BIOMODIFICATION OF KENAF USING WHITE ROT FUNGI

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White rot fungi can be used as a pretreatment of biomass to degrade lignin. It also alters the structure of the lignocellulosic matter, thus increasing its accessibility to enzymes able to convert polysaccharides into simple sugars. This study compares the ability of two species of white rot fungi, *Pycnoporous sanguineus and Oxyporus latemarginatus* FRIM 31, to degrade lignin in kenaf chips. The white rot fungi were originally isolated from the tropical forest in Malaysia. Kenaf chips were first inoculated with each fungus separately using corn steep liquor as a fungal growth promoter. The kenaf chips were inoculated with white rot fungus for a period of 1, 2, 4, 8 and 16 weeks, after which they were observed under the scanning electron microscope (SEM). Chemical analyses were conducted following TAPPI Standard Methods and Fourier Transmission Infra Red (FTIR). SEM observations showed evidence of fungal colonization. When calculating weight loss, both *P. sanguineus* and *O. latemarginatus* FRIM 31 showed the greatest reduction. Amounts by mass of cellulose, hemicelluloses, extractives, and lignin in the treated kenaf chips all were lowered. The results show that *O. latemarginatus* FRIM 31 had a greater ability to degrade lignin when compared to *P. sanguineus*.

Keywords: Delignification; Kenaf; Pretreatment; Pycnoporous sanguineus; Oxyporus latemarginatus

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INTRODUCTION

Fungi act as decomposers in the forest and play an important role in the ecosystem. Most of the fungi are able to attack wood cell wall components, and each has differences in behavior. Decay fungi can be classified into three major groups, the white rot, brown rot, and soft rot fungi; among these, the white rot fungi have the ability to degrade lignin (Pointing et al. 2003; Ward et al. 2004). This can be shown by the fact that wood materials that have been decayed by white rot fungi are pale in colour, brittle, spongy, soft, and fibrous in texture (Eriksson et al. 1990; Schwarze 2007). The ability to degrade lignin has prompted interest in using white rot fungi for bioconversion of lignified tissues, such as biopulping and biobleaching (Istek et al. 2005). The biomechanical pulping ability of *Ceriporiopsis subvermispora* appears to be superior to that of others in terms of energy savings and improvements in the strength properties (Akhtar et al. 1993). Most of the white rot fungi are from family of basidiomycetes, and some of them belong to the ascomycetes (Schmidth 2006). White rot fungi have been distinguished according to two different degradation patterns: simultaneous degradation and selective delignification (Fuhr et al. 2011).

White rot fungi are introduced to lignicellulose biomass through a process called pretreatment. In this context, pretreatment can be defined as a means of breaking down the structure of lignocellulosic material, mainly comprised of cellulose, hemicelluloses, and lignin prior to further processing (nee' Nigam et al. 2009). Lignin is a recalcitrant substance that acts as a seal in lignocellulosic matter, associated with hemicelluloses and cellulose, reducing the accessibility of the carbohydrates for further transformation processes. Therefore, white rot fungi have been used as biological agents in pretreatment to degrade the lignin and disrupt the crystalline structure of cellulose.

During simultaneous degradation, all the wood cell wall components, including lignin and polysaccharides, are broken down at approximately the same rate (Schwarze et al. 2000). Experiments with *C. subvermispora* grown on *Eucalyptus grandis* wood chips under solid state fermentation revealed that hydrolytic and oxidative processes occurred during the entire course of biodegradation process, rendering the wood chips more susceptible to the pulping process (Ferraz et al. 2003). Both types of degradation can be associated with the same fungus, although they are distinctly different (Eriksson et al. 1990; Blanchette et al. 1998; Halis 2010). Biodegradation by *P. sanguineus* has been carried out in previous studies. *P. sanguineus* was found to produce laccase as the sole phenoloxidase (Pointing and Vrijmoed 2000; Lu et al. 2007). In contrast to *P. sanguineus*, *O. latemarginatus* secreted lignin peroxidase and manganese peroxidase but not laccase (Dhouib et al. 2005). Laccase, lignin peroxidase, and manganese peroxidase are important enzymes in delignification and have been widely used in industrial application such as dye-decolouration, bioremediation, biopulping and biobleaching. The aim of this study was to compare of two tropical basidiomycetes *P. sanguineus* and *O. latemarginatus* FRIM 31, which were isolated from the Malaysian forest. Favourable fibre properties and low lignin content has made it a popular alternative plant to replace wood in paper and composite making.

EXPERIMENTAL

Fungi Cultivation

The white rot fungi, *P. sanguineus* and *O. latemarginatus* FRIM 31, used in this study were obtained from the Forest Research Institute of Malaysia (FRIM) centre. The isolates were grown in Potato Dextrose Agar (PDA) slants following the regular procedure, and incubated at 28°C for 6 days in anamorphic stages, after which the mycelium and basidiospores are normally produced on the PDA slants. Cultures were stored at 4°C until further use.

Fungal Pretreatment on Kenaf Chips

Kenaf (*Hibicus cannabinus, L)* cultivar V36 was obtained from the Malaysian Agricultural Research and Development Institute (MARDI), Malaysia. Whole stalks of kenaf were chipped into small particles using a chipper mill. Kenaf chips were then dried to 5% moisture content and screened to even size. Solid-state cultivation was carried out in 30 mm x 20 mm x 60 mm aluminum trays. A total of 300 g of oven-dry kenaf chips were sterilized at 121°C for 30 mins, after which 100 mL of 4% Corn Steep

Liquor (CSL) were added after cooling. The kenaf media was then inoculated with basidiospores suspension. Incubation periods were 1, 2, 4, 8, and 16 weeks for the species of white rot fungi. After incubation, the kenaf chips were washed with boiling water to sterilize them and remove the mycelia. Kenaf chips were then oven-dried at 60°C for 24 hours and weighed to determine the weight loss.

Enzyme Assays

Crude enzyme samples were extracted by added 10 mL of 10 mM acetate buffer solution (pH 5.0) to each flask with shaking for 20 mins at 25°C in an incubation shaker. The contents were filtered through Whatman filter paper No. 1 (150 mm). The filtrate was centrifuged 10000 rpm at temperature 4°C and kept as supernatant for enzyme determination (Arora et al. 2002).

Lignin peroxidase (LiP)

LiP was determined in reaction mixture containing 0.1M sodium tartrate (pH3.0), 0.16mM azure B, and 0.5 mL of culture supernatant. The reaction was initiated by adding 2 mM H_2O_2. Absorbance was read after 1 minute at 651 nm. One unit of enzyme activity was expressed as Optical Density (O.D) decrease of 0.1 units per minutes per mL (Arora and Gill 2001).

Manganese peroxidase (MnP)

MnP was determined in a reaction mixture containing 100 mM sodium tartrate buffer (pH 4.5), 0.4 mL $MnSO_4$, 1mM 2,6-dimethoxyphenol (2.6-DMP), and 0.1 mL of culture supernatant. The reaction was initiated with 0.1 mM H_2O_2. Absorbance was read after 3 minutes at 469 nm. One unit of enzyme activity was expressed as O.D decrease of 0.1 units per minute per mL.

Laccase

Laccase activity was determined in a reaction mixture consisting of 1 mM sodium acetate buffer (pH 5.0), 0.5 mM 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), and 1.5 mL of culture supernatant. The absorbance was read at 420 nm, and enzyme activity was defined as amount of enzyme catalyzing production of 1μmol of colored product per minute per mL (Li et al. 2008)

Observation through Scanning Electron Microscope (SEM)

Kenaf chips treated with white rot fungi were cut into sections of 1 cm x 1 cm and fixed in 4% glutaraldehyde at 4°C. After the primary fixation, specimens were washed with 0.1 M sodium sacodylate buffer for 3 times of 30 mins each. Specimens were then post-fixed in 1% osmium textroxide for 2 h at 4°C, followed by washing in 0.1 M sodium cacodylate buffer for 3 times of 30 mins each. A series of dehydration steps were performed by immersing the specimens into acetone with increasing concentrations: 35%, 50%, 75%, and 95% of 30 minutes each, and 100% for 3 times of 1 h. Specimens were dried in a critical point dryer for 30 mins. Dried specimens were then mounted onto the stub and coated with gold in sputter coater and examined under scanning electron microscope (PHILIPS XL 30 ESEM).

Chemical Composition Analysis

Chemical composition analyses were carried out using TAPPI Standard Methods. The procedure for water solubility followed T207 om-93, and the ethanol-acetone solubility followed T204 cm-97 for 6 hours. Holocellulose and alpha cellulose were determined following the T9 wd-76 and T203 cm-99 procedures, respectively. Klason lignin content was determined by hydrolyzing the carbohydrate in 72% sulphuric acid, as stated in T222 om-88. All tests were carried out in triplicates, and ANOVA and tukey multiple-range test were conducted.

Chemical Changes by Fourier Transmission Infra-Red Analysis

Treated kenaf chips were wood-milled and oven-dried to reduce the moisture content (MC) to 5%. FTIR spectra of treated sample and control were measured by direct transmittance using the Golden Gate Single Reflection Diamond Attenuated Total Reflectance (DTAR P/N 10500 series) techniques. Spectra were recorded using a Perkin Elmer Spectrum 2000 FTIR spectrometer equipped with a DTGS detector. The conditions of analysis were as follows: resolution 8 cm^{-1}, co-adding 5 scans, and a frequency range of 550 to 1800 cm^{-1} (Faix 1992). Peak and height area were measured by OMNIC Software version 1.2a (Nicolet Instruments Corp., USA).

RESULTS AND DISCUSSION

Microscopic Observations

Using SEM, the extents of degradation caused by the white rot fungi to the structural cell wall of kenaf chips were observed. Degradation of *O. latemarginatus* was observed after 1 week of incubation (Fig. 1A-B). At 16 weeks a yellowish mycelia mat was heavily spread on the entire surface of kenaf chips. In transverse section, *O. latemarginatus* hyphae were observed penetrating into the vessel lumen, and fibres were destroyed (Fig. 1A). Evidence of penetration of hyphae in the vessel can be clearly seen in radial section, where the hyphae grew through the cells from the pit on the wall. Ray parenchyma cells were colonized and eroded to form large bore holes (Fig. 1B). After 16 weeks of incubation, extensive degradation was observed in the sample (Fig. 1C-D). In transverse section, large cavities were noted, resulting from simultaneous removal of all cell wall components (Fig. 1C). Erosion on ray parenchyma cells had become enlarged, and the vessel elements were detached due to advanced degradation (Fig 1D).

Degradation on kenaf occurred in the first week for *P. sanguineus* (Fig. 2E-F). The orange colour mycelia formed a thick mat-like structure on the surface and wrapped kenaf chips tightly starting at 4 weeks, and at this period the kenaf chips were hard to detach from the trays used for treatment (Halis 2010). Numerous bore holes appeared in the early stages of decay, and *P. sanguineus* were obviously formed in ray parenchyma and fibres. Hyphae penetrated from cell to cell via pit structures into cell lumens toward the middle lamella. In transverse section it can be seen that vessel and fibre lumens were filled up with hyphae (Fig. 2E). Erosion was apparent from the formation of bore holes on fibre walls (Fig. 2F). Advanced degradation appeared after 16 weeks of incubation (Fig. 2G-H). With the increase of incubation period, bore holes became progressively

larger (Fig 2G). Extensive delignification resulted in collapse and detachment due to loss of cell wall rigidity (Fig. 2 H). High erosion also caused thinning of the cell wall.

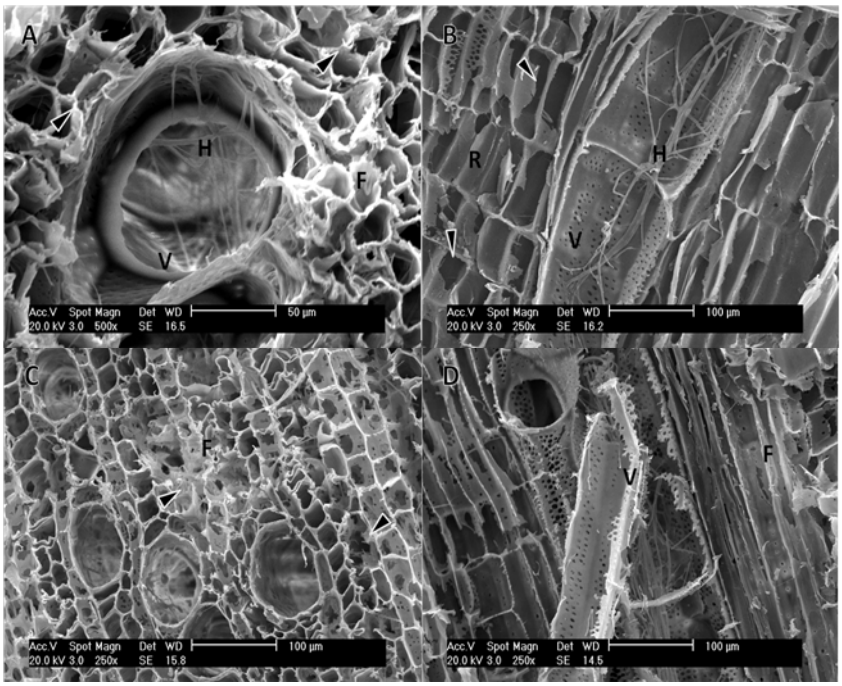


Fig. 1. SEM of kenaf chips decayed by *O. latemarginatus* FRIM 31 after 1 week **(A-B)** and 16 weeks of incubation **(C-D)**. **A:** Transverse section showing hyphae (H) colonizing vessel (V) lumens. Destruction (arrowhead) can be seen on the fibres (F). **B:** Radial section showing invasion of hyphae (H) into vessel (V). Large holes on ray parenchyma (R) wall (arrowhead). **C:** Advance erosion of fibres (F) (arrowhead). **D:** Detached vessel (V) elements. Erosion of fibres (F) with large holes.

Enzymes Activity

Production of the three major ligninolytic, laccase (Lac), lignin peroxidase (LiP) and manganese peroxidase (MnP) enzymes by each of the fungi were detected over the different incubation period. *P. sanguineus* secretes Lac as the sole ligninolytic enzyme (Pointing and Vrijmoed 2000; Munusamy et al. 2008). The activity of Lac produced by *P. sanguineus* was observable after one week of incubation (Fig. 3). Low Lac was detected on week 4; however, its activity increased the following week and reached a maximum at week 16 (0.0066 U/mL).

O. latemarginatus FRIM 031 was found to secrete LiP and MnP in decaying kenaf, but no Lac was detected (Fig. 4). This result is similar to the findings of Dhouib et al. (2005), in which *O. latemarginatus* with the strains CTM 10133 and CTM 10136 produced a high rate of MnP, a trace of LiP, but no Lac activity. MnP activity was found maximum at the early stages of incubation (0.0035 U/mL), then decreased at week 2 and showed an increase during subsequent observations, but again presented a decrease from week 8 onwards. LiP activity attained the maximum of 0.0006 U/mL after 8 weeks of degradation by this fungus. A similar pattern of activity was observed; LiP found reduced its activity at week 2 and during the later stages of degradation.

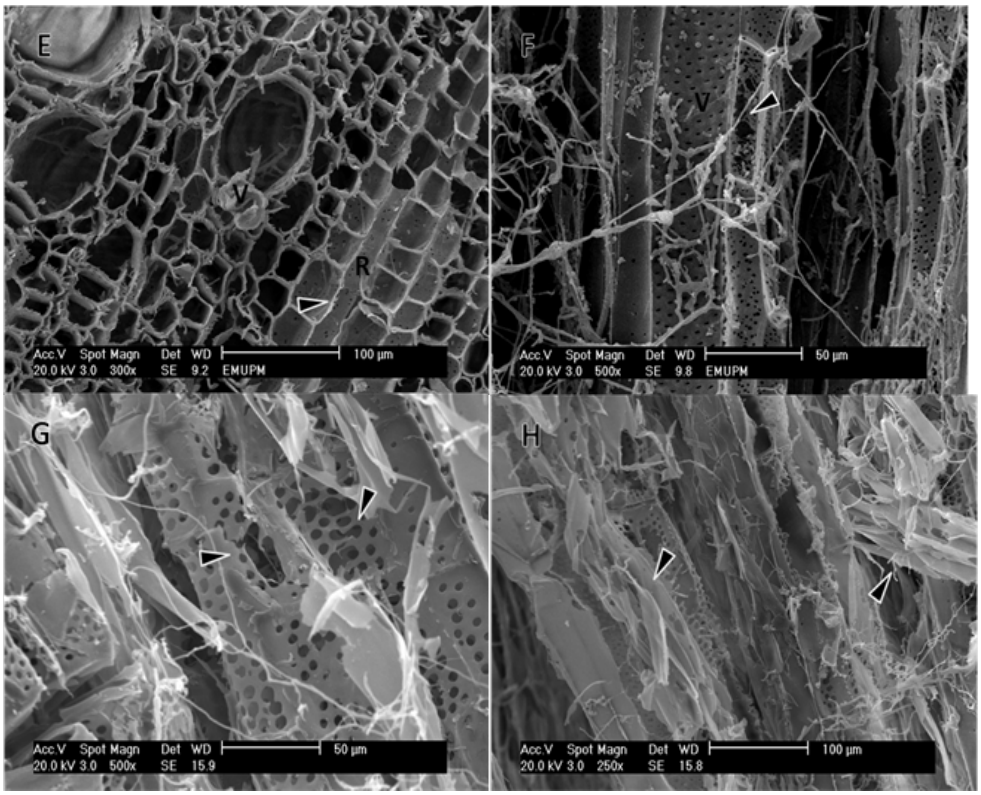


Fig. 2. SEM of kenaf chips decayed by *P. sanguineus* after 1 week **(E-F)** and 16 weeks incubation **(G-H)**. **E:** Transverse section showing penetration of hyphae into vessel (V) lumens. Minute bore holes found in ray parenchyma (R) cells (arrowhead). **F:** Vessel (V) remnant with abundant of hyphae. Large holes formed in fibres wall. **G:** Large holes in ray parenchyma cells (arrowhead). **H:** Collapsed and detached of fibre (arrowhead).

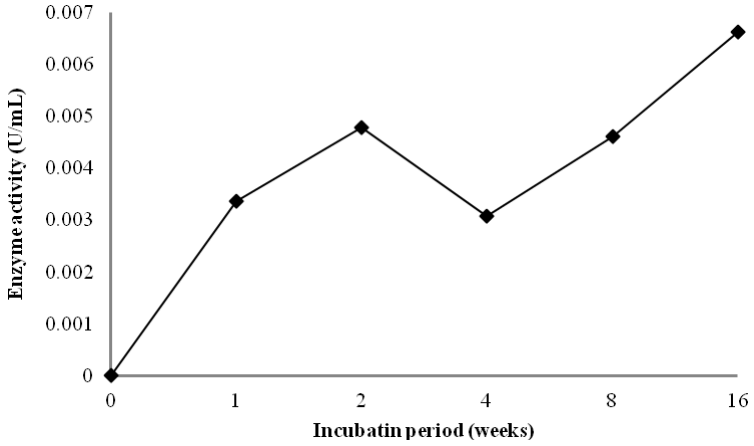


Fig. 3. Production of Lac (–◆–) by *P. sanguineus* over incubation periods varying from week 1 to 16

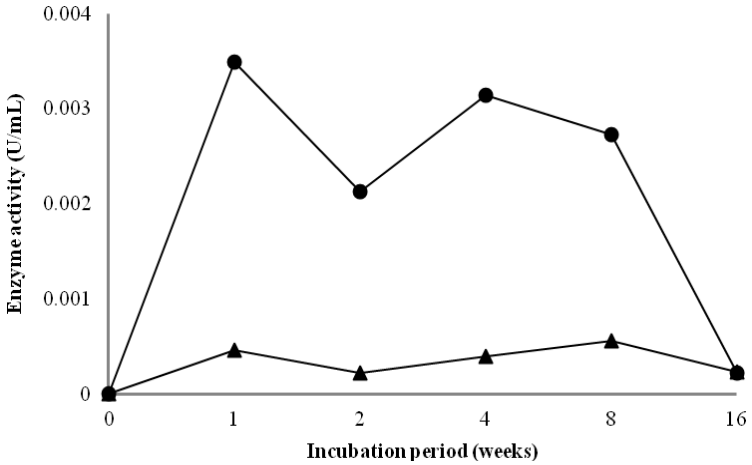


Fig. 4. Production of LiP (–●–) and MnP (–▲–) by *O. latemarginatus* FRIM 31 over incubation periods varying from week 1 to 16

Weight Losses

Weight loss can be considered as an indicator of fungal degradation. Results in Fig. 5 show that *P. sanguineus* and *O. latemarginatus* had similar trends of weight loss from 1 week to 4 weeks of incubation, but at 8 weeks *O. latemarginatus* showed greater weight loss than *P. sanguineus*. Differences in kenaf degradation may result by different biochemical systems, especially on the enzymatic activities. Weight loss of kenaf chips treated by *P. sanguineus* in 4 weeks was 30.18%, which was similar to the reported values 29.11% (Halis, 2010), 34.9% in 12 weeks on *Figus sylvatica* (Pointing et al. 2003), and 59.05% on poplar wood for 22 weeks (Luna et al. 2004). Weight loss of kenaf chips treated by *P. chrysosporium* and *C. versicolor* in 4 weeks exposure were 11.69% and 10.09%, respectively (Halis 2010). Brutia pine chips treated with *C. subvermispora* lost 6.13% within 4 weeks of incubation (Copur and Tozluoglu 2007). Thus, *P. sanguineus* and *O. latemarginatus* can be classified as aggressive white rot fungi that degrade large portions of wood components, resulting in high weight loss.

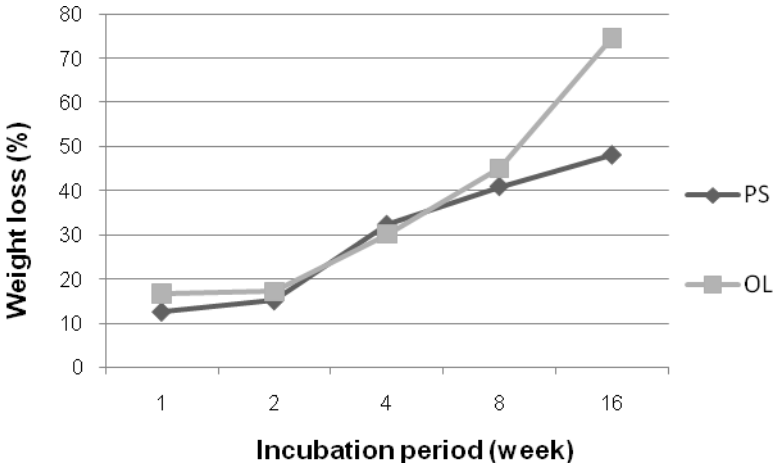


Fig. 5. Weight loss of kenaf treated by *P. sanguineus* (PS) and *O. latemarginatus* FRIM 31 (OL)

Chemical Composition Analysis

Table 1 exhibits the changes in chemical composition of treated kenaf over the incubation period. After 16 weeks of incubation, white rot fungi had demonstrated the ability to degrade all of the wood chemical components, including lignin, cellulose, and hemicelluloses. *P. sanguineus* was found to degrade holocellulose and lignin from week 1 to 8, but the contents of these components were increased after 16 weeks of incubation. The observed increase of lignin may be because of removal cellulose and other biodegradable components, resulting in relative increase in polyphenolic compounds (van Heerden et al. 2008).

O. latemarginatus showed evidence of degrading all cell wall components from week 1 to week 16 of the incubation period. Cellulose content of kenaf after being subjected to *O. latemarginatus* for 16 weeks showed a drastic decrease of about 28.17%, corresponding to a high weight loss. Solubility of hot and cold water, alcohol-acetone solvent, and ash content of kenaf chips by treated of *O. latemarginatus* showed gradually increases. The rising of solubility can be attributed to soluble sugar and glucose as products of carbohydrate biodegradation (Hunt et al. 1998; Halis 2010). Statistical analysis showed that the differences were significant at 0.01 to 0.001 confidence levels in chemical composition of fungi treated kenaf chips, except for percentage of cellulose of kenaf chips that had been inoculated with *P. sanguineus*. The Tukey multiple-range test showed significant differences of lignin content between week 1, 2, 4, 8, and 16 weeks (Table 1) for pretreatment by *O. latemarginatus*, but in the case of pretreatment by *P. sanguineus*, only 8 weeks treatment showed a difference from other incubation periods.

FTIR Spectra

Many well-defined "fingerprint" characteristics were observed in the region of wave numbers ranging between 1800 and 800 cm^{-1}, indicating changes in the chemistry and structure of carbohydrates and lignin in the wood. The peaks in the fingerprint region are assigned accordingly: 1738 cm^{-1} for unconjugated C=O in hemicelluloses, 1596 cm^{-1} for aromatic skeletal vibration plus C=O stretch, 1505 cm^{-1} for aromatic skeletal vibration in lignin, 1462 cm^{-1} for aromatic C-H deformation, 1375 cm^{-1} for C-H deformation in lignin and carbohydrates, 1268 cm^{-1} for guaiacyl ring breathing plus C-O stretch in lignin, 1244 cm^{-1} for syringyl ring plus C-O stretch in lignin and xylan, 1158 cm^{-1} for C-O-C vibration in cellulose and hemicelluloses, 1122 cm^{-1} for aromatic skeletal and C-O stretch, and 898 cm^{-1} for C-H deformation in cellulose.

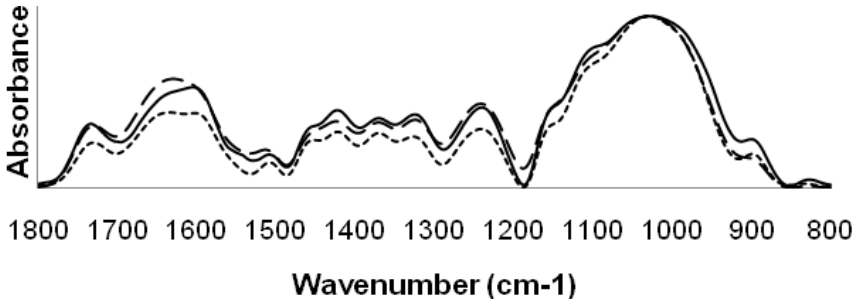
Figure 6a shows that there was a significant decrease from untreated kenaf to 16 weeks for *P. sanguineus* decayed kenaf in intensities of polysaccharide bands at 1738 cm^{-1} and 1375 cm^{-1} but no obvious changes at band 1158 cm^{-1} from untreated kenaf to 1 week treated kenaf; increases were found from 1 week to 16 weeks treated kenaf at band 898 cm^{-1}. It can be observed that the spectrum for kenaf decayed for 1 week at bands 1596 cm^{-1} and 1505 cm^{-1} had no apparent changes compared with the undecayed kenaf spectrum, but they showed a decrease when compared with 16 weeks decayed kenaf spectrum. Intensities of absorption bands at 1426 cm^{-1} and 1122 cm^{-1} showed a decrease, while bands at 1268 cm^{-1} and 1244 cm^{-1} exhibited an increase with increasing exposure time to *P. sanguineus*.

Table 1. Chemical Analyses of Kenaf Chips Innoculated by *P. sanguineus* and *O. latemarginatus* over Incubation Period

Species		HW solubility (%)	CW solubility (%)	EA solubility (%)	Lignin (%)	Holocellulose (%)	Cellulose (%)	Ash (%)
P. sanguineus		ANOVA						
	MS	26.8991	15.6077	0.2077	25.6653	146.3702	102.3620	2.6525
	F-ratio	41.42***	37.18***	9.62**	10.2**	62.55***	6.38^{ns}	28.02***
	Treatments	Tukey test						
	1 week	5.3582c	2.3922bc	1.3576a	14.358a	63.385b	67.687a	2.4975b
	2 weeks	6.9061bc	1.3342c	1.4857a	13.465a	64.882b	57.610ab	2.4696b
	4 weeks	7.9267b	3.2431b	1.2262ab	13.516a	61.908bc	56.912b	2.4306b
	8 weeks	12.438a	7.1756a	1.4455a	7.73b	58.974c	55.608b	4.0544a
	16 weeks	4.9778c	2.2508bc	0.8341b	15.067a	77.124a	52.035b	4.2957a
O. latemarginatus		ANOVA						
	MS	61.2567	47.1914	5.0993	8.1033	295.1144	428.6255	2.6497
	F-ratio	83.85***	238.49***	14.13***	25.71***	2118.64***	596.14***	917.35***
	Treatments	Tukey test						
	1 week	5.1576c	2.4716c	0.9115c	13.8331a	81.6916a	55.0062ab	1.89654d
	2 weeks	4.6854c	2.1925c	1.0361bc	12.7885ab	80.2746b	55.4853ab	1.84951d
	4 weeks	7.9348b	5.4692b	1.5365bc	11.6155bc	75.3026c	55.5432a	2.37739c
	8 weeks	13.7439a	10.3067a	2.5685ab	10.5064dc	73.8154d	53.2267b	3.51291b
	16 weeks	13.9704a	10.1492a	4.0369a	9.7927d	56.8514e	28.1704c	3.88288a

^{ns} means statistically non-significant difference at 0.05 confidence level
**Statistically significant difference at 0.01 confidence level
***Statistically significant difference at 0.001 confidence level
[a] Within a column, means bearing same letter are not significantly different (P<0.05)
HW Hot water
CW Cold water
EA Ethanol-acetone

(a)



(b)

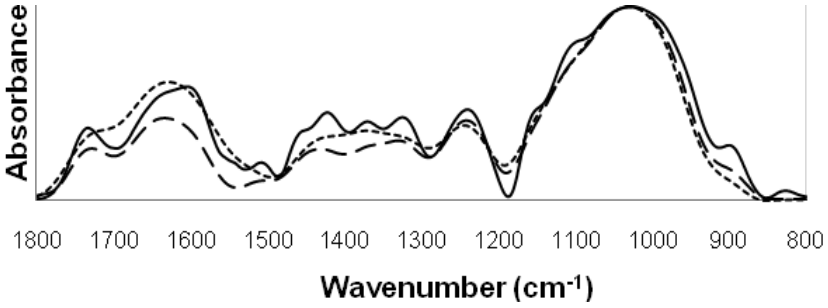


Fig. 6. FTIR spectra of kenaf decayed by (a) *P. sanguineus* and (b) *O. latemarginatus* for 1 week (— —) and 16 weeks (-----) with untreated kenaf (——)

As seen in the spectra of kenaf decayed by *O. latemarginatus* (Fig. 6b), carbohydrates bands at 1738 cm^{-1}, 1375 cm^{-1}, and 1158 cm^{-1} increased in their intensities, but a decrease in intensity is shown at band 898 cm^{-1}. For lignin bands, intensities increased at bands 1596 cm^{-1}, 1505 cm^{-1}, 1462 cm^{-1}, and 1268 cm^{-1} but decreased at band 1244 cm^{-1}, and no distinct changes were apparent at band 1122 cm^{-1} between the two degrees of decay. From the FTIR spectral results, *P. sanguineus* showed simultaneous degradation the lignin and carbohydrates components in kenaf, whereas *O. latemarginatus* showed selective decay, in which lignin was degraded preferentially.

CONCLUSIONS

From the SEM examination it can be concluded that all the cells of fibres, vessels, and ray parenchyma are decayed by the white rot fungi. Kenaf chips were degraded noticeably during the first week of pretreatment, and extensive degradation occurred during the incubation period to 16 weeks for *P. sanguineus* and *O. latemarginatus*. Weight loss of kenaf chips after being treated with both white rot fungi indicated increasing degradation as the incubation period was prolonged. Drastic degradation occurred after pre-treatment of 2 weeks. *P. sanguineus* secreted Lac as the sole ligninolytic enzyme, while *O. latermarginatus* FRIM 031 produces two types of enzymes, LiP and MnP. Based on chemical composition analysis, *O. latemarginatus* was shown to be a potential candidate for delignification and may even have better performance than *P. sanguineus*. Significant degradation of lignin by *O. latemarginatus* was apparent from ANOVA analysis. However, *O. latemarginatus* still is considered as a new white rot fungus in biodegradation or biodelignification; therefore further research needs to be done on it. Future research will focus on optimizing the pretreament conditions for this fungus. There is a need to identify and evaluate not only ligninolytic enzymes, but also enzymes involved in degrading carbohydrates.

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