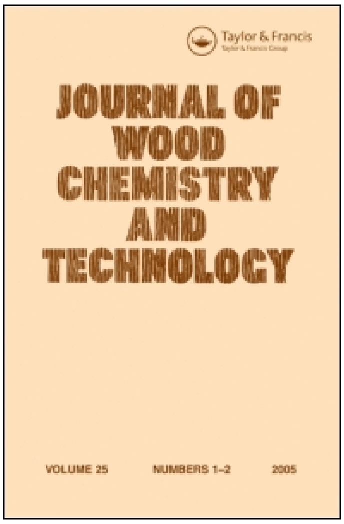

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Lignin Degradation by *Flavodon flavus* (Klotzsch.) Ryv. and *Schizophyllum commune* Fr. on *Mangifera indica* and *Syzygium cumini* Woods

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Abstract: The lignin degradation by *Flavodon flavus* (Klotzsch) Ryv. and *Schizophyllum commune* Fr. on *Mangifera indica* and *Syzygium cumini* wood, changes in the chemical composition of the degraded wood, and production of extra-cellular lignocellulolytic enzymes were analyzed. White rot fungi *F. flavus* and *S. commune* selectively degraded the lignin of *S. cumini* rather than the holocellulose component, whereas simultaneous degradation of lignin occurred in the case of *M. indica*. After 90 days of pretreatment with *F. flavus*, total weight loss was 29% and loss in lignin content was 25.7% in *M. indica* wood. However, 8% loss of holocellulose was caused by *S. commune* in *S. cumini* wood. Extracellular enzymes from *F. flavus* such as ligninase and cellulase showed higher activity in degradation of *M. indica* wood than in *S. cumini* wood. Weight loss and changes in chemical composition of *M. indica* and *S. cumini* woods showed good correlation with enzyme activity in lignocellulose degradation. Woods of *S. cumini* showed resistance to the white rot fungi could be due to the presence of polyphenols.

Keywords: Cellulolytic enzymes, lignin degrading fungi, lignin modifying enzymes, *Mangifera indica*, *Syzygium cumini*, white rot

INTRODUCTION

Cellulose is the major component (~50%) of wood substance by weight and lignin constitutes about 15–25% of the weight in hardwood biomass. Lignin is the second most abundant renewable organic polymer on earth. Wood and other lignocellulosics materials are used as a renewable resource for the production

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of paper products, feeds, chemicals, and fuels. There has been an increasing research emphasis on the fungal degradation of lignin.[1,2] White rot–causing *Basidiomycetous* members are important in any forest ecosystem since they are the only fungi capable of degrading all cell wall components of wood. Therefore, many white-rot fungi were considered either simultaneous or selective degradation of various components.[3,4] White-rot fungi are the most efficient lignin- degrading organisms described to date.[5,6] Among the white-rot fungi, *Phanerochaete chrysosporium* are the well-studied lignin-degrading microbes in nature and the majority of the studies focused on its lignin-degrading enzymes.[7–9] The major families of fungal lignin-modifying enzymes (LMEs) that participate in lignin degradation are laccases, manganese-dependent peroxidases (MnPs), and lignin peroxidases (LiPs).[10,11] The basic mechanism for fungal degradation of lignin was explained with the discovery of certain extra-cellular fungal peroxidases that are capable of cleaving carbon–carbon bonds in dimeric lignin model compounds.[12–15] The hydroxyl radicals derived from hydrogen peroxide (H2O2) were involved in lignin degradation by *P. chrysosporium.*[16]

Recently, there has been a growing interest in studying the lignin-modifying enzymes of a wider array of white-rot fungi, not only from the standpoint of comparative biology but also with the expectation of finding better lignin-degrading systems for use in various biotechnological applications such as bio-bleaching and the pulp and paper industry.[17–20,10] In bio-pulping the pretreatment of wood chips with white-rot fungi enhances the subsequent pulping step and substantially reduces the refining energy consumption.[21] In search of white-rot fungi with better lignin-degrading systems for use in bio-pulping and bio-bleaching processes the efforts were made in isolation and identification of lignin-degrading fungi. The studies were continued on their ability to degrade the lignin and cellulose in the case of *M. indica* and *S. cumini* wood. In the present investigation, the studies were carried out to determine the production of lignocellulolytic enzymes from two timber-degrading fungi, namely *Schizophyllum commune* and *Flavodon flavus.*

EXPERIMENTAL

Isolation

Fruiting bodies of the two fungi were collected from natural habitat, growing on wooden logs of *M. indica* and *S. cumini.* Wood chips or fruiting bodies measuring $5 \times 5 \times 1$ mm were aseptically removed from the decaying wood samples and transferred to petri plates containing 2% malt extract agar medium supplemented with streptomycin sulphate (250 μg/ml). The plates were incubated at $25 \pm 2^{\circ}$C for 7 days. Each colony thus obtained was transferred to a new agar slant. The wood decaying fungi were identified based on macroscopic (e.g., size, color, number of pores/mm, length of tubes), microscopic (presence/absence of structures, dimensions, vegetative, and reproductive characters),[22] and cultural characters.[23] The isolates were identified as *S. commune* and *F. flavus* causing white rot in wooden logs of *M. indica* and *S. cumini.* Other fungal isolates were strain a and b of *F. flavus* and *S. commune.*

In vitro Decay Test

A weight loss experiment was performed by a modified method of ASTM.[24] Fungi were grown on 2% malt extract agar for 1 week prior to inoculation in decay chambers. All wood blocks (2 × 1 × 1 cm) were cut from wood logs, dried at 80°C for 48 h in a recirculating oven and weighed. Wood blocks were soaked in distilled water for 1 h to get 80 to 85% humidity, blotted dry and sterilized at 120°C, 6.895 kPa for 1 h in an autoclave. Blocks were then inoculated in 250 ml Borosil glass decay chambers containing 50 ml of malt extract (3 blocks per chamber). Six decay chambers for each wood type were inoculated with one isolate. Six blocks were used for weight loss data. Three blocks were used for chemical analysis. Six uninoculated wood blocks of each wood species served as controls. The decay chambers were incubated at 27 ± 2°C and 90% relative humidity for 20, 45, and 90 days. After completion of the incubation period, the wood blocks were taken out and the mycelium removed from the surface. They were oven dried at 80°C for 48 h and weighed to determine the weight loss.

Chemical Analysis of Wood

Prior to chemical analysis of the wood, both control and decayed wood blocks were ground and passed through a 40-mesh-sized screen. Estimation of cellulose was performed using the method suggested by Yemn and Willis.[25] Acetic–nitric reagent (3 ml) was added to 0.5 g of the sample in a test tube and mixed well. The test tubes were then placed in a water bath at 100°C for 30 min. After cooling, the samples were centrifuged for 15–20 min at 5000 rpm and the supernatants were discarded. The residue obtained was washed with distilled water and 10 ml of 67% sulfuric acid was added to it. Then it was allowed to stand for 1 h. One milliliter of the aforementioned solution was diluted to 100 ml with distilled water. One milliliter of this solution was taken in three different test tubes. Ten milliliters of anthrone reagent was added to it and mixed well. After that, the tubes were heated in boiling water bath for 10 min, cooled, and it was measured at 630 nm using a spectrophotometer (Systronics Spectrophotometer 106). A blank was obtained by using anthrone reagent and distilled water. A standard curve was prepared by using D-glucose (1 mg/ml) with different concentrations treated with anthrone reagent.

Estimation of lignin content was performed using the method suggested by Dill and Kraepelin.[26] Flasks containing 1 g of ethanol-benzene extracted wood meal and 20 ml of H2SO4 (72%) were gently shaken in a water bath at 30°C for 1 h. The acid was then diluted with H_2O to 4% (wt/vol), and the samples were autoclaved at 121°C for 30 min. The lignin that settled overnight was quantitatively collected by filtration through a crucible, washed free of acid with hot water, and dried. The lignin content was calculated as a percentage of oven-dried, non-extracted wood meal. Both the experiments (estimation of lignin and cellulose) were done three times. One-way ANOVA was performed to determine significance difference at $p > 0.05$ level.

Bavendamm Test

The activity of laccase was estimated by the method of Bavendamm.[27] Each fungal strain was inoculated onto the PDA medium containing 0.1% (w/v) tannin acid or gallic acid. After cultivation for 2 weeks, the lignin-degrading enzymatic activities of the fungi were qualitatively evaluated by observing the color changes in the media.

Enzymatic Test

The screening of two white-rot fungi for their lignin- and cellulose-degrading enzymatic activity were evaluated by substituting tannic acid (for ligninases) and carboxy methylcellulose (for cellulases) in 3% malt extract agar medium.[28] The pH of the medium was adjusted to 5 with 1 N NaOH and 1 N HCl. Chloramphenicol (75 mg) was added prior to sterilization, to avoid bacterial contamination, except in the case of lignin- degrading enzymatic activity where tannic acid itself acts as a bactericidal agent. The petri plates were inoculated with fungal mycelium and incubated at 28°C for 7 days. Three replicates were maintained for each set of observations. The enzymatic activities were evaluated by observing the zone of clearance formed, if any, by flooding the plates with visualizing dye Congo red for 15 min[29] for detecting the cellulolytic activity. Lignin-degrading enzymatic activity was assessed by observing the dark brown–colored zone around the fungal colony.

RESULTS AND DISCUSSION

In vitro Decay Test

The percentage weight loss in the wooden blocks due to the decay by two white-rot fungi are shown in Table 1. In 90 days, a 29 to 26.85% weight loss

Table 1. Percentage weight loss and decay resistance of *M. indica* and *S. cumini* wood blocks infected with *S. commune* and *F. flavus*

Wood species	Decay fungi	Incubation period (days)	% weight loss*
Mangifera indica	*Flavodon flavus*	20	1.09 ± 0.01
		45	9.55 ± 0.02
		90	29.00 ± 0.07
	Schizophyllum commune	20	1.88 ± 0.01
		45	10.45 ± 0.02
		90	26.85 ± 0.5
Syzygium cumini	*Flavodon flavus*	20	1.77 ± 0.03
		45	9.12 ± 0.05
		90	23.68 ± 0.09
	Schizophyllum commune	20	2.06 ± 0.01
		45	10.45 ± 0.04
		90	23.74 ± 0.03

*Average percentage weight loss determined from five replicates after respective incubation period. ± Results were significant at $p < .05$ level by one way ANOVA.

was noted in the wooden blocks of *M. indica* decayed by *F. flavus*, whereas in *S. cumini,* the percentage weight loss due to decay by the two white-rot fungi was 23%. In the first 20 days, the weight loss caused by *F. flavus* was 1.77% in *S. cumini*, whereas in *M. indica* it was only 1%. Both the fungi produced almost similar weight loss in the wood of *M. indica* and *S. cumini* under laboratory conditions (Table 1). *S. commune* showed 0.5–0.7% weight loss in *Pinus radiata* wood for 126 days[30] but our results show a weight loss of 1.88–26.85% in *M. indica* and 2.66–23.74% in *S. cumini*. Birch sapwood decayed by *S. commune* showed 22.4% weight losses in 90 days.[31] In this study, *M. indica* wood showed 26.85% and *S. cumini* wood showed 23.74% weight loss in 90 days. Although the *in vitro* wood decay test cannot be taken as absolute evidence for the behavior of lignin-degrading fungi, they are useful to determine their wood-destroying properties.

Based on percentage weight loss, the American Society for Testing Materials[24] classified the resistance of wood. Highly resistant wood showed weight loss of zero to 10%, resistant wood shows weight loss of 11 to 24%, moderately resistant wood showed 25 to 44% weight loss, and nonresistant wood showed 45% or greater weight loss. According to this classification, the wood blocks of *M. indica* showed moderate resistance to white-rot-causing fungi *F. flavus* and *S. commune* at 90 days of incubation, whereas wood blocks of *S. cumini* showed resistance to both fungi in 90 days of incubation. As incubation period increases the percentage weight loss also increases in both the cases. White-rot-causing fungi *F. flavus* shows moderate resistance in *M. indica* whereas it shows resistance in *S. cumini* due to the presence of polyphenol.[32,33]

Chemical Analysis of Wood

Chemical analysis of *M. indica* and *S. cumini* wood blocks indicated the removal of lignin and cellulose (Table 2). For 20 and 45 days of degraded samples, the percentage loss of lignin was more in *S. cumini* than in *M. indica*, whereas percentage loss of cellulose was more in *M. indica* than in *S. cumini*. *S. commune* caused 20% loss of lignin content within 90 days of introduced decay in both *M. indica* and *S. cumini*. Similiarly, *F. flavus* brought about a loss of 25% lignin in both *M. indica* and *S. cumini*. The loss of cellulose was more in *S. commune*–infected *M. indica* wood compared to *S. cumini* wood whereas cellulose loss was also comparatively more in *F. flavus*–infected *M. indica* wooden blocks. From the overall result, both the fungi showed higher percentage loss of lignin in *S. cumini* whereas there was a high percentage loss of cellulose in *M. indica* wood. *S. cumini* wooden blocks were found to be more resistant to the fungal attack compared to the *M. indica* wood due to the presence of polyphenolic compounds. When a brown-rot-causing fungi *Polyporus palustris* was infected

Table 2. Percentage loss of Klason lignin (KL), Chlorite holocellulose (CHC), and Klason lignin and Holocellulose ratios in control and decayed woods of *M. indica* and *S. cumini*

Wood species	Decay fungi	Incubation period (days)	Loss in KL* (%)	Loss in CHC* (%)	%KL:%CHC*
Mangifera indica	*Flavodon flavus*	*Control	50	50	0.25:0.25
		20	5.57 ± 0.1	12 ± 0.16	0.06:0.12
		45	15.10 ± 0.2	12.2 ± 0.32	0.11:0.09
		90	25.7 ± 0.5	14 ± 0.24	0.10:0.05
	Schizophyllum commune	20	2.0 ± 0.2	10 ± 0.24	0.01:0.05
		45	12.72 ± 0.1	13 ± 0.40	0.02:0.03
		90	20.89 ± 0.1	15 ± 0.32	0.10:0.03
Syzygium cumini	*Flavodon flavus*	*control	60	30	0.18:0.09
		20	11.53 ± 0.2	6 ± 0.32	0.19:0.09
		45	20.58 ± 0.3	8 ± 0.24	0.16:0.06
		90	25.19 ± 0.6	11 ± 0.40	0.19:0.08
	Schizophyllum commune	20	6.4 ± 0.3	4 ± 0.16	0.10:0.06
		45	16.37 ± 0.2	5 ± 0.32	0.13:0.04
		90	20.14 ± 0.5	8 ± 0.20	0.18:0.06

*Percentage loss of klason lignin, chlorite holocellulose, and ratios of the percent of each component are the three replicates. Uninoculated wooden blocks were incubated for 90 days to act as a control.

± Results were significant at $p < .05$ level by one way ANOVA.

Table 3. Ligninolytic and cellulolytic activity of different isolates of *Flavodon flavus* and *Schizophyllum commune*

Fungi/isolate	Ligninolytic (Zone of clearance in cm) #	Cellulolytic (Zone of clearance in cm)#
Flavodon flavus	9.0 ± 0.01	8.0 ± 0.03
F.f.[a]	4.5 ± 0.03	9.0 ± 0.1
F.f.[b]	6.5 ± 0.04	9.0 ± 0.02
Schizophyllum commune	1.0 ± 0.02	8.5 ± 0.05
S.c.[a]	2.0 ± 0.01	9.0 ± 0.07
S.c.[b]	1.5 ± 0.03	7.0 ± 0.12

[a,b] isolates numbers were collection numbers.

hallow zone of clearance in cm was determine from the three replicates of ligninolytic and cellulolytic test plates.

± Results were significant at $p < .05$ level by one way ANOVA.

to *M. indica* wood shavings for considerable periods, approximately 40 to 50% lignin loss was observed in two years.[34] In the present study, when the *Mangifera* wood blocks were infected with white-rot-causing fungi for 90 days, the utilization of lignin was 26% by *F. flavus* and 20% by *S. commune.*

Adaskaveg et al.[35] observed selective delignification and simultaneous decay in oak wood infected with *Ganoderma* isolates. In oak wood, for simultaneous decay, the ratio of Klason lignin (%KL) to Chlorite Holocellulose (%CHC) obtained was 1:1 by *G. meredithiae*; for moderate amount of delignification the ratio was 1.5:1 by *G. zonatum*; and for high amount of delignification 2.5 to 5:1 by *G. colossum* and *G. oregonense*. In the present article, both white-rot fungi showed moderate amount of delignification in *S. cumini*–infected wood, whereas both fungi showed simultaneous decay in *M. indica*–infected wood. After 90 days of incubation, both the white-rot fungi degraded a moderate amount of lignin in *M. indica* wooden blocks, while in *S. cumini* a moderate amount of delignification was shown by *F. flavus* and *S. commune* showed a high amount of delignification. When *S. commune* was grown on liquid media containing ^{14}C-lignin-labeled wood, the degradation of lignin was low and variable.[36] In this study *S. commune* showed high delignification capacity in *S. cumini* and moderate delignification capacity in *M. indica* wood. The solid waste (pomace) from olive oil processing was subjected to delignification by *P. chrysosporium*, *Oxysporus* sp., *S. commune*, *Hyphoderma* sp., or *Ganoderma* sp. The levels of ligninase or laccase secreted and the extent of lignin degradation judged the relative activity of the species. The *Oxysporus* sp. (ca. 69%) and *S. commune* (ca. 53%) gave significantly higher levels of breakdown of the lignified material than the other isolates.[37] The present study supports the aforementioned result that *S. commune* has the ability to produce lignin-degrading enzymes for degradation of lignocellulosics materials.

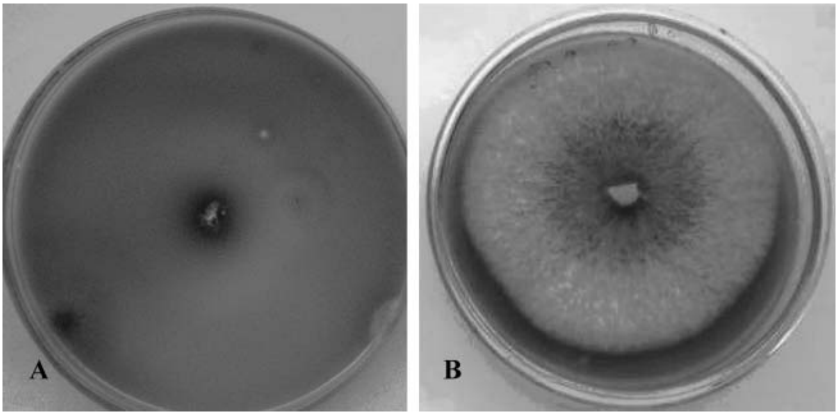

Figure 1. Ligninolytic activity of *Schizophyllum commune* (A) and *Flavodon flavus* (B) petriplates showing zone of clearance.

Enzymatic Test

In the present study, all *S. commune* and *F. flavus* strains showed positive reactions to tannic acid used in the Bavendamm test. The data from the Bavendamm test provided evidence for the presence of laccase activity in this fungus (Table 3). De Vries et al.[38] studied the production of extra-cellular laccases from *S. commune*. The present study confirms this finding by the aforementioned test. *F. flavus* (strain 312), isolated from decaying sea grass from a coral lagoon off the west coast of India, mineralized nearly 24% of ^{14}C-labeled synthetic lignin to ^{14}CO2 in 24 days.[39] When grown in low-nitrogen medium (2.4 mM N) this fungus produced three major classes of extra cellular lignin-modifying enzymes (LMEs): manganese-dependent peroxidase (MNP), lignin peroxidase (LIP), and laccase.[39] The present study reveals that all evaluated strains of *F. flavus* show strong positive reactions to tannic acid. So all strains of *F. flavus* produce lignin-modifying enzymes to degrade the lignin in *M. indica* and *S. cumini* woods.

Six fungal isolates of two white-rot fungi, *F. flavus* and *S. commune*, were the common producers of lignin- and cellulose-degrading enzymes. *F. flavus* isolates showed highest lignin-degrading enzymatic activity up to 9 cm zone of clearance whereas *S. commune* isolates had the lowest lignin-degrading enzymatic activity (1.5 cm zone of clearance) (Figure 1). Both the white-rot fungi were able to produce highest cellulolytic activity up to 9 cm zone of clearance. The results obtained led us to conclude that the isolates of *F. flavus* possessed high lignin-degrading capacity whereas isolates of *S. commune* possessed low lignin-degrading capacity (Figure 2).

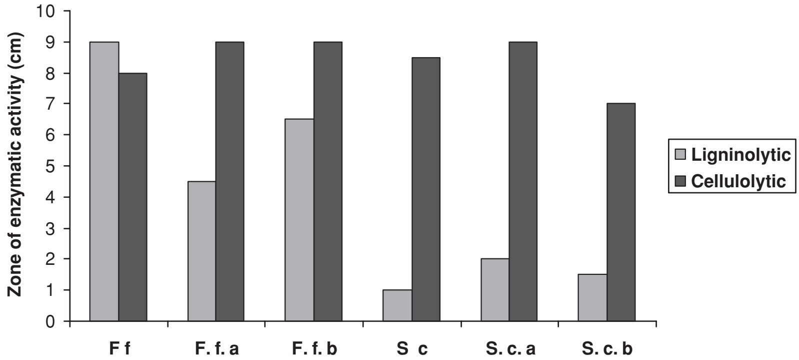

Figure 2. Histogram showing the ligninolytic and cellulolytic activity of white-rot fungal isolates. # F f – *Flavodon flavus*, F. f. a – *Flavodon flavus* a, F. f. b – *Flavodon flavus* b, S c – *Schizophyllum commune*, S. c. a – *Schizophyllum commune* a, S. c. b - *Schizophyllum commune* b.

CONCLUSIONS

The results of the present study allow the following conclusions to be drawn about the ligninolytic activity of *F. flavus* and *S. commune* on *M. indica* and *S. cumini* wood.

- White-rot fungi selectively degrade the lignin in *S. cumini* wood whereas simultaneous decay was observed in *M. indica* wood.
- *M. indica* wood shows moderate resistance while *S. cumini* wood shows resistance to the white-rot-causing fungi, namely *F. flavus* and *S. commune.*
- Natural decay resistance of *S. cumini* to wood decay is due to the presence of polyphenols.
- Based on the ratio of % Klason lignin to % Holocellulose the lignolytic activity of *S. commune* is more in the case of *S. cumini* wood decay when compared to *F. flavus.*
- The delignification capacity of *S. commune* is greater than that of *F. flavus*.
- The lignocellulolytic activity of *F. flavus* isolates is greater than that of *S. commune* isolates.
- The isolates of *F. flavus* possess high lignin-degrading capacity whereas isolates of *S. commune* have low lignin-degrading capacity.

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