabietic acid, sterols relative to β-sitoste-rol, steryl esters and waxes relative to cholesteryl oleate and triglycerides relative to tristearin. Heptadecanoic acid was used as internal standard. A mixture of these standard compounds with a concentration range between 0.1 and 1 mg ml^{-1} was used to elabor-ate a calibration curve for the quantitation of wood extractives.

In a second step, individual resin acids, long chain fatty acids and sterols were identified by gas chromatography-mass spectrometry (GC-MS). Analyses were performed using a modification of the analytical procedure described by Örså and Holmbom (1994). The latter analytical procedure is not suitable for the identification of high boiling point constituents including those in the steryl ester and triglyceride fraction. A gas chromatograph (Varian Star 3400) with an ion trap detector (Varian Saturn 2000) and a high temperature capillary column (15 m × 0.25 mm DB-5HT; 0.1 mm film thickness, J&W Scientific) were used. Helium was the carrier gas. The samples were injected with an autoinjector (Varian 8200) directly onto the column using a septum-equipped programmable injection (SPI) system. The initial temperature of the injector was 120 °C, and 0.1 min after the injection was increased to 380 °C at a rate of 200 °C min^{-1} and maintained for 10 min at this temperature. The column temperature program was as follows: started at 120 °C, held for 1 min, increased to 380 °C at a rate of 10 °C min^{-1} and held for 5 min at this temperature. The temperatures of the ion trap and the transfer line were set at 200 °C and 300 °C, respectively.

Klason lignin (acid insoluble) and acid soluble lignin contents were measured after digesting milled wood with 72 % sulfuric acid as described in method T222 of the Technical Association of the Pulp and Paper Industry (TAPPI, Atlanta, GA., USA). The acid soluble lignin was estimated by measuring the absorbance at 280 nm using 20.21 $g^{-1}cm^{-1}$ as the extinction coefficient (Fengel and Wegener 1989b). Holocellulose was estimated from the difference in total weight less the sum of Klason lignin, acid soluble lignin and acetone-soluble extractives.

Results and Discussion

Figure 1 compares the degradation of total extractives (acetone extracts) in sapwood and heartwood from Scots pine by two sapstain fungi, *Ophiostoma ainoae* and *Ceratocystis allantospora*, and two white rot strains, *Bjerkandera* sp. and *Funalia trogii*. The initial extractive content determined in the sapwood and heartwood fraction was 31.0 and 51.0 mg per g (o.d.) wood, respectively. Except for *O. ainoae*, all fungi decreased significantly the extractive

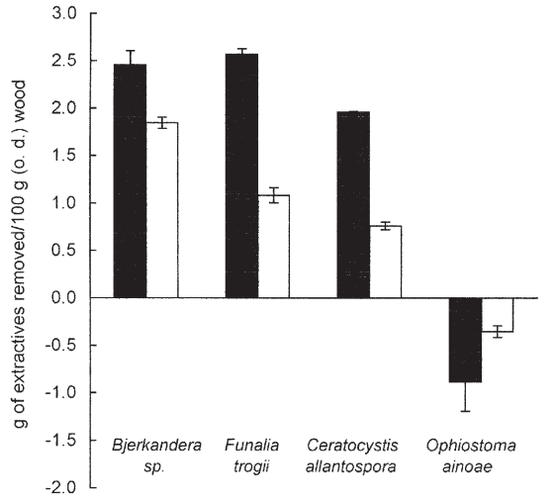

Fig. 1. Elimination of total extractives following incubation of Scots pine sapwood (filled bar) and heartwood (empty bar) by two white rot and two sapstain fungal strains for 6 weeks.

contents present in both sapwood and heartwood. Fungal treatment led to higher removal of total extractives in sapwood as compared to heartwood. The fungal degradation of sapwood extractives ranged from 25.7 to 19.6 mg per g (o.d.) wood. In heartwood, *Bjerkandera* sp. removed somewhat lower amounts of extractives than in sapwood, 24.6 and 18.4 mg per g (o.d.) wood, respectively. In contrast, the degradation of total extractives attained by *F. trogii* and *C. allantospora* in heartwood was less than half of that determined in sapwood. The sapstain fungus *O. ainoae* led to an increase in the extractive contents present in both sapwood and heartwood. The formation of degradation products extractable in acetone as a result of the fungal attack of wood may account for the observed increase in the extractive fraction. The identity of such compounds is as yet unknown as they were not comprised among the lipophilic extractive classes (*ie*., resin acids, free long chain fatty acids, triglycerides, sterols, steryl esters and waxes) detected under the chromatographic conditions applied in this study.

Table 1. Chemical composition of the acetone-extractives from the Scots pine (*Pinus sylvestris*) sapwood and heartwood used in this study

Extractive Constituents	Sapwood (mg g^{-1} (o.d.) wood)	Heartwood (mg g^{-1} (o.d.) wood)
Fatty acids	3.96 ± 1.00[a]	2.15 ± 0.60
Resin acids	8.92 ± 0.94	31.09 ± 0.94
β-Sitosterol	0.16 ± 0.02	0.27 ± 0.02
Waxes	1.60 ± 0.30	0.43 ± 0.10
Steryl esters	1.20 ± 0.20	0.48 ± 0.10
Triglycerides	7.30 ± 2.00	1.02 ± 0.20

[a] Standard deviation values.

The composition of acetone extracts from Scots pine sapwood is shown in Table 1. Major fractions in the sapwood extractives were resin acids, triglycerides and long chain fatty acids, representing 38.5 %, 31.5 % and 17.1 % of the total acetone extractives. Waxes, steryl esters and sterols were detected in minor quantities. Detailed analysis of sapwood extractives by GC-MS showed that the composition of individual free long chain fatty acids, resin acids, and sterols was according to the literature (Hafizoglu 1983). The major free fatty acid constituents identified were oleic, linoleic, linolenic and palmitic acids. Abietic, dehydroabietic, palustric and different isomers of pimaric acid were predominant in the resin acid fraction. The sapwood sterols consisted mainly of β-sitosterol, with traces of stigmastanol.

The percentages of the various extractive fractions removed from sapwood by incubation with the various fungal strains are presented in Table 2. Triglycerides and waxes were nearly depleted by fungal treatment of pine sapwood. Likewise, the content of free long chain fatty acids in pine sapwood was substantially reduced by 78 to 97 %. White rot fungi removed 81 to 90 % of the resin acids

Table 2. Degradation of the main classes of compounds in the acetone extractives of Scots pine sapwood following 6-weeks of incubation with several wood-inhabiting fungi. The fungal elimination of each extractive class is expressed as total loss (mg g^{-1} (o.d.) wood) and, as relative loss (% loss relative to abiotic control)

Extractive Classes	Elimination of Sapwood Extractives							
	Bjerkandera sp.		*Funalia troggi*		*Ophiostoma ainoae*		*Ceratocystis allanstospora*	
	Total[a]	Relative[b]	Total	Relative	Total	Relative	Total	Relative
Fatty acids	3.84	97	3.77	95	3.09	78	3.49	88
Resin acids	8.03	90	7.22	81	0.00	0	2.13	24
β-sitosterol	0.11	69	0.08	50	0.00	0[c]	0.00	0[d]
Waxes	1.60	100	1.60	100	1.49	93	1.51	94
Steryl esters	1.20	100	1.20	100	1.20	100	0.95	79
Triglycerides	7.30	100	7.30	100	7.30	100	7.30	100

[a] Total loss in each extractive class (mg g^{-1} (o.d.) wood); [b] Relative loss (%); [c] the amount was increased by 1.20 mg g^{-1} (o.d.) wood.; [d] the amount was increased by 0.43 mg g^{-1} (o.d.) wood.

Table 3. Degradation of the main classes of compounds in the acetone extractives of Scots pine heartwood following 6-weeks of incubation with several wood-inhabiting fungi. The fungal elimination of each extractive class is expressed as total loss (mg g^{-1} (o.d.) wood) and, as relative loss (% loss relative to abiotic control)

Extractive Classes	Eliminations of Heartwood Extractives							
	Bjerkandera sp.		*Funalia troggi*		*Ophiostoma ainoae*		*Ceratocystis allanstospora*	
	Total[a]	Relative[b]	Total	Relative	Total	Relative	Total	Relative
Fatty acids	1.87	87	0.82	38	0.56	26	0.97	45
Resin acids	15.54	50	0	0	0	0	5.91	19
β-sitosterol	0.05	18	0.11	41	0	0[c]	0.00	0[d]
Waxes	0.32	74	0.19	44	0.29	67	0.05	12
Steryl esters	0.30	62	0.28	58	0.28	58	0.08	17
Triglycerides	0.82	80	1.02	100	1.02	100	0.61	60

[a] Total loss in each extractive class (mg g^{-1} (o.d.) wood); [b] Relative loss (%); [c] the amount was increased by 0.27 mg g^{-1} (o.d.) wood; [d] the amount was increased by 0.19 mg g^{-1} (o.d.) wood.

in sapwood, whereas, sapstain strains only decreased the resin acid content by 24 % or less. The degradation of the steryl ester fraction by the various fungal strains ranged from 79 % to 100 %. Finally, the fungal removal of the main sterol detected, β-sitosterol, varied widely depending on the fungal treatment. *Bjerkandera* sp. and *F. trogii* degraded up to 69 % and 50 % of the β-sitosterol in sapwood, respec-tively. A distinguishing characteristic of the sapstain fungi is that they did not degrade β-sitosterol under the solid state fermentation conditions selected. On the contrary, the β-sitosterol content was increased by 0.4 and 1.2 mg per g (o.d.) sapwood after incubation with *C. allantospora* and *O. ainoae*, respectively. The results suggest that although sapstain fungi are capable of hydrolyzing β-sitosteryl esters, and most likely other minor acyl-sterols present in sapwood, they lack the ability to degrade complex extractive constituents such as β-sitosterol.

The chemical composition determined in the extractives of the Scots pine heartwood used in this study is listed in Table 1. Resin acids were present in high concentrations and accounted for 88 % of the total extractives. Although the total amount of resin acids in heartwood was substantially higher than in sapwood, the main resin acid constituents identified were similar. Triglycerides and long chain fatty acids, which were important compounds in pine sapwood, were found to be much less abundant in heartwood. The elimination of total extractives attained by incubation of Scots pine heartwood with the various fungal strains is presented in Table 3. The ability of the two white rot strains to degrade heartwood extractives was found to be very different. *Bjerkandera* sp. degraded twice as much resin acids in heartwood than in sapwood, 15.5 and 8.0 mg per g (o.d.) wood, respectively. In contrast, *F. trogii* left resin acids untouched in heartwood. In general, *F. trogii* caused low degradation of heartwood extractives, while all the extractive constituents present in sapwood were almost depleted. Similarly to *Bjerkandera* sp., the sapstain fungi *C. allantospora* degraded higher amounts of resin acid in heartwood, where other easily available carbon sources were scarce. As already observed in sapwood, *O. ainoae* did not degrade resin acids in heartwood.

The extent of extractive degradation by the various fungal strains was considered in relation to the total weight losses determined on sapwood and heartwood. Figure 2 shows that all fungal treatments led to higher weight losses in sapwood as compared to heartwood.

The white rot fungus *F. trogii* caused up to 4.9 % weight loss on sapwood, and only 0.6 % on heartwood. Smaller differences were determined with the white rot strain *Bjerkandera* sp. that led to 5.3 % and 4.0 % weight losses for sapwood and heartwood, respectively. As expected, wood attack by the white rot fungi was not limited to the degradation of the extractive fraction. Lignin and holocellulose losses were also recorded in the decayed samples. The consumption of holocellulose in sapwood and heartwood samples by these white rot fungi was low (less than 1.4 % in 6 weeks). The percentage of lignin removed was by comparison high, particularly in sawpood samples. After six weeks of solid state fermentation, 11.5 % and 5.4 % decreases in Klason lignin were observed in pine sapwood treated with *Bjerkandera* sp. BOS55 and *F. troggi*, respectively.

Heartwood exhibited resistance to degradation by the sapstain fungi. In fact, *C. allantospora* did not cause any significant loss of weight on pine heartwood. On the other hand, *O. ainoae*, caused total weight losses that were almost twice as high for sapwood compared to heartwood, 3.6 % and 2.1 %, respectively. The low weight losses of heartwood caused by the sapstain fungi can partly be explained by the limited ability shown by these microorganisms to degrade the highly abundant extractive components, resin acids. The poor colonization of heartwood could also be related to fungal inhibition due to the large extractive content in this wood fraction.

In order to evaluate the possible inhibitory effect of extractives to the various test microorganisms, sapwood and heartwood samples devoid from apolar extractives by previous extraction with acetone were subjected to fungal

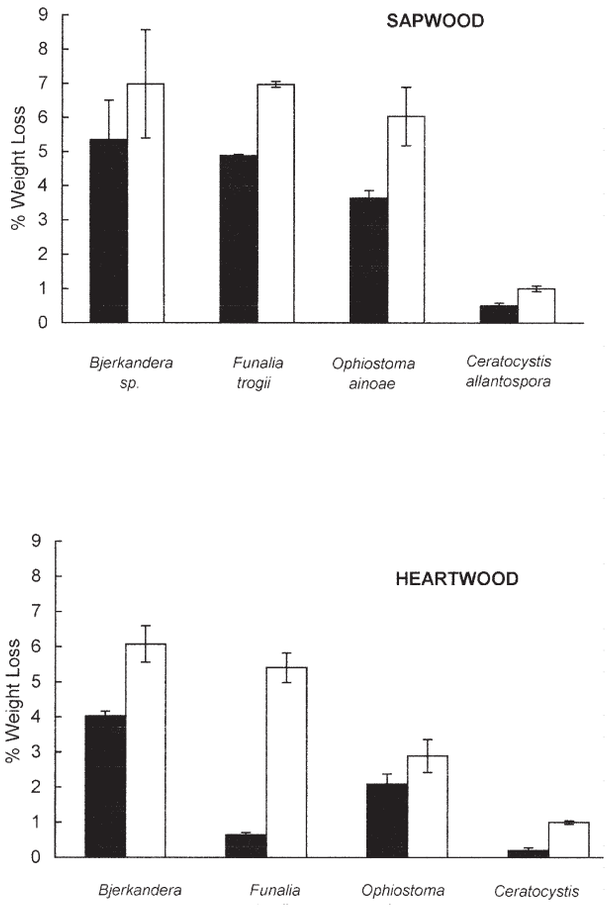

Fig. 2. Effect of lipophilic wood extractives on the decay of Scots pine wood by several wood-inhabiting fungi. Top panel: Relative weight losses caused by a 6-week fungal treatment of sapwood (filled bars) and extractive-free sapwood (open bars); Lower panel: Relative weight losses caused by 6-week fungal treatment of heartwood (filled bars) and extractive-free heartwood (open bars).

treatment. The mass losses determined for the non-extracted wood and acetone-extracted wood after a 6-week treatment with the different strains are compared in Figure 2. Fungal inhibition by heartwood extractives was evidenced by the considerable increase in the fungal-mediated weight losses determined in the extractive-free samples. Sapwood extractives also proved to be somewhat toxic, since the removal of extractives enhanced sapwood attack by the various fungi.

Interestingly, the sapstain fungus *O. ainoae* caused moderate weight losses in extractive-free sapwood and heartwood amounting to 6.0 % and 2.9 %, respectively. Such weight losses can not be explained unless the fungus is capable of degrading cellulose, hemicellulose, or lignin. Although sapstaining fungi are generally considered unable to decay wood cell walls, some *Ophiostoma* spp. have been reported to cause extensive degradation of poorly lignified cells (*eg.* ray parenchyma cells) (Brush *et al*. 1994). Also, some sapstain fungi are known to exhibit soft rot activity under specific environmental conditions (Fengel and Wegener 1989 c; Zabel and Morrell 1992 b). Soft rot fungi include different ascomycetes and deuteromycetes causing a variety of decay types at the wood cell wall level (Eaton and Hale 1993 b). Chemical analysis of control- and fungal-treated samples confirmed that *O. ainoae* accomplished a degradation of 6.1 % and 3.8 % of the holocellulose content in sapwood and heartwood, respectively. In contrast, incubation with this fungal isolate did not cause any significant alteration of the amount of lignin in the sapwood and heartwood samples. Lignin and holocellulose losses determined in wood samples attacked by *C. tenella* were negligible.

Considerably differences in the tolerance to toxic extractives was displayed by the various fungal strains. The high levels of extractives in heartwood were particularly toxic to *F. trogii* as indicated by the large improvement in wood weight losses after heartwood extraction with acetone. Being the major extractive constituents in Scots pine heartwood, resin acids probably accounted for the toxicity exerted by heartwood towards the fungal strains considered in his study. Resin acids were previously reported to cause severe inhibition of other wood-inhabiting fungi (Micales and Hans 1994; Eberhardt *et al*. 1994).

In general, the results indicated that triglycerides and free long chain fatty acids represented the first carbon-source among the constituents of the acetone extracts in Scots pine wood. This finding is in agreement with previous reports on the biodegradation of softwood extractives by the well-known sapstain fungi *O. piliferum* and *O. piceae* (Gao *et al.* 1994; Brush *et al.* 1994; Farrell *et al.* 1993). The fungal strains assayed in this study also reduced substantially the amounts of waxes and steryl esters in pine wood, which is in clear contrast to the low biodegradation reported for the commercial sapstain strain Cartapip (Chen *et al.* 1994). Triglycerides, waxes and long chain fatty acids largely contribute to the formation of pitch deposits (Fischer and Messner 1992; Allen 1988). Therefore, all the strains studied here seem to be promising for the biological control of pitch. However, the resin acid removal varied significantly among the fungal strains tested. This fact is especially relevant since the highly concentrated extractive fraction in pine heartwood consists mainly of resin acids. Although white rot fungi were very advantageous in the removal of resin acids in sapwood, *F. trogii* should be discarded as a potential strain for softwood depitching due to the low tolerance exhibited by this fungus to the high resin acids levels present in pine heartwood. On the other hand, *Bjerkandera* sp. was not significantly inhibited by the high levels of resin acids and, degraded effectively all extractive constituents in heartwood. Therefore, the white rot fungus *Bjerkandera* sp. strain BOS55 results particularly interesting for pitch control in softwood species. In this respect, it should be noted that rapid and extensive degradation of the extractives in beech wood and hemp stem wood by this strain was recently reported (Mester *et al*. 1998). The applicability of white rot fungi in pulpwood depitching would greatly depend on their effects on pulp properties and pulp yield. Fungal attack of cellulose in pulp fibers is highly undesirable as it causes reduction in pulp

yields and pulp strength properties. Limited attack of cellulose during beech wood decay by *Bjerkandera* sp. strain BOS55 was recently reported (Mester *et al*. 1998), what enhances the potentials of this strain in biotechnology for pulp and paper industry.

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