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Screening of *Ophiostoma* Species for Removal of Eucalyptus Extractives

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Abstract: *Ophiostoma* species have been demonstrated to metabolize wood extractives and be useful to the pulp and paper industry. In order to have new isolates for the Asian market, *Eucalyptus camaldulensis* logs were harvested from forest sites in central Taiwan and 28 strains of the *Ophiostoma* genus were isolated from them. These strains were subsequently inoculated onto Eucalyptus wood chips to evaluate their effects on weight losses of wood and the amounts of acetone extractives degraded. At the same time, Gas Chromatography-Mass Spectroscopy (GC-MS) analysis was conducted and by using calibration curves and a database of GC-MS mass spectra, changes in lipophilic compounds were analyzed. Fatty acids, hydrocarbons, sterol compounds, sterol esters, and triglycerides were significantly reduced after two weeks' inoculation by the fungal strains. The results show that six of the strains were capable of reducing the lipophilic fractions by more than 60% in a two-week treatment. DNA of the most effective strains were analyzed and found to be a variant of *Ophiostoma querci.*

Keywords: *Eucalyptus camadulensis*, *Ophiostoma*, lipophilic, pitch

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INTRODUCTION

Organic solvent extractable substances from wood are complex fractions consisting of fats, waxes, resin acids, free and esterified sterols, alcohols, terpenoids, and phenolics. These substances often interact during the pulping and papermaking processes to cause serious pitch troubles, causing decreased quality in paper products and hampering production. Compositions of the extractives vary depending on tree species and seasons of wood harvesting. Lipophilic extractives in particular exert strong negative impact on manufacturing processes, causing so-called pitch deposits on pulp and paper and on paper machine parts.[1]

The primary industrial plantation tree species nowadays are pine, poplar, and eucalypt. Among these, the eucalypt wood is a major raw material for paper pulp manufacturing in countries from Asia, Europe, Brazil, and other world regions. Eucalypt species used as pulpwood provides the highest yield and the best pulp quality during kraft cooking and totally chlorine free (TCF) bleaching.

Pitch problems, however, are dramatically enhanced when the elementary chlorine free (ECF) bleaching sequences are substituted by TCF sequences for the manufacture of high-quality eucalypt kraft pulps. Furthermore, process effluents containing wood extractives may be toxic and harmful to the environment.[2–5]

In order to design a rational strategy for the efficient control of extractive-derived problems in these high-quality eucalypt pulps, different studies have been carried out during the past few years. For example, chemical analyses of *E. globulus* wood extractives[6–8] and pitch deposits formed during pulping and bleaching,[4,5,9,10] with the aim of identifying those extractive compounds responsible for deposit formation, and the search for methods to remove or control the degree of deposition of these problematic compounds.

Conventional pitch control methods include measures such as extensive debarking, seasoning of wood, and use of pitch control agents to disperse, detackify, and sequester the pitch. Biological control such as using bacteria,[11] fungi,[8,12,13] lipolytic,[13–17] and oxidative[18–20] enzymes to degrade pitch and resin from wood and pulp have also been used, some in laboratories and some in continual industrial use.[21] However, the aforementioned methods are aimed for commercial utilization purposes; for instance, enzymes (such as lipase commercialized by Novo Nordisk as Resinase™) or organisms (such as *Ophiostoma piliferum* strains commercialized by Clariant as Cartapip™), however, are useless for the control of pitch in eucalypt kraft pulping. Also, pitch control treatment using an albino strain of *O. piliferum* was found to reduce content of pitch in softwood.[12,22–24] It is, however, rather ineffective in removing the lipophilic compounds of eucalypt, as the free sitosterol content tended to increase with the treatments.[8] In the present study, the aim of this work was to screen suitable wood inhabiting fungi capable of decreasing

extractive content in eucalypt wood, thus enabling the prevention of pitch trouble.

MATERIALS AND METHODS

Fungal Strains

Twenty-eight strains of fungi were isolated according to the methods of Duncan et al.[25] from cut logs of Murray red gum (*Eucalyptus camaldulensis*) trees harvested from a plantation in central Taiwan. After harvesting the chipped wood was air-dried for a week before proceeding for treatments. *O. floccosum* and the Cartapip™ 97 were kindly provided by University of Waikato, New Zealand, and Parrac Ltd., New Zealand, respectively.

Wood Chips

E. camaldulensis wood chips (air-dried) were made 1–2 cm^2 in size, and sterilized by autoclaving at 121°C for 15 min.

Fungal Treatments of Wood Chips

The sterilized *E. camaldulensis* wood chips, 100 g, were placed into 500 mL serum flasks and 30 mL sterilized water containing individually each fungal strain (ca. colonies of 2×10^6 cells) was inoculated onto the wood chips. Each inoculation was done in triplicate, and incubated at 25°C and 75%RH under darkness for 2 weeks. Control wood chips were treated with the same amount of water, and also left to incubate for 2 weeks. After 2 weeks' incubation, wood chips were subsequently sterilized and washed with double distilled water. Weight losses of the wood chips were measured.

The Sample Preparation

After two weeks' incubation, air-dried wood chips were milled using a Wiley Mill and extracted three times with acetone at room temperature for 48 h. The extractives were evaporated to dryness and then redissolved in chloroform for quantitative determination and chromatographic analysis of the lipophilic fractions, which are the chloroform extractables from the acetone extractives of wood. Subsequently, GC and GC-MS analyses were performed as described in the following section.

GC Analyses

The GC analyses were performed with a Hewlett-Packard model HP 5890 GC equipped with a flame ionization detector by using a high-temperature,

polyimide-coated, fused-silica capillary column (5 m by 0.25 mm; type DB5-HT; film thickness, 0.1 μm; J & W Scientific). The injector and detector temperatures were 270 and 380°C, respectively. The oven temperature was programmed to increase from 100°C (holding 1 min) to 380°C (holding 3 min) at a rate of 10°C/min. Samples were injected in the splitless mode. Helium was used as the carrier gas. A mixture of standard compounds (palmitic acid, sitosterol, cholesteryl oleate, and triheptadecanoin) was used to construct a calibration curve for quantitation of wood extractives at concentrations ranging from 0.1 to 1 mg/ml. The correlation coefficient was greater than 0.99 in all cases. Peaks were quantified by determining areas.

GC-MS Analyses

The method of Gutiérrez et al.[9] was adopted. GC-MS analyses were performed with a Hewlett-Packard HP 6890/HP5973 by using a type DB-5HT capillary column (15 m by 0.25 mm; film thickness, 0.1 micrometers; J & W Scientific). The GC analysis parameters were the ones listed above and the MS was operating (full scan mode: scan time: 0.3 s, mass range was *m/z* 30–800) in the EI mode at 70 eV. All test data are the average of triplicate analyses. Compounds were identified by comparing their mass spectra with mass spectra in the Wiley and NIST libraries and, when possible, by using standards.

DNA Extraction

Cultures were grown 3–7 days in potato dextrose agar (PDA, Difco) subculture for DNA extraction. We selected the edge of filamentous fungi colony from a culture plate. We suspended the cells in 200 μl PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems) in a 2 ml microcentrifuge tube, and vortexed the sample for 10 to 30 sec. We heated the sample for 10 min at 100°C in a water bath, and spun the sample for 3 min at 16000 × g. We transferred the supernatant into a new microcentrifuge tube, and the supernatant was then ready for a polymerase chain reaction (PCR).

PCR Amplification and DNA Sequencing

Primers used for amplification of the internal transcribed spacer region of the DNA were ITS-4 combined with ITS-1F. PCR amplification conditions were 94°C initial denaturation for 5 min, 35 cycles of 94°C denaturing, 50°C annealing, and 72°C extension, each at 60s. The final extension was 72°C for 5 min, and kept at 4°C until DNA sequencing was conducted. PCR was conducted by the protocol of using reagents from Yeastern Biotech. Co.Ltd., Taipei, Taiwan.

RESULTS AND DISCUSSION

Lipophilic Extractives in Eucalypt Wood and Pulp

Table 1 shows changes in the amounts of wood extractives after autoclaving and after kraft pulping. Wood chips contained 0.82% (wt.% on dry wood) of acetone (at room temperature) fraction. The control wood chips, on the other hand, had only 0.58% cold acetone extractives, 80% of which is chloroform extractable, or lipophilic fraction. The wood extractive content and the amount of lipophilic extractives were similar to those of Gutiérrez et al.[7,8] and Rencoret et al.[26] After pulping, only 0.11% (with respect to dry wood) of cold acetone extractive remained, most of which belonged to lipophilic fraction. Thus, most of the polar components dissolved during pulping and only lipophilic fraction remained. The lipophilic fraction is often blamed for causing pitch trouble and deposition problems.[1]

Observation on the components of the lipophilic fraction and their contents are shown in Table 2. The results clearly show that during the pulping process, 60% of hydrocarbon was removed, and most (ca. 90%) of the fatty acid could be removed as well. Ester compounds, such as steryl esters, would be totally hydrolyzed in the alkaline cooking liquor, while more than half of the sterols remained in the pulp.

Figures 1 and 2 show the comparison of the GC-MS chromatograms of lipophilic compounds in wood chips and kraft pulp of *E. camadulensis*. The identified compounds and their contents are listed in Table 3. For wood chips, the most abundant lipophilic compounds were steryl ester and triglycerides, which constituted 57% of all lipophilic fractions. These results are similar to the observations of Gutiérrez et al.,[7,29] Rencoret et al.,[26] Freire et al.,[27] and Gutiérrez and del Río.[28] However, these compounds were relatively rare in the kraft pulp. This was due to their saponification and hydrolysis in the

Table 1. Changes in amounts of wood extractives after autoclaving and kraft pulping wood chips (wt.% on dry wood)

Extractives	Wood chips[a]	Control[b]	Pulp[c]
Acetone extractive	0.82	0.58	0.11 (0.25)[d]
Total lipophilics	0.66	0.47	0.1 (0.22)
Polar compounds	0.16	0.11	0.01 (0.02)

a: Unsterilized *Eucalyptus camaldulensis* wood chips.

b: Wood chips sterilized at 121°C for 15 min and treated same as in fungal treatment except without addition of fungi.

c: Pulping condition: Liquor/dry wood = 4, sulphidity 25%, active alkali 16%, pulping temperature 160°C, pulping time 3 hr (to temp. 160°C, 90 min, at temp. 160°C, 90 min).

d: Wt.% base on pulp.

Table 2. Changes in compounds of acetone extractives after autoclaving and pulping of *E. camadulensis* (mg/100 g wood chip)

Compound	Wood	Control	Pulp[a]
hydrocarbons	6.7	15.0	3.3
fatty acids	48.8	50.6	4.1
fatty alcohols	0.2	trace[b]	3.1
waxes	5.0	trace	—[c]
steroid hydrocarbons	17.2	12.8	0.5
sterols	117.9	113.6	67.4
steroid ketones	18.1	79.4	3.1
squalene	6.5	1.1	0.7
α-tocopherol	12.5	6.3	1.4
steryl esters & triglycerides	377.2	41.8	7.4
others	6.1	74.2	1.4
unidentified	42.4	75.1	7.8
Total	661.3	470.0	100.0

a: Pulping condition: Liquor/dry wood = 4, sulphidity 25%, active alkali 16%, pulping temperature 160°C, pulping time 3 hr (to temp. 160°C, 90 min, at temp. 160°C, 90 min).
B: trace < 0.1 mg/100 g.
c: not detected.

alkaline cooking liquor. These results are similar to the observations of several other references.[4,5,10,30–36] Sterols were the second most abundant compound in wood, making up 18% of all lipophiles. Eleven of these had been identified, and β-sitosterol was the most abundant sterol, which tended to remain in the pulp as well. Although two compounds each of the steroid ketones and steroid hydrocarbons were found in wood, there were only trace amounts of these in

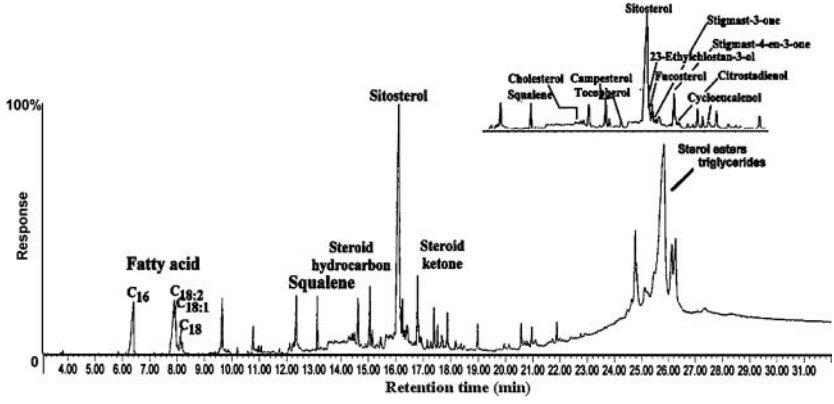


Figure 1. Composition of lipophilic extractives of *E. camaldulensis* wood.

Table 3. Compositions of lipophilic substances from acetone extractives of *E. camdulensis* wood and kraft pulp (mg/100 g wood chips)

Compound Identified	A	B[a]
Fatty acids	**48.8**	**4.1**
n-hexadecanoic acid	18.2	3.0
n-heptadecanoic acid	0.2	—[c]
9,12-octadecadienoic acid	20.1	—
9-octadecadienoic acid	6.5	1.1
octadecanoic acid	3.8	—
Waxes	**5.0**	**—**
hexadecanoic acid esters	5.0	—
Hydrocarbons	**6.7**	**3.3**
n-tetratriacontane	1.2	0.9
n-heneicosane	0.6	0.6
n-tetradecane	1.1	0.7
n-eicosane	1.1	0.6
n-octacosane	2.7	0.5
Fatty alcohols	**0.2**	**3.1**
9-octadecen-1-ol	0.2	0.7
1-eicosanol	—	1.3
1-dotriacontanol	—	1.1
Steroid hydrocarbons	**17.2**	**0.5**
stigmasta-3,5-diene	13.7	0.5
stigmasta-4,22-diene	3.5	—
Sterols	**117.9**	**67.4**
ergosta-7,22-dien-3-ol	4.2	1.7
(3β,22Z)-chola-5,22-dien-3-ol	2.5	—
campesterol	3.9	0.7
β-sitosterol	96.7	59.5
ergostanol	trace[b]	—
23-ethylcholestan-3-β-ol	trace	—
fucosterol	2.4	—
cycloeucalenol	4.2	2.7
stigmastanol	—	1.2
cholest-8(14)-en-3-ol	2.7	1.6
citrostadienol	1.3	—
Steroid ketones	**18.1**	**3.1**
stigmasta-3-one	3.7	—
stigmast-4-en-3-one	14.4	3.1
Squalene	**6.5**	**0.7**
Tocopherols	**12.5**	**1.4**
α-tocopherol	12.5	1.4
Steryl esters & triglycerides	**377.2**	**7.4**

(Continued on next page)

Table 3. Compositions of lipophilic substances from acetone extractives of *E. camdulensis* wood and kraft pulp (mg/100g wood chips) *(Continued)*

Compound Identified	A	B[a]
Others	**6.1**	**1.4**
dehydrodieugenol	1.3	—
1-octadecanethiol	1.4	—
syringaldehyde	—	0.5
2-hexadecyloxyethanol	0.7	—
tetradecyl oxirane	2.0	0.9
hexadecyl oxirane	0.7	—
Unidentified	**42.4**	**7.8**
Total	**661.3**	**100.0**

A: Wood extractives B: Pulp extractives.

a: Pulping condition: Liquor/dry wood = 4, sulphidity 25%, active alkali 16%, pulping temperature 160°C, pulping time 3 hr (to temp. 160°C, 90 min, at temp. 160°C, 90 min).

b: trace < 0.1 mg/100g. c: not detected.

the pulp. Five hydrocarbons were also identified in wood, but 40% of these still appeared in pulp. Though present in low amounts in wood, fatty alcohols tended to increase in the pulp. This was deemed to be the result of hydrolysis products.

Removal of Lipophilic Extractives by *Ophiostom*a Strains

Table 4 and Figure 3 show the results of treating wood chips by inoculating them with several strains among the 28 fungal strains (mostly tentatively identified as belonging to the *Ophiostoma* spp) that showed strong pitch-degrading

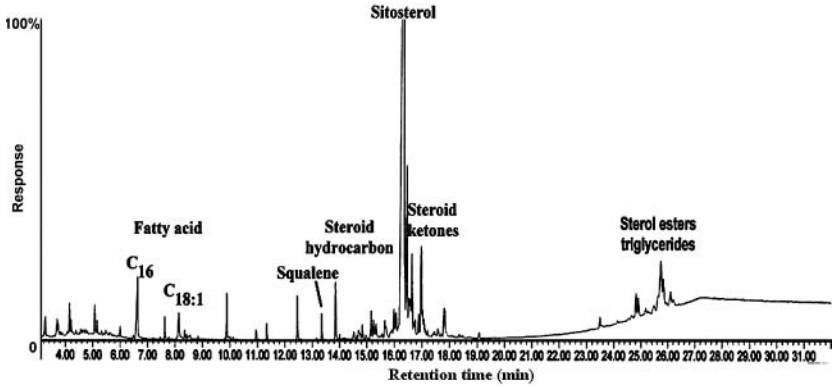


Figure 2. Composition of lipophilic extractives of kraft pulp from *E. camaldulensis.*

Table 4. Quantitative analysis of the lipophilic extractives after 2 weeks fungal treatments of. *E. camaldulensis* wood chips (mg/100 g wood chips)

Compound	Control	*O. floccosum*	Cartapip™97	C1-8	C1-12	C1-14	C1-17	C3-2	C3-3
hydrocarbons	15.04	4.73	4.31	4.45	3.52	3.91	4.26	3.39	3.75
fatty acids	50.62	18.41	16.72	12.80	13.67	13.12	9.66	15.60	7.60
fatty alcohols	trace[a]	—[b]	—	1.90	2.47	2.52	2.25	2.99	—
waxes	trace	—	—	—	—	—	—	—	—
steroid hydrocarbons	12.83	4.91	4.56	3.23	3.74	3.87	2.24	3.63	1.85
sterols	113.60	42.95	46.72	42.98	53.89	53.44	42.66	71.54	42.08
steroid ketones	79.43	30.42	34.20	29.28	31.18	31.72	25.19	40.24	25.28
squalene	1.08	trace	0.44	0.50	0.22	0.36	0.41	0.55	0.27
α-tocopherol	6.30	2.41	1.88	1.66	1.07	1.93	0.94	2.97	0.90
sterol esters & triglycerides	41.83	16.02	13.34	8.18	8.01	9.81	8.05	12.85	9.42
others	74.21	27.65	30.29	23.25	25.09	28.33	18.48	31.55	16.59
unidentified	75.10	32.50	37.50	33.70	29.60	33.50	28.10	37.70	32.30
Total	470.04	180.00	189.95	161.902	172.46	182.51	142.23	223.01	140.04

a. trace < 0.1 mg/100 g.
b. not detected.

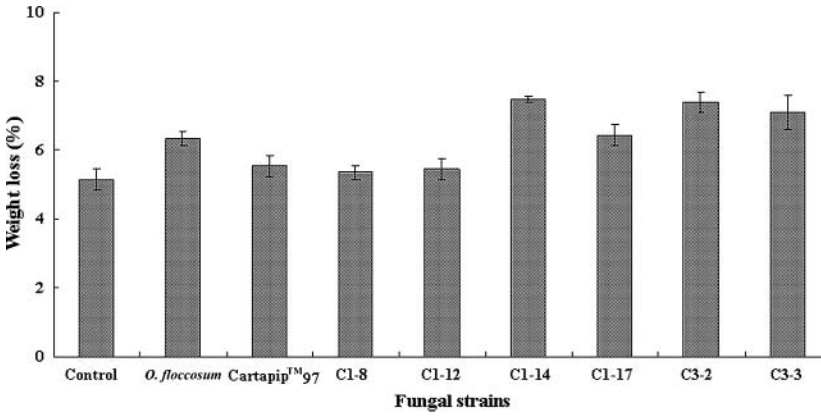


Figure 3. Weight losses after 2 weeks of fungal treatment at 25°C and 75% RH.

capabilities. After two weeks of fungal treatment, the weight losses and changes in acetone extractive contents were recorded. Figure 3 shows comparisons of the control group with the various fungal treatments. The weight losses due to fungal metabolism were merely 1–3%, indicating that these fungi won't cause massive wood degradation, which rendered them suitable as candidate for depitching applications. Figure 4 shows that, with regard to cold acetone extractives, there was maximal 40% reduction after the fungal treatment for 2 weeks but, with regard to the lipophilic components, the maximal reduction reached nearly 70%. Hence, some strains of fungi were highly effective in degrading lipophiles in wood chips.

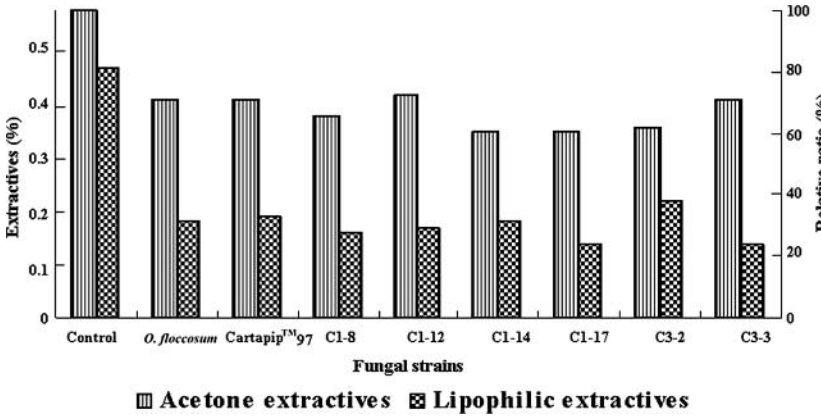


Figure 4. Reduction of wood extractives after the fungal treatment.

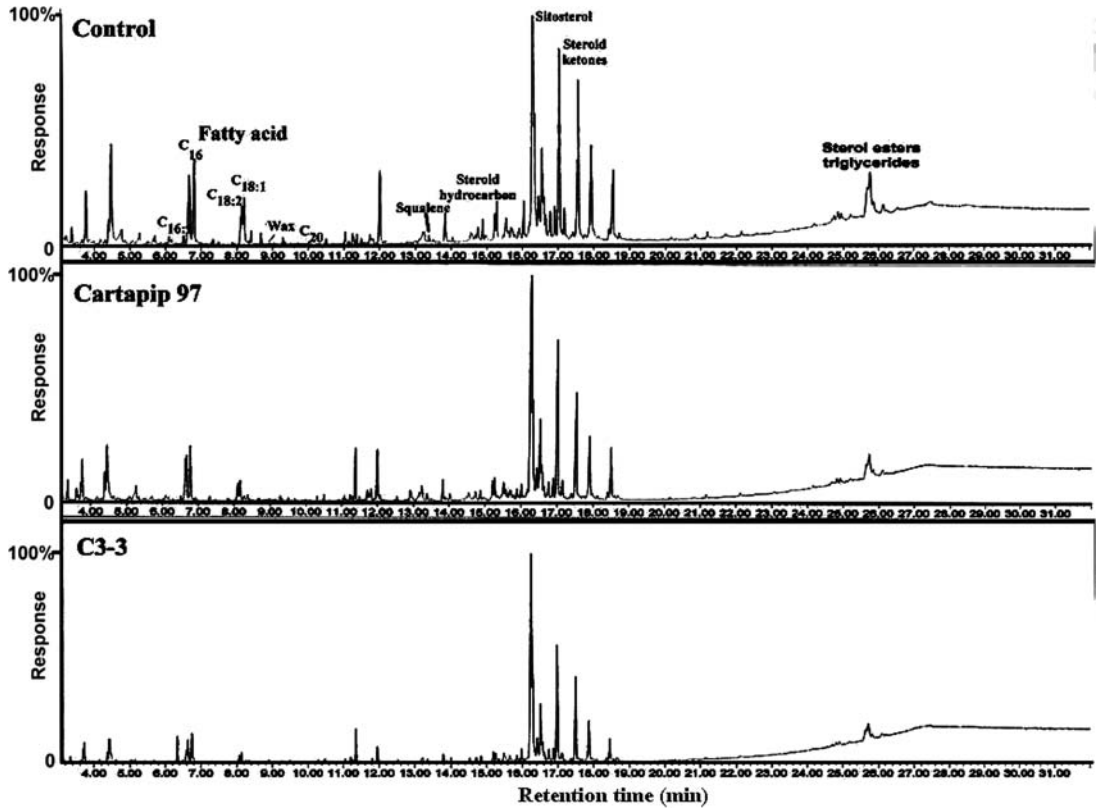


Figure 5. Comparisons of compositions of lipophilic extractives of *E. camaldulensis* woodchips after fungal treatment.

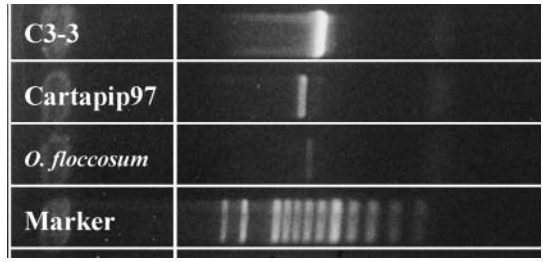


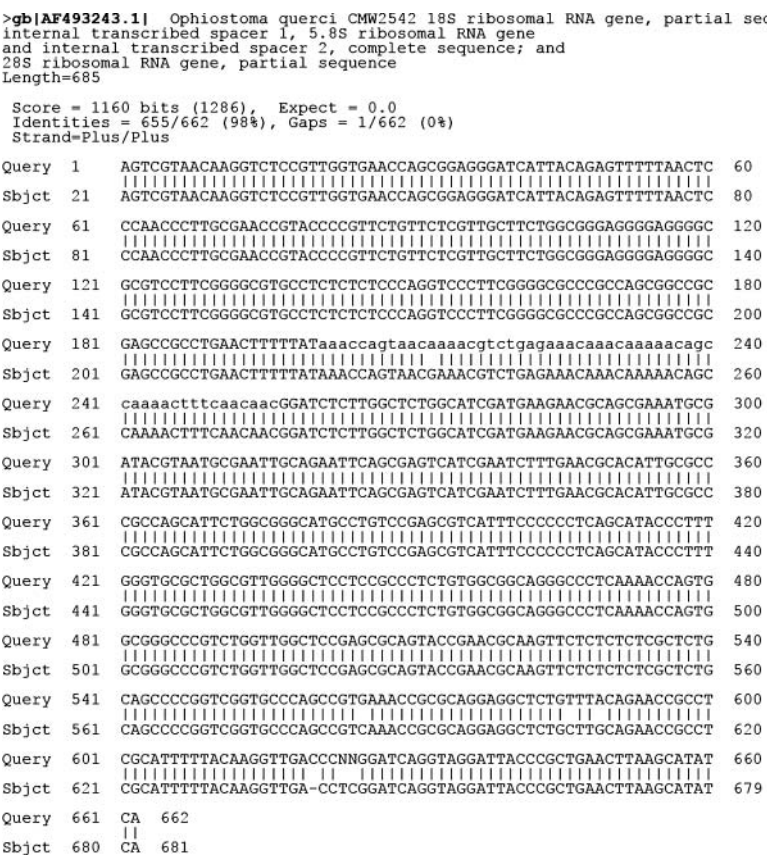
Figure 6. The electrophoresis graphs of fungal PCR preparations.

Figure 5 shows the treatment results of using the fungal C3–3 strain in comparison to the control group and the Cartapip^{TM} 97 treated wood. Treatment using the C3–3 strain showed marked reduction of fatty acids and steryl hydrocarbons and steryl ketones and steryl esters also showed significant reductions. Only the sterol components showed lesser degrees of reduction. Table 4 shows the effects of treating wood chips using various fungal strains on the changes in their lipophilic component contents. The best reduction in total amounts was achieved by a strain designated C-3–3; after two weeks incubation, 70% of the lipophiles were destroyed. Nearly all component parts showed high amplitudes of reduction. Sterols had the highest residual contents, but their removal rates still reached 63%, indicating a high efficacy of pitch degradation by the fungus. These results compared more favorably to those of Gutiérrez et al.[8] using a strain of *O. piliferum* to remove lipophilic compounds of eucalypt. This was because *O. piliferum* performed poorer in removing free sitosterol content of the wood. These results bode well for a successful screening of fungi with high capability of removing pitch components of hardwood chips before they were used for pulping. Prevention of pitch trouble through inoculation of these strains is feasible providing they are capable of dominance in the chip pile when applied.

Fungal DNA Extraction and Sequencing

The fungal strain designated C3–3 was extracted for its DNA and upon PCR amplification its DNA sequence was determined. A BLAST search revealed similarity between C3–3 and *O. querci* (GenBank AF493243.1) was 98.9%. Its identity was established as the closest match to *O. querci* (Figures 6 and 7).

Furthermore, we compared the *O. querci* with other fungi of the *Ophiostoma* genus and with other fungi, such as white-rot, brown-rot, soft-rot, sapstain species, etc., as to their capabilities of degrading lipophilic compounds of heartwood. It degraded triglycerides and free fatty acids at rates similar to those of Gutiérrez et al.[8] and Rocheleau et al.,[37] while it degraded sterol esters at rates



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>gb|AF493243.1|  Ophiostoma querci CMW2542 18S ribosomal RNA gene, partial sequence;
internal transcribed spacer 1, 5.8S ribosomal RNA gene
and internal transcribed spacer 2, complete sequence; and
28S ribosomal RNA gene, partial sequence
Length=685

 Score = 1160 bits (1286),  Expect = 0.0
 Identities = 655/662 (98%), Gaps = 1/662 (0%)
 Strand=Plus/Plus

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Query  601  CGCATTTTTACAAGGTTGACCCNNGGATCAGGTAGGATTACCCGCTGAACTTAAGCATAT  660
            |||||||||||||||||| ||  ||||||||||||||||||||||||||||||||||||
Sbjct  621  CGCATTTTTACAAGGTTGA-CCTCGGATCAGGTAGGATTACCCGCTGAACTTAAGCATAT  679

Query  661  CA  662
            ||
Sbjct  680  CA  681
```

Figure 7. The DNA sequencing of C3–3 was established as closest match to *Ophiostoma querci.*

better than those of Chen et al.[38] and Leone and Breuil,[39] and were similar to those of Gutiérrez et al.[8] and Martínez-Íñigo et al.[40] Comparing to the Cartapip™, *O. querci* could effectively degrade triglycerides, free fatty acids, sterol esters, and free sterols; however, the Cartapip™ has limited capacity in removing free sterols of broadleaf trees. Based on these results, it appears that *O. querci* has excellent capability in degrading the lipophilic compounds of broadleaf species. Its free sterol-degrading efficacy was also superior to that of Cartapip™. Hence, the species could be considered as a candidate of treatment to remove pitch components of hardwood chips before they were used for pulping.

CONCLUSIONS

The following salient points are observed:

1. When *E. camadulensis* wood chips are pulped using kraft process, more than 90% of theacetone extractives belong to lipophilic fractions. Lipophilic fractions from wood and pulp have compounds such as hydrocarbons, waxes, sterols, steryl ketones, steryl hydrocarbons, steryl esters and triglycerides, fatty acids, fatty alcohols, and other compounds.
2. Twenty-eight strains of suspected *Ophiostoma* spp. fungi were obtained from the cut logs of *E. camadulensis* and used for a depitching study. Among the strains, 6 are capable of reducing lipophilic fractions of wood more than 60%. Of these, the strain C3–3 showed the best degradation efficacy, removing about 70% of all lipophilic content in 2 weeks of incubation. The DNA sequencing result suggested that the strain was an *O. querci.*
3. After inoculation of *O. querci* on wood chips for 2 weeks, lipophilic fractions undergo marked reduction, which varied by the components. *O. querci* strain showed marked reduction of fatty acids and steryl hydrocarbons, and steryl ketones and steryl esters also showed significant reductions. Only the sterol components showed lesser degrees of reduction. Although many sterols remain in the wood, their removal rates still reached 63%, indicating a high efficacy of pitch degradation by the *Ophiostoma querci*. These results bode well for a successful screening of fungi with high capability of removing pitch components of hardwood chips before they were used for pulping.

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