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The Degradation of Wood and Pulp by Wood-degrading Fungi

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ABSTRACT

Degradations of pine, yellow poplar and sweet gum by two fungi, *Pycnoporus cinnabarinus* and *Trichophyton rubrum* LSK-27 were investigated. *P. cinabarinus* degraded pine block samples much faster than *T. rubrum* LSK-27, whereas *P. cinnabarinus* and *T. rubrum* LSK-27 degraded yellow poplar and sweet gum at almost the same rate. In an effort to get a better understanding of how fungi degrade lignin in wood, contents of various functional groups were analyzed. After three-months of degradation of pine flour by these fungi, the following changes were observed: an increase in condensed phenolic OH group and carboxylic acid group content, a decrease in the guaiacyl phenolic OH content, and little change of aliphatic OH grou p content. Further studies in the degradation of pine flour by *P. cinnabarinus* indicated that the increase in condensed phenolic OH group content and the decrease in guaiacyl phenolic OH group content occurred in the first month of the degradation. The changes of functional group contents in the degradation of unbleached softwood kraft pulp by *P. cinnabarinus* had the same trends as those in the degradation of pine flour. That is, structural alteration of lignin due to the kraft pulping process had little effect on how *P. cinnabarinus* degraded lignin.

Keywords: *Pycnoporus cinnabarinns, Trichophyton rubrum LSK-27, lignin, functional group*

1. Introduction

Lignin is the second most abundant renewable biopolymer on earth and its biodegradation is of great importance for the global carbon cycle. Selective biodegradation of lignin has drawn particular attention because lignin is one of the major obstacles for the efficient utilization of lignocellulosic materials in many industries such as the pulp and paper industry and the animal feed industry. White-rot fungi are the only microorganisms able to selectively and efficiently degrade lignin in natural environments. However, the

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mechanisms by which white-rot fungi degrade lignin are still poorly understood. It is believed that three types of potentially ligninolytic enzymes, lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase, are involved in the fungal degradation of lignin. Extensive studies reveal that these enzymes degr ade lignin in different ways. For instance, LiP is able to degrade non-phenolic lignin substructures that account for 90% of all lignin structures in wood, whereas MnP and laccase are only able to degrade phenolic lignin structures that accounts for only about 10% of all lignin structures in wood (1). White-rot fungi secrete one or more of these three enzymes for lignin degradation, which suggests that there is likely more than one mechanism for fungal degradation of lignin.

It has been using the white rot fungus *Pycnoporus cinnabarinus* as a model white-rot fungus for studying mechanisms of fungal degradation of lignin because of its simple ligninolytic enzyme system. Only one of the three ligninolytic enzymes, laccase, is produced in the degradation of lignocellulosic materials by *P. cinnabarinus* although the fungus can degrade lignin efficiently (2-5). It is well established that laccase can only oxidize non-phenolic lignin substructures in the presence of a redox mediator (6-8). Our recent studies revealed that *P. cinnabarinus* was able to degrade non-phenolic lignin substructures (9). However, identification of a natural mediator compound that enables laccase to degrade non-phenolic lignin substructures remains elusive despite extensive efforts (10). We also investigated newly isolated wood-degrading fungus, *Trichophyton rubrum* LSK-27, for its ability to degrade lignin (11,12).

In this study, we investigated the abilities of *P. cinnabarinus* and *T. rubrum* LSK-27 to degrade wood and unbleached kraft pulp. We analyzed the changes in various functional group in pine and kraft lignin during the degradation of wood and unbleached kraft pulp by these fungi.

2. Materials and Methods

2.1 Microorganisms

P. cinnabarinus, strain PB (ATCC 204166) and *T. rubrum* LSK-27 were used in this study. *T. rubrum* LSK-27 was isolated from a decayed hardwood chip pile in the vicinity of Chonnam, Korea. This fungus was identified through a GC-FAME (gas chromatography-fatty acid methyl ester) method (Microbe Inotech Labs, Inc., St. Louis, MO). When searched against the database of Microbe Inotech Labs, Inc., the most similar fungal species for this fungus was *Trichophyton rubrum.*

P. cinnabarinus and *T. rubrum* LSK-27 were separately inoculated on PDA plates and incubated at 30℃ for 5 days before inoculated on wood blocks. *P. cinnabarinus* on a PDA plate was inoculated on autoclaved long-grain polished rice (60 g/l flask) for production of fungal spores. After cultivation at 30℃ for 8 days, the fungal spores were extracted with sterile 0.9% (w/v) NaCl solution, filtered with cheesecloth to remove the rice, and extensively washed with sterile 0.9% (w/v) NaCl solution (4). The spore suspension contained about 3 x 107 spore/ml and was used for the degradation of pine wood flour.

2.2 Degradation of wood blocks and pine flour by two fungi

Glass chambers (300 ml) were half-filled with moist garden loam followed by addition of 50 ml tap water. An Alder feeder block (3 cm

× 3 cm × 2 cm) was put on the surface of the soil and autoclaved at 121℃ for 45 min. After it was cooled down to room temperature, the feeder block in the soil-containing chamber was inoculated with a PDA disc (6 mm) taken from the hyphal tip in a fungus-growing PDA plate, and incubated at 30℃ until the fungus had thoroughly colonized the feeder. A wood block specimen (1 cm × 1 cm × 1cm) cut from pine sapwood, sweet gum or yellow poplar was dried at 105℃ overnight, cooled in a desiccator, and then weighed. All wood block specimens were autoclaved at 121℃ for 45 min, cooled in a desiccator and placed on the corner of the feeder block in the chamber. The yellow poplar and sweet gum block specimens were incubated for 2 months at 30℃, while the pine block specimens were incubated for 3 months at 30℃. After the mycelia were carefully removed, the decayed wood block specimens were rinsed with water, dried at 105℃, and then weighed to determine the weight loss. Nine replicates were investigated for each fungus on each wood species.

Pondarosa pine sapwood flour (30 mesh) was extracted with acetone/water (v/v, 9/1) and dried under vacuum. The moisture content was determined as 9.8 %. A mixture of wood flour (9.07 g oven-dried) and de-ionized water (10 ml) was autoclaved in 250 ml Erlenmeyer flask at 121℃ for 1 h. The autoclaved wood flour was inoculated with the *P. cinnabarinus* spore suspension (5 ml), and the moisture content of the wood flour adjusted to 80% with sterilized water. The culture was then incubated at 30℃ for pre-determined times.

Twelve PDA discs (6 mm) of the pre-incubated *T. rubrum* LSK-27 were inoculated with 250 ml of cultivation medium in a 1-L flask and incubated at 30℃ in a shaker (120-150 rpm, 18 mm). The culture medium (per liter) for *T. rubrum* LSK-27 contains 30 g glucose, 10 g Bacto-peptone, 1.5 g KH_2PO_4, 0.5 g $MgSO_4$, 20 mg $CuSO_4$, 50 mg $MnSO_4$ and 10 mg thiamine-HCl. The fungal mycelia were filtered with filter paper, washed with sterilized water and then homogenized in 40 ml water. Mycelial suspension (5 ml) was inoculated on pine flour (3.61 g, oven-dried) in a 125 ml flask and the moisture content adjusted to 83%.

After a predetermined incubation time at 30℃, fungal mycelia were carefully removed from wood flour, and wood flour was dried under vacuum at 40℃ for further analyses.

2.3 Degradation of unbleached softwood kraft pulp by *P. cinnabarinus*

Unbleached softwood kraft pulp (USKP) from Douglas fir was obtained from Pope and Talbot (Halsey, Oregon) and washed with deionized water until the effluent was neutral and colorless. USKP (8.0 g, oven-dried) was autoclaved at 121℃ for 40 min, cooled down at room temperature and then inoculated with 3 ml of *P. cinnabarinus* spore suspension (ca. 9 x 10^7 spore/ml). The moisture content of the culture was adjusted to 80% and the culture was incubated statically at 30℃. After a pre-determined incubation time, mycelia were carefully removed from pulp. The treated pulp was analyzed for kappa number.

2.4 Isolation of lignin from wood and pulp

Lignin in pine flour was isolated before and after the fungal degradation with an acidolysis method, i.e., the pine flour samples (10 g) were refluxed under nitrogen for 2 h with 200 ml 0.1 M HCl in dioxane/water (85/15, v/v) at solvent/wood weight ratio 20:1.13) The dioxane-water extract was neutralized with solid sodium bicarbonate. Dioxane in the

extract was removed through a rotary evaporator, and lignin left in the solution was precipitated in water (500 ml, pH 2-3). The precipitated lignin was collected by centrifugation, washed with acidified water (pH 2-3) and then freeze-dried.

The pulp samples before and after the fungal degradation were air-dried and milled with Wiley Mill to flour (30 mesh). Lignin in pulp was isolated with the acidolysis method described previously. Lignin extracted from pine flour and the milled pulp before fungal degradation was used as the control.

2.5 Quantitative analysis of lignin functional groups with ^{31}P NMR

Lignin structures were characterized with ^{31}P NMR according to established methods (13,14). Accurately weighed lignin (40 mg/ml) together with cholesterol (3.5 mg/ml, an internal standard) and chromium acetylacetonate (5.0 mg/ml, relaxation reagent) were dissolved in a mixture of pyridine/$CDCl_3$ (1.6/1) in a 4-ml vial with magnetic stirring. The phosphorylation agent 2-chloro-4, 4, 5, 5-tetramethyl-1, 3, 2-dioxaphospholane (100 $\mu\ell$) was added and stirred for 20 min. The resulting solution was then transferred to a 5 mm NMR tube for the acquisition of ^{31}P NMR spectra. The ^{31}P NMR spectra were acquired by the inverse gated decoupling sequence on a Bruker 300 MHz NMR spectrometer (10,14). All chemical shifts in this paper were referenced by the reaction product of water with the phosphorylation agent, which gives a sharp ^{31}P NMR signal at 132.2 ppm in pyridine/$CDCl_3$ (15,16). The integral value of the internal standard (^{31}P NMR signals at 146.1 ppm) was used to calculate the amount of various hydroxyl groups. The peaks of quantitative ^{31}P NMR spectra were assigned as follows: aliphatic alcoholic units 149.6-146.4 ppm; condensed phenolic units 145.7-141.6 ppm; guaiacyl phenolic units 141.6-140.1 ppm; and carboxylic acids 136.8-135.8 ppm.

3. Results and Discussion

3.1 Weight loss of decayed wood blocks

After three months of degradation, the weight losses of pine blocks caused by *P. cinnabarinus* and *T. rubrum* LSK-27 were 87.6% and 39.2%, respectively (Table 1). Because lignin content in pine is at least 30%, a weight loss of 87.6% implies that *P. cinnabarinus* efficiently degrade all wood components including lignin.

Table 1. Weight loss of pine blocks caused by wood-degrading fungi after a three-month incubation*

	P. cinnabarinus	*T. rubrum* LSK-27	Control
Weight loss (%)	87.6±2.7	20.8±1.5	2.3±0.5

*data were the mean values of nine wood samples.

The ability of these two fungi to degrade the hardwoods, yellow poplar and sweet gum, was also investigated (Table 2). The weight losses of yellow poplar blocks after two months of degradation by *P. cinnabarinus* and *T. rubrum* LSK-27 were 30.1% and 39.2%, respectively. Statistically, *P. cinnabarinus* and *T. rubrum* LSK-27 degraded yellow poplar blocks at the same rate. It is perplexing that *P. cinnabarinus* degraded pine blocks much more efficiently than *T. rubrum* LSK-27, whereas *P. cinnabarinus* and *T. rubrum* LSK-27 degraded hardwood blocks (yellow poplar and sweet gum) at the same rate.

Table 2. Weight loss of hard wood blocks caused by wood-degrading fungi after a two-month incubation*

	P. cinnabarinus	*T. rubrum* LSK-27	Control
Yellow ceder	30.1±9.5	39.2±1.5	0.9±0.3
Sweet gum	24.8±4.6	28.4±4.4	0.23±0.1

*data were the mean values of nine wood samples.

3.2 Fungal modification of lignin structures in wood

Activities of three ligninolytic enzymes, lignin peroxidase, manganese peroxidase, and laccase were detected during the incubation of pine flour with *T. rubrum* LSK-27, whereas only laccase activity was detected with *P. cinnabarinus* (data not shown). It appeared that these two fungi used different ligninolytic enzyme systems for lignin degradation. To gain a better understanding of the mechanism by which these fungi degraded lignin, we investigated the structural changes of lignin during the fungal degradation of wood. Pine flour samples rather than pine block samples were used for the fungal degradation experiments so that lignin could be directly and efficiently extracted with dioxane/water from the decayed wood residue without a post-degradation grinding because the grinding may cause structural changes in the lignin. As shown in Fig. 1, aliphatic hydroxyl groups in lignin mainly refer to those hydroxyl groups of compound I. Compound II is a representative lignin substructure with condensed phenolic hydroxyl groups. Compound III is a representative lignin substructure containing guaiacyl phenolic hydroxyl groups.

Extracted lignin samples were analyzed with ^{31}P NMR for the content of various functional groups. When compared with the control, the contents of condensed phenolic OH and carboxylic acid groups increased, the content of guaiacyl phenolic OH groups decreased, and the contents of aliphatic OH groups remained statistically the same after a three-months degradation of pine flour by *P. cinnabarinus*. For *T. rubrum* LSK-27, the trends in functional group changes were the same as those for *P. cinnabarinus*, i.e., the contents of the aliphatic OH groups were basically the same, the contents of condensed phenolic OH and carboxylic acid groups slightly increased; and the content of the guaiacyl phenolic hydroxyl groups decreased. Pine lignin is predominately derived from coniferyl alcohol. In pine lignin, about 10% of lignin substructures are phenolic, i.e., those substructures with a guaiacyl phenolic hydroxyl group, whereas about 90% of lignin substructures are non-phenolic, i.e., the guaiacyl phenolic hydroxyl group is covalently linked to the lignin matrix. It is well established

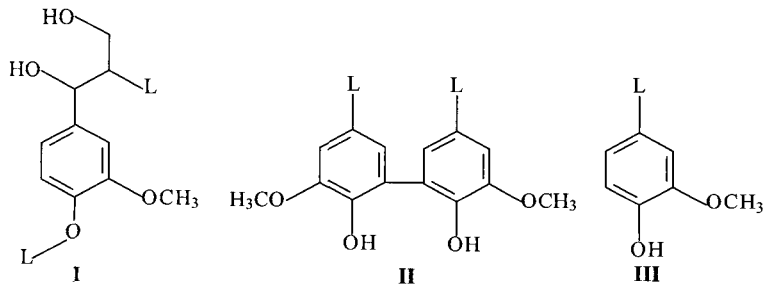

Fig. 1. Representative lignin substructures (L=lignin matrix).

Table 3. The contents of various functional groups(mmol/g lignin) after a three-month degradation of pine flour by three fungi as determined by ^{31}P NMR techniques*

	Aliphatic OH	Phenolic OH		
		Condensed	Guaiacyl	R-COOH
Control	3.80±0.37	0.35±0.20	1.19±0.04	0.07±0.04
P.c[a]	4.31±0.34	0.54±0.10	0.78±0.03	0.20±0.04
T.[b] LSK-27	3.78±0.34	0.39±0.02	0.80±0.01	0.16±0.01

* data were the mean values of nine wood samples.
a *P.c* refers to *Pycnoporus cinnabarinus*
b *T.* refers to *T. rubrum*

that a phenolic hydroxyl group is easy to oxidize. In other words, a phenolic lignin substructure is easier to oxidize than a non-phenolic lignin substructure with the exception that condensed lignin structures are especially difficult to degrade because of the carbon-carbon linkages between two phenylpropane units. The ^{31}P NMR results indicated that two fungi used in this study all caused decreases in the guaiacyl phenolic OH group content and increases in the condensed phenolic hydroxyl group content, which implied that these fungi first degraded the easily oxidizable lignin substructures with guaiacyl phenolic OH group and left the condensed lignin substructures behind regardless of the ligninolytic enzymes they secreted for lignin degradation. Little change of the aliphatic OH groups suggested that the oxidization of the phenylpropane side chain in the pine lignin was not the important action mode in the degradation of lignin by these fungi. The increase in the carboxylic acid content probably resulted from the ring-opening reactions in the oxidation of the guaiacyl phenolic substructure.

The previously proposed action modes for the fungal degradation of lignin were derived from a three-month fungal degradation of pine flour. Additional studies were performed to determine the effect of incubation time, i.e., the degree of wood degradation. Results in Table 4 revealed that the incubation time had little effect on the change of the aliphatic OH content. The condensed phenolic OH content significantly increased in the first month of the incubation, remained at the increased level during the second and the third months of the incubation, and then decreased after the fifth month of the incubation. In contrast with the significant increase of the condensed phenolic structures, there was a significant reduction of the guaiacyl phenolic OH content after one

Table 4. Effects of incubation time on the contents of various functional groups (mmol/g lignin) in the degradation of pine flour by *P. cinnabarinus**

Incubation time (month)	Aliphatic OH	Phenolic OH		
		Condensed	Guaiacyl	R-COOH
Control	3.80±0.37	0.35±0.20	1.19±0.04	0.07±0.04
1	3.74±0.33	0.54±0.10	0.81±0.04	0.17±0.04
2	3.75±0.33	0.58±0.00	0.80±0.00	0.21±0.03
3	4.31±0.34	0.54±0.10	0.78±0.03	0.20±0.01
5	3.71±0.14	0.38±0.01	0.67±0.00	0.20±0.01

*data were the mean values of two wood samples.

months incubation. The guaiacyl phenolic OH content remained unchanged between one month and three months of incubation. However, the guaiacyl phenolic OH content further decreased when the pine flour samples were incubated with *P. cinnabarinus* for five months. Carboxylic acids content gradually increased in the first two months of the fungal degradation and remained constant thereafter.

It appeared that some easily accessible guaiacyl phenolic substructures were preferentially degraded in the first month, which led to the increase in the relative content of the condensed phenolic substructures. Lignin is a complex polymeric material. Guaiacyl phenolic substructures are widely distributed in the lignin matrix. Therefore, some guaiacyl phenolic substructures were not readily accessed by the fungus *P. cinnabarinus*, which accounts for the detection of a significant amount of the guaiacyl phenolic OH group even after five months of degradation.

It is not clear whether all lignin substructures including the predominant non-phenolic lignin substructures and the condensed phenolic substructures were degraded at approximately the same rate after the readily accessible guaiacyl phenolic substructures were degraded in the first month of the incubation. The small changes in the guaiacyl phenolic OH group content during one to three months of the fungal degradation appeared to suggest that the fungal degradation of guaiacyl phenolic substructures were controlled by the accessibility of these substructures to the fungus. The relatively constant level of the condensed phenolic substructures appeared to suggest that the condensed phenolic substructures were also degraded at the time when non-phenolic lignin substructures were degraded.

3.3 Fungal modification of lignin structures in unbleached softwood kraft pulp

The structures of lignin in unbleached softwood kraft pulp (USKP) were significantly altered during the Kraft pulping process. We investigated whether the alteration of lignin structures had effects on the action modes of degradation for P. cinnabarinus (Table 5). In contrast to the initial increase in the condensed phenolic OH group content in the degradation of pine flour, the condensed phenolic OH group content gradually decreased in the degradation of USKP. The aliphatic OH group content first increased and then decreased during the fungal degradation of USKP, which is also contrary to the small changes observed in the aliphatic OH group content in the fungal degradation of pine flour. The trends of the content change for guaiacyl phenolic OH group and carboxylic acids group during fungal degradation were the same for both pine flour and USKP. It is worth noting that both the increased rate of loss of

Table 5. The contents of various functional groups (mmol/g lignin) in the degradation of bleached softwood kraft pulp by *P. cinnabarinus**

Incubation time(day)	Aliphatic OH	Phenolic OH		
		Condensed	Guaiacyl	R-COOH
Control	2.18±0.04	0.76±0.02	0.68±0.01	0.21±0.04
25	2.72±0.04	0.72±0.01	0.48±0.00	0.28±0.01
40	2.37±0.26	0.60±0.15	0.33±0.04	0.46±0.19

* data were the mean values of three pulp samples.

the guaiacyl phenolic OH group content and the decreased rate of loss of the carboxylic acid group content in the fungal degradation of USKP were higher than those in the fungal degradation of pine flour. This is probably due to the fact that the guaiacyl phenolic substructures in USKP were more accessible than those in pine flour.

Treatment of USKP with the fungus *P. cinnabarinus* led to decrease in Kappa number (Table 6), which implied that the fungus selectively degraded lignin. The Kappa number decreased from 23.34 to 5.46 after a 25-day-incubation. However, an increase of the incubation time from 25 to 40 days resulted in little change of the Kappa number. The fungus was also able to degrade cellulose and hemicelluloses although it preferentially degraded lignin. When the lignin content was low, the fungal degradation rates for carbohydrates increased, which might account for the small change in Kappa number when the incubation time increased from 25 days to 40 days.

Table 6. Kappa number of pulp before and after treatment of unbleached softwood kraft pulp by *P. cinnabarinus

Incubation time (day)	Kappa number
Control	23.34±0.10
25	5.46±0.30
40	5.67±0.39

* data were the mean values of three pulp samples.

4. Conclusions

In an effort to gain a better understanding of how white-rot fungi degrade lignin, we investigated the abilities of *P. cinnabarinus* and *T. rubrum* LSK-27 to degrade wood and analyzed the changes of various functional group contents in pine lignin and kraft lignin during the degradation of wood and unbleached kraft pulp by these fungi.

P. cinnabarinus degraded pine blocks much more efficiently than *T. rubrum* LSK-27, whereas *P. cinnabarinus* and *T. rubrum* LSK-27 degraded hardwood blocks (yellow poplar and sweet gum) at the same rate. It appeared that these two fungi used different ligninolytic enzyme systems for lignin degradation. Activities of three ligninolytic enzymes, lignin peroxidase, manganese peroxidase, and laccase were detected during the incubation of pine flour with *T. rubrum* LSK-27, whereas only laccase activity was detected with *P. cinnabarinus*.

Extracted lignin samples were analyzed with ^{31}P NMR for the content of various functional group. The ^{31}P NMR results indicated that two fungi used in this study all caused decreases in the guaiacyl phenolic OH group content and increases in the condensed phenolic hydroxyl group content, which implied that these fungi first degraded the easily oxidizable lignin substructures with guaiacyl phenolic OH group and left the condensed lignin substructures behind regardless of the ligninolytic enzymes they secreted for lignin degradation. Little change of the aliphatic OH groups suggested that the oxidization of the phenylpropane side chain in the pine lignin was not the important action mode in the degradation of lignin by these fungi. The increase in the carboxylic acid content probably resulted from the ring-opening reactions in the oxidation of the guaiacyl phenolic substructure. The changes of functional group contents in the degradation of unbleached softwood kraft pulp by *P. cinnabarinus* had the same trends as those in the degradation of pine flour. That is, structural alteration of lignin due to the kraft pulping

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