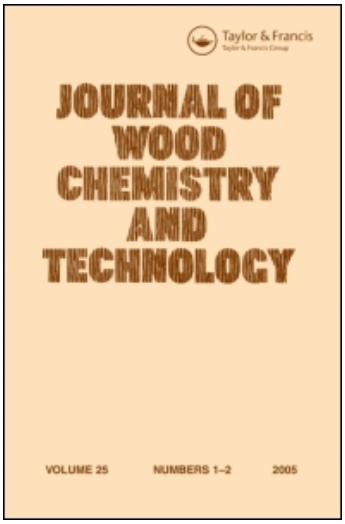

Journal of Wood Chemistry and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597282

Evaluation of the Lignolytic Effect of the White-Rot Fungi Ceriporiopsis Sp, Pleurotus Sp, and Phlebia Sp on Industrial Pinus Radiata Logs

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To cite this Article Tapia, Cristián , Jimenéz, Ivonne , Medina, Jorge , Sapag-Hagar, Jaime , González, Javier and Carmona, René(2005) 'Evaluation of the Lignolytic Effect of the White-Rot Fungi Ceriporiopsis Sp, Pleurotus Sp, and Phlebia Sp on Industrial Pinus Radiata Logs', Journal of Wood Chemistry and Technology, 25: 1, 81 — 94
To link to this Article: DOI: 10.1081/WCT-200058243
URL: http://dx.doi.org/10.1081/WCT-200058243

Journal of Wood Chemistry and Technology, 25: 81–94, 2005

ISSN 0277-3813 print/1532-2319 online
DOI: 10.1081/WCT-200058243

Evaluation of the Lignolytic Effect of the White-Rot Fungi Ceriporiopsis Sp, Pleurotus Sp, and Phlebia Sp on Industrial Pinus Radiata Logs

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Abstract: Pretreatment of pinus *Radiata* logs with the white-rot fungi *Ceriporiopsis sp* (9C), *Pleurotus sp* (9P), and *Phlebia sp* (24P), under field conditions was studied. The lignin content was evaluated by gravimetric techniques (Klason lignin), FTIR spectroscopy, and gas chromatography. A control sample and samples treated for 90 days with each of the fungi were analyzed. The ability of the fungi to degrade the lignin was in the order *Phlebia sp* (24P) > *Ceriporiopsis sp* (9C) > *Pleurotus sp* (9P).

Keywords: Lignin biodegradation, white rot fungi, *Pinus radiata*, FTIR, GC

The authors thank FONDEF for financial support through research grant project No. D97I-2032.

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INTRODUCTION

Biological pulping of wood has the potential to improve the quality of the pulp and the properties of the resultant paper, and reduce the energy costs and the environmental impact relative to traditional pulping operations. The technology has been focused on the white-rot fungi, which have complex extracellular ligninolytic enzyme systems that have potential to selectively remove or alter the wood lignin, facilitating preparation of the cellulose fibers. Scale-up to industrial process requirements present challenges that are difficult to simulate in the laboratory or pilot-scale tests.[1] The white-rot fungi exhibit two gross patterns of decay: (a) a simultaneous decay, in which the cellulose, hemicelluloses, and lignin are removed more or less simultaneously, and (b) delignification, in which the lignin and hemicelluloses are removed from the cellulose.[2] Chile is an important world cellulose producer, with 2 million tons of kraft pulp from Pinus Radiata wood in 2002.[3] Partially because of the pollution potential of the kraft process in Chile research on white-rot fungi has been pursued for over 10 years. *Ceriporiopsis sp* is a well-known fungus that exhibits great ligninolytic activity when inoculated on wood chips of *Radiata* pine. It shows Mn peroxidase and laccase activity.[4] *Ceriporiopsis sp* caused a weight loss of 23.7 $\pm$ 0.9%, a lignin loss of 49.8, and a glucose loss of 3.3% in *Pinus Strobus L.*[5] The high ratio of lignin to glucose loss, that is, 12.5, demonstrates that it is a selective delignifier for pine. Early studies with chips of *Radiata* pine treated with two types of white-rot fungi—*Phlebia sp* and *Ceriporiposis sp*—showed that *Phlebia sp* was more effective for delignification, but that it left less residual hemicelluloses.[6] Typical industrial logs of *Radiata* pine treated with *Pleurotus sp* under laboratory conditions (23°C and 95% of relative humidity), showed an effective delignification compared with a control group.[7] The purpose of this work was to evaluate the pretreatment of pinus *Radiata* logs with the white-rot fungi *Ceriporiopsis sp* (9C), *Pleurotus sp* (9P), and *Phlebia sp* (24P) under field conditions. The lignin content was evaluated by gravimetric techniques (Klason lignin), by FTIR spectroscopy, and by gas chromatography (GC).

EXPERIMENTAL

Materials

The wood samples were from the inner and outer parts of a control log and logs treated for 90 days with *Ceriporiopsis sp* (9C), *Pleurotus sp* (9P), and *Phlebia sp* (24P).

Solid Inoculum Preparation

The fungi (9C, 9P, and 24P) were obtained from the Biodegradation and Wood Laboratory, Forest Sciences Faculty, University of Chile. Each fungus was inoculated over a solid medium (sawdust) under 70–80% relative humidity and were stored in erlenmeyer flasks in an incubation chamber for 3 weeks. The incubation chamber was kept at the ideal temperature for each fungus: 22°C for 9C, 25°C for 9P, and 32°C for 24P. The samples were thoroughly mixed twice a week. After 3 weeks the inoculated samples showed the optimum growing conditions for each fungus, and the hypha of each fungus were selected after an antibiosis assay with their antagonists. In order to obtain a sufficient amount of each fungus for the field study, one sample of 160 kg of fresh *Radiata* pine chips coming from the feed assigned to the industrial digestors was thoroughly mixed and classified. These chips were separated into four samples of approximately 10 kg each. To three of the samples 1% white-rot fungi (9C, 9P, and 24P) was applied with the aid of a sprayer. River water was used for diluting the fungus. One sample was maintained without fungus as a control.

Application of the Solid Inoculum to the Logs

Debarked logs, 2.44 m long with a diameter between 10–20 cm, were used. The logs were supplied by "Celulosa Arauco y Constitución" (CELCO Plant, Chile). Logs taken at random from 1.54 m^3 of solid wood were inoculated at a 3% dose with the solid innoculum. The fungi were dispersed by hand over the logs. Afterwards the logs were covered with plastic sheets to maintain their moisture content.

Sampling Procedure

A control sample and samples exposed to the fungi for 90 days were analyzed. The samples were taken from a couple of logs selected at random from the 1.54 m^3 of solid wood. Discs with a thickness of 0.05 m were cut at 0.15 m from each end of the selected logs. From each disc samples were obtained from the outer and inner zones. The samples were ground in a Wiley mill using a 100 mesh sieve.

Klason Lignin Determinations

The Klason lignin was determined according to TAPPI test method T222 om-88.[8] The analyses were run in triplicate.

FTIR Analysis

The ground samples were mixed with powdered KBr (1:100) and pressed into 10 mm pellets with a Graseby Spacecab press. The infrared spectra from 4000 to 700 cm^{-1} were obtained on a Bruker Model ISF N° 28 FTIR spectrometer operated at a nominal resolution of 1 cm^{-1}. Two samples of the outer and inner sections of the wood treated with 9P and 9C, two samples of the outer and inner sections of the control wood, and one sample of the outer and inner sections of the wood treated with 24P were analyzed. In the case of the outer sample of the wood treated with 9P the analysis was repeated. Some of spectra were void of absorbances at some of the wavelengths of interest, that is, 897, 1267, 1510, and 1734 cm^{-1}.

Gas Chromatographic Analysis

The procedure to quantify the monomers present in the lignin by GC was the reductive cleavage method described by Lou and Ralph[9] to break the α- and β-aryl ether bonds. The degraded products were dissolved in methylene chloride and 1–2 μL of this solution was used for GC analysis. The degraded monomers were quantitatively determined using a Hewlett Packard 5890 Series II plus instrument: column, 0.32 mm × 30 m HP-5 (Hewlett Packard); H_2 carrier gas, 1 mL/min; 30:1 split ratio; injector 220°C; and FID detector, 300°C. The analyses were temperature-programmed: initial temp. 140°C, hold 1 min; 3°C/min to 240°C, hold 1 min; 30°C/min to final temp 310°C, hold 17 min. Total run time 50 min.

The G_t or G_c content is expressed as:

$$G_{t,c} = \frac{(AG^*_{t,c}T^*R)}{(FG^*_{t,c}A^*_tM)} \times (100) \qquad \frac{(\mu g\ G_{t,c})}{(\text{sample mg})}$$

where:

$AG_{t,c}$: trans or cis Guaiacyl area (G_t)

T: Tetracosane (internal standard, IS) Mass (0.4 mg)

R: Attenuation relationship: $\frac{\text{Tetracosane attenuation}}{\text{Guaiacyl}(G_{t,c})\ \text{attenuation}}$: 2

FG_t: Response factor trans or cis Guaiacyl ($G_{t,c}$): 1.85

A_t: Tetracosane area; M: Sample mass (mg); A_t: Tetracosane area; M: Sample mass (mg).

The inner and outer samples of each treated wood (9P, 9C, and 24P) and the control wood were analyzed. Each analysis was carried out in duplicate for the control wood and the wood treated with 24P. The samples of the wood treated with 9P and 9C were analyzed in triplicate.

RESULTS AND DISCUSSION

Figure 1 shows the Klason lignin contents of the inner and outer sections of the control logs and the logs treated with fungi 9P and 9C. Literature values for the Klason lignin of Pinus Radiata wood range between 25 and 28%.[10] No significant differences were found between the sections for each fungus (9Pout/9Pin: 27.4 ± 1.4%/25.1 ± 0.7%; 9Cout/9Cin: 24.5 ± 1.0%/23.9 ± 0.3%, n = 3, $p = 0.05$), between the woods treated with 9P and 9C fungi (9P/9C: 26.2 ± 1.2%/24.2 ± 0.5%, n = 6, $p = 0.05$), or between the treated woods and the control (9P/control: 26.2 ± 1.2%/26.1 ± 2.9%; 9C/control: 24.2 ± 0.5%/26.1 ± 2.9%, n = 6, $p = 0.05$). However, based on the mean values of the Klason lignin, it appeared that there was a slight delignification effect of the 9C fungus.

The lignin in the samples of the logs treated with fungus 9C, 9P, and 24P was also evaluated by infrared spectroscopy (FTIR) of the extractives-free wood. As the part of the purpose was to evaluate if there was an orientation for the attack of the fungus, the IR analysis was used for the different sampling zones. The spectra of the samples of the inner sections of the control wood were compared with the corresponding samples of the fungally treated woods. A similar comparison was done for samples of the

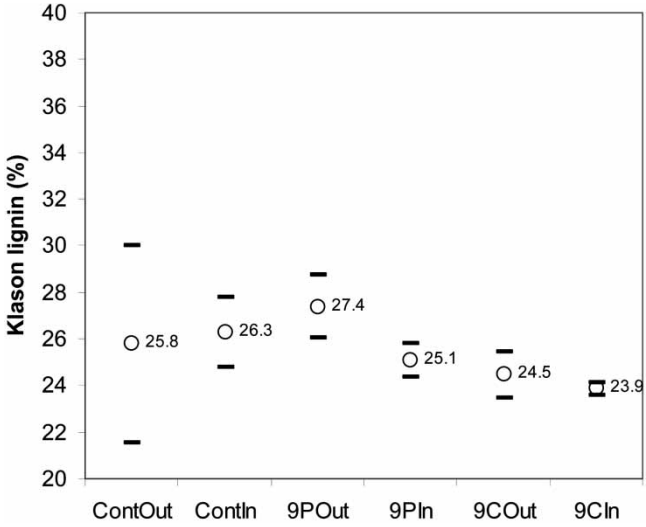

Figure 1. Klason lignin of the control log and logs treated with fungi 9P and 9C. **ContOut**: outer sample of control log; **ContIn**: inner sample of control log; **9POut**: outer sample of logs treated with *Pleurotus sp*; **9PIn**: inner sample of logs treated with *Pleurotus sp*; **9COut**: outer sample of logs treated with *Ceriporiopsis sp*; **9CIn**: inner sample of logs treated with *Ceriporiopsis sp*. Each point represents the mean of three samples. Each bar represents the 95% confidence interval.

outer sections of the woods. The absorption bands[11] that give the best structural information are as follows: 1734 cm^{-1} related to the stretching vibration of the carbonyl group in the glucuronoxylans, which is a good indicator of what happens to the polyoses; 1510 cm^{-1} associated with the lignin aromatic ring vibration; 1267 cm^{-1} associated with the guaiacyl ring; and 897 cm^{-1} associated to the glucose units present in cellulose.

Figure 2 shows the lignin removal effect of fungi 9P, 9C, and 24P, in the outer part of the log. There was a statistically significant reduction in the absorption band at 1267 for 9P: 0.65 $\pm$ 0.04 cm^{-1} (n = 15, $p = 0.05$); 9C: 0.63 $\pm$ 0.08 cm^{-1} (n = 10, $p = 0.05$); and 24P: 0.58 $\pm$ 0.05 cm^{-1} (n = 5, $p = 0.05$); relative to the control sample: 0.82 $\pm$ 0.06 cm^{-1} (n = 10, $p = 0.05$). There was also a statistically significant reduction in the absorption band at 1510 cm^{-1} for 9P: 0.72 $\pm$ 0.04 cm^{-1} (n = 20, $p = 0.05$); 9C: 0.72 $\pm$ 0.08 cm^{-1} (n = 15, $p = 0.05$); and 24P: 0.62 $\pm$ 0.04 cm^{-1} (n = 5, $p = 0.05$); relative to the control sample: 0.85 $\pm$ 0.03 cm^{-1} (n = 10, $p = 0.05$). There were no statistically significant differences in the delignification effect between logs

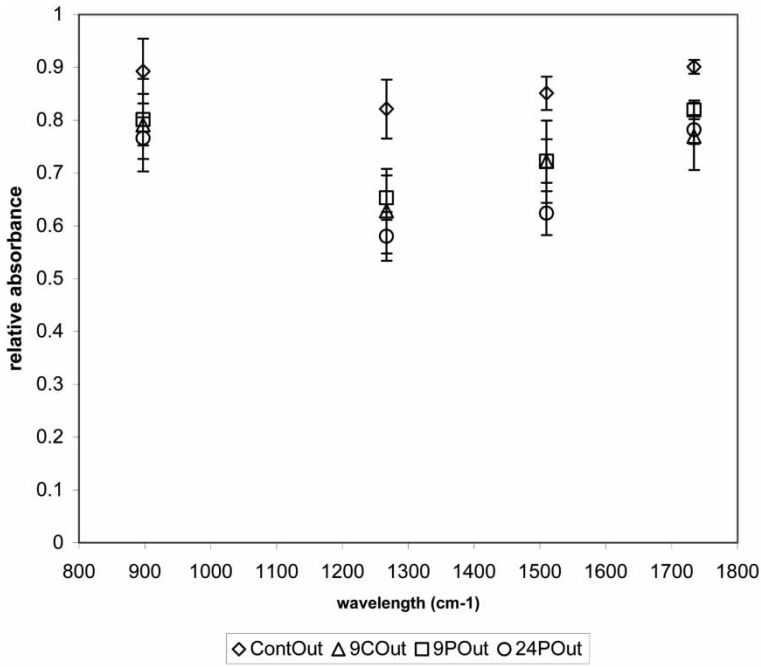

Figure 2. Relative FTIR absorbance values of the samples obtained from the outer section of the logs. **ContOut**: outer sample of control log (n = 10); **9COut**: outer sample of logs treated with *Ceriporiopsis sp* (n = 15); **9POut**: outer sample of logs treated with *Pleurotus sp* (n = 20); **24POut**: outer sample of logs treated with *Phlebia sp* (n = 5). Each point represents the mean. Each bar represents the 95% confidence interval.

treated with the different fungi, but the average values for the 24P fungus were lower compared to that of 9P and 9C fungi.

There was also a significant effect on the polyoses because there was a statistically significant reduction in the absorption band at $1734\,cm^{-1}$ for 9P: $0.82 \pm 0.02\,cm^{-1}$ ($n = 20$, $p = 0.05$); 9C: $0.77 \pm 0.06\,cm^{-1}$ ($n = 15$, $p = 0.05$); and 24P: $0.78 \pm 0.03\,cm^{-1}$ ($n = 5$, $p = 0.05$); relative to the control sample: $0.90 \pm 0.01\,cm^{-1}$ ($n = 10$, $p = 0.05$). The absorption band related to glucose ($897\,cm^{-1}$) was not significantly different between the fungi and the control, although the average values for logs treated with fungi were lower that that of the control log, that is 9P: $0.80 \pm 0.05\,cm^{-1}$ ($n = 20$, $p = 0.05$); 9C: $0.79 \pm 0.09\,cm^{-1}$ ($n = 15$, $p = 0.05$); and 24P: $0.77 \pm 0.04\,cm^{-1}$ ($n = 5$, $p = 0.05$); versus the control: $0.89 \pm 0.06\,cm^{-1}$ ($n = 10$, $p = 0.05$).

Figure 3 shows the relative IR spectra in the $1200–1800\,cm^{-1}$ range for the samples from the outer part of the logs. The figure illustrates the large decrease in the bands associated with lignin (1510 and $1267\,cm^{-1}$), and polyoses ($1734\,cm^{-1}$) for the 24P fungus, relative to the control.

Figure 4 shows that the lignin removal effect of 9P and 9C fungi does not extend to the inner part of the logs, because the absorption bands at 1257 and

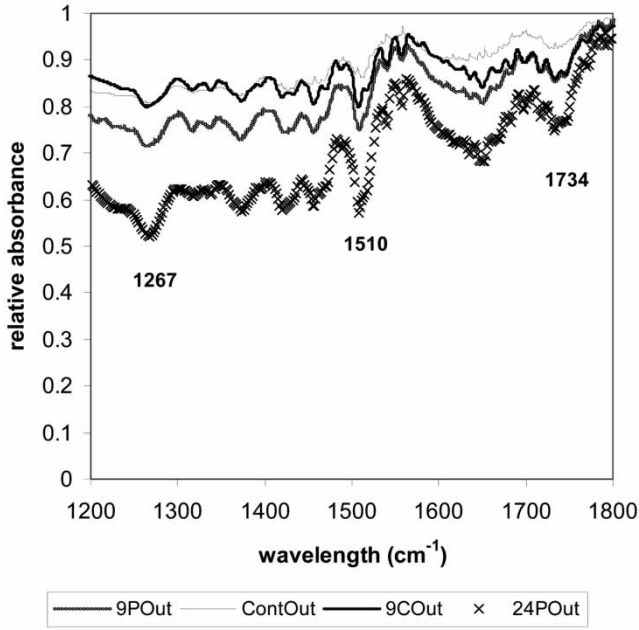

Figure 3. IR spectra between $1200–1800\,cm^{-1}$ for samples from the outer section of the logs. **ContOut**: outer sample of control logs; **9POut**: outer sample of logs treated with *Pleurotus sp*; **9COut**: outer sample of logs treated with *Ceriporiopsis sp*; **24POut**: outer sample of logs treated with *Phlebia sp*.

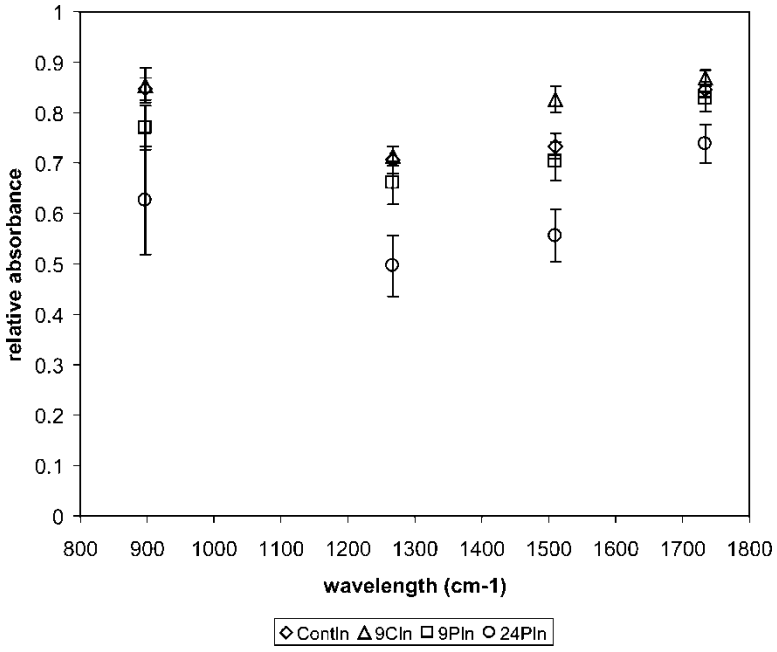

Figure 4. Relative absorbance values of the samples obtained from the inner part of the logs. ContIn: internal sample of control log; 9CIn: inner sample of logs treated with *Ceriporiopsis sp*; 9PIn: inner sample of logs treated with *Pleurotus sp*; 24PIn: inner sample of logs treated with *Phlebia sp*. Each point represents the mean. Each bar represents the 95% confidence interval. The sample populations were: ContIn n = 10, 9PIn n = 15, 9CIn n = 15, and 24PIn n = 5.

1510 cm^{-1} do not show values significantly lower than that of the reference. In addition, neither the cellulose nor polyoses were affected because there were no significant differences in the bands at 897 and 1734 cm^{-1} compared to the control. The 24P fungus was more aggressive than the 9P and 9C fungi, because it was able to attack the lignin and polyoses at the inner portion of the log. Thus, logs treated with 24P fungus showed significantly lower absorption bands at 1267, 1510, and 1734 cm^{-1} (1267 cm^{-1} values: 24P: 0.50 $\pm$ 0.06 cm^{-1} (n = 5, $p = 0.05$); 9P: 0.66 $\pm$ 0.04 cm^{-1} (n = 15, $p = 0.05$); 9C: 0.71 $\pm$ 0.02 cm^{-1} (n = 15, $p = 0.05$); control: 0.71 $\pm$ 0.03 cm^{-1} (n = 10, $p = 0.05$); 1510 cm^{-1} values: 24P: 0.56 $\pm$ 0.05 cm^{-1}; 9P: 0.70 $\pm$ 0.04 cm^{-1}; 9C: 0.83 $\pm$ 0.03 cm^{-1}; control: 0.73 $\pm$ 0.03 cm^{-1}; 1734 cm^{-1} values: 24P: 0.74 $\pm$ 0.04 cm^{-1}; 9P: 0.83 $\pm$ 0.03 cm^{-1}; 9C: 0.87 $\pm$ 0.02 cm^{-1}; control: 0.85 $\pm$ 0.02 cm^{-1}).

Thus, the FTIR analyses of the extractive-free wood treated with 9P, 9C, and 24P fungi, showed that the fungi degraded the guaiacyl fraction of lignin at the outer position of the log but, except for the 24P fungus, did not affect the lignin in the inner part of the log.

In order to further examine this conclusion the samples were analyzed by the GC technique described in the Methods section. This procedure can be used to analyze the guaiacyl component of the lignin as its cis and trans isomers. To establish the retention time and then quantify the cis and trans forms of the coniferyl alcohol diacetate, designated Gc and Gt, respectively, the reported retention times[9] were considered, and, additionally, a standard was prepared from coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol), which was acetylated with acetic anhydride/pyridin. The retention times reported for Gc and Gt and those obtained for the standard are presented in Table 1. Table 1 indicates that there was good agreement between the retention times of the guaiacyl cis an trans isomers described by Lu and Ralph[9] and those obtained for the standard under the chromatographic conditions used in our laboratory.

Then, using the same sampling as in the FTIR study, the G_c and G_t contents were determined for fungally treated (9P, 9C, and 24 P) logs (outer, inner) relative to the control log. The results, as presented in Figure 5, clearly show that the G_t content is significatively lower in the wood treated with the 9C ($4.44 \pm 0.40\,\mu g/mg$, $n = 6$, $p = 0.05$) and 24P fungi ($4.40 \pm 0.44\,\mu g/mg$, $n = 4$, $p = 0.05$) compared to the control ($6.64 \pm 0.89\,\mu g/mg$, $n = 4$, $p = 0.05$). The monolignol coniferyl alcohol diacetate is mainly in its trans form (see Figure 6). That is why the G_t value has greater analytical value in checking the effect of the fungus on the lignin. Figure 7 shows the monolignol contents in the inner section of log. Again the G_t content is significatively lower for the logs treated with 9C ($4.03 \pm 0.29\,\mu g/mg$, $n = 6$, $p = 0.05$), and 24P fungi ($1.49 \pm 0.19\,\mu g/mg$, $n = 4$, $p = 0.05$) compared to the control log ($5.99 \pm 0.70\,\mu g/mg$, $n = 4$, $p = 0.05$). These results indicate that these fungi degrade the lignin as much in the inner as in the outer sections of the log. In order to corroborate the results indicated by the IR and GC analyses, that is, that fungi 9C and 24P are effective at delignification, the laccase and manganese peroxidase enzymes of these fungi were evaluated according to the method used by Ruttimann-Johnson.[12]

Table 1. Retention times (t_r) for G_c and G_t, internal standard, and those reported by Lu and Ralph[9]

Guaiacyl isomers	t_r reported[9]	Standard coniferyl alcohol diacetate (t_r) X ± IC (95%, n = 3)
G_c	19–21 min	19.6 ± 0.11 min
G_t	23–25 min	25.1 ± 0.24 min
Tetracosane Internal standard	35 min	35.9 ± 0.01 min

G_c: guaiacyl trans; G_t: guaiacyl cis.

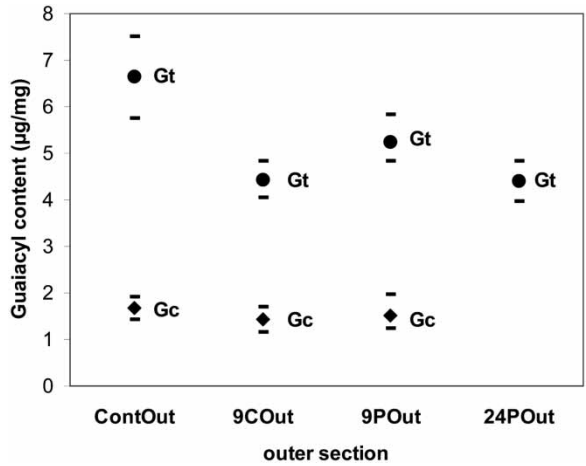

Figure 5. Guaiacyl content in the outer section of log determined by GC analysis of samples from the control log, and the logs treated with the 9P, 9C, and 24P fungi. G_c: guaiacyl cis; G_t: guaiacyl trans; ContOut: outer sample of control log; 9POut: outer sample of logs treated with *Pleurotus sp*; 9COut: outer sample of logs treated with *Ceriporiopsis sp*; 24POut: outer sample of logs treated with *Phlebia sp*. Each point represents the mean. Each bar represents the confidence interval with a significance level of 0.05. The size of sample populations were: ContOut n = 4, 9COut n = 6, 9POut n = 6, 24POut n = 4.

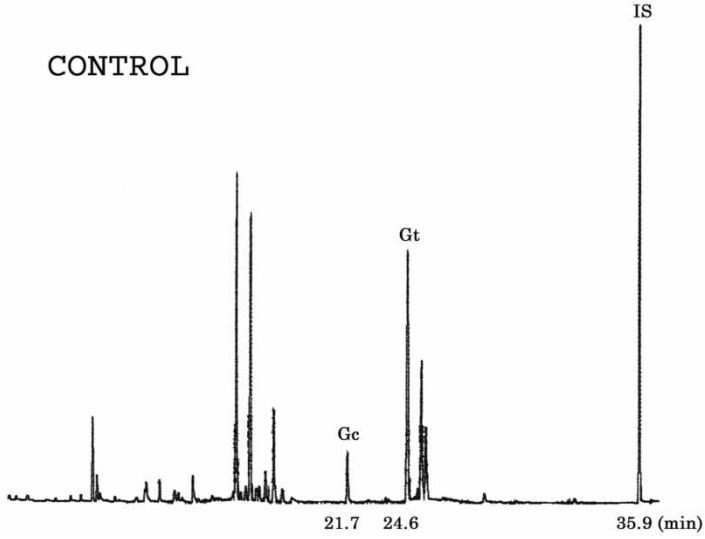

Figure 6A. GC Analysis of the outer section of the control log. G_c: guaiacyl cis; G_t: guaiacyl trans; IS: tetracosane internal standard.

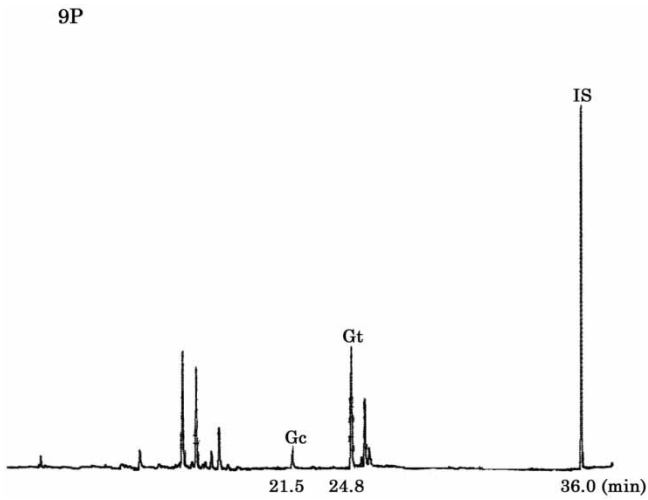

Figure 6B. GC Analysis of the outer section of the log treated with fungus 9P, *Pleurotus sp*. G_c: guaiacyl cis; G_t: guaiacyl trans; IS: tetracosane internal standard.

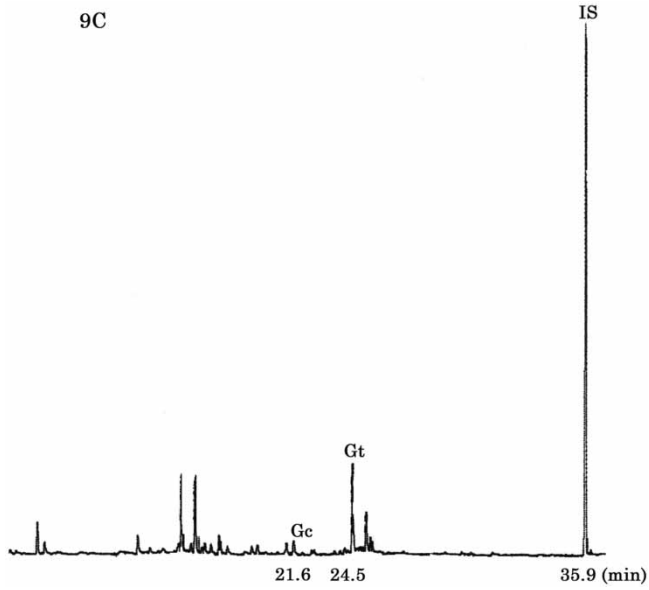

Figure 6C. GC Analysis of the outer section of the log treated with fungus 9C, *Ceriporiopsis sp*. G_c: guaiacyl cis; G_t: guaiacyl trans; IS: tetracosane internal standard.

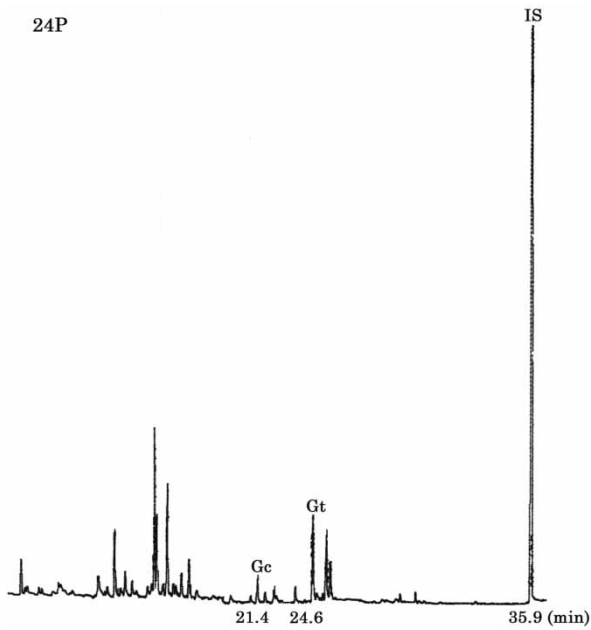

Figure 6D. GC Analysis of the outer section of the log treated with fungus 24P, *Phlebia sp*. G_c: guaiacyl cis; G_t: guaiacyl trans; IS: tetracosane internal standard.

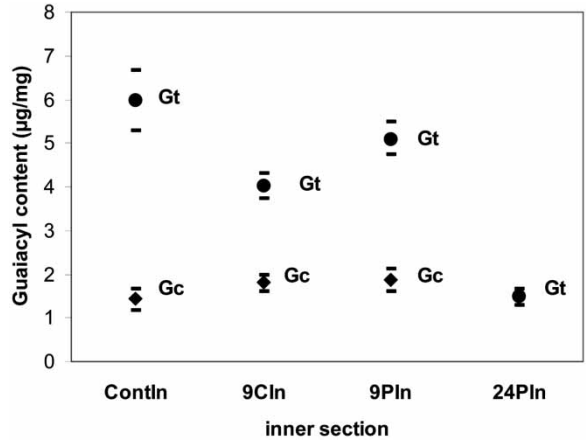

Figure 7. GC Analysis of the inner sections of the logs. G_c: guaiacyl cis; G_t: guaiacyl trans; ContIn: inner sample of the control logs; 9PIn: inner sample of logs treated with *Pleurotus sp*; 9CIn: inner sample of logs treated with *Ceriporiopsis sp*: 24PIn: inner sample of logs treated with *Phlebia sp*. Each point represents the mean. Each bar represents the 95% confidence interval. The sample populations were: ContIn n = 4, 9CIn n = 6, 9PIn n = 6, 24PIn n = 4.

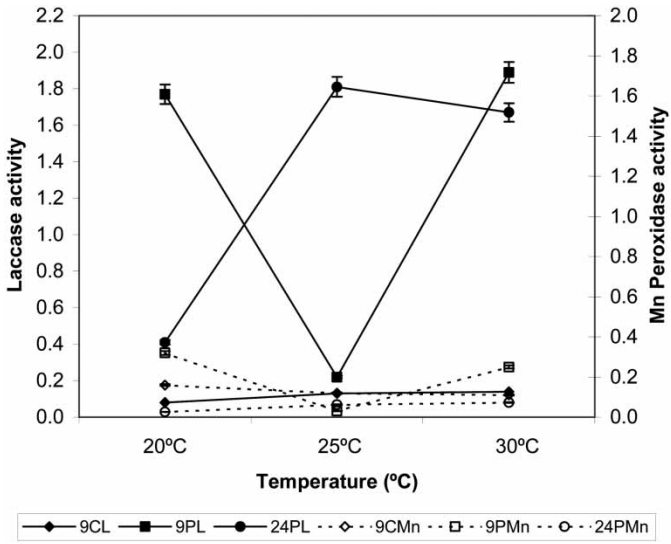

Figure 8. Laccase and manganese peroxidase activity of the 9P, 9C, and 24P fungi, at different temperatures. 9CL: laccase activity of *Ceriporiopsis sp*; 9PL: laccase activity of *Pleurotus sp*; 24PL: laccase activity of *Phlebia sp*; 9CMn: Mn peroxidase activity of *Ceriporiopsis sp*; 9PMn: Mn peroxidase activity of *Pleurotus sp*; 24PMn: Mn peroxidase activity of *Phlebia sp*. Each point represents the mean of three samples. Each bar represents the standard deviation.

Figure 8 shows the laccase and manganese peroxidase for 9C, 9P, and 24P fungi in the solid inoculum. Clearly it can be observed that laccase activity is greater than the Mn peroxidase activity, and that the laccase of 24P and 9P fungi is greater than that of 9C fungus. Moreover, it is seen that laccase activity of these fungi is greatly dependent on temperature. As the biodegradation studies of logs were done under field conditions, the temperature of biodegradation was not controlled. According to the FTIR data, the temperature in the logs should have been around 25°C due to the greater degradation observed in the logs treated with 24P.

CONCLUSIONS

Treatment of *Radiata* pine logs with the fungus *Ceriporiopsis sp* (9C) resulted in a lower percentage of Klason lignin relative to the control logs. FTIR analysis indicated a reduction in the 1510 and 1267 cm^{-1} bands, associated to guaiacyl component of lignin, in the outer part of the log compared to the control. GC analysis showed a decrease of the guaiacyl component in both the outer and inner sections of the logs. In the case of fungus *Pleurotus sp* (9P) FTIR analysis only indicated a significative decreasing of

the 1510 and 1267 cm^{-1} bands in the outer section of the logs relative to the control logs. The fungus *Phlebia sp* (24P) caused a significative reduction of the lignin, as evaluated by FT-IR and GC, in both the inner and outer sections of the treated log compared with the control logs. Fungus 24P had the greatest laccase activity at around 25°C, and the 9P and 9C fungi had similar but reduced laccase activities. Considering all of the data, the lignin degrading capability of the fungi is in the order 24P > 9C > 9P.

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