The Pretreatment Effect of Single Culture White Rot Fungi on the Anatomical Structure of Betung Bamboo

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Abstract

This study was to investigate the changes of morphological, macroscopic and microscopic characteristic on betung bamboo fiber during single culture of white rot fungi pretreatment. Fresh, and bark less 2 years old betung bamboo (*Dendrocalamus asper*) chips, 1.6 cm in length were inoculated by 10% of white-rot fungi inoculums stock for 30 and 45 days in room temperature. This study used three kinds of fungi i.e *Trametes versicolor, Pleurotus ostreatus* and *Phanerochaete chrysosporium*. After the incubation period was finished the chips were separated by maceration process (Schutze method) to analyze the fiber dimension and its derived value. The fibers were then observed the macroscopic and microscopic structure by optical microscope. The pretreatment caused the decreasing of fiber length, cell wall thicknesses, while the lumen and fiber diameter were since in versa. The improvement on fiber derived value except muhlsteph ratio was found in this study. Bamboo pretreated by *T. versicolor* for 30 days showed the best fiber dimension and fiber derived value and the treated bamboos were predicted to produce pulp with superior grade quality (grade I).

Key words: bamboo structure, betung bamboo, fiber dimension, white rot fungi

Introduction

Bamboo belongs to the family of grasses and it is the most widely used non wood lignocellulose for the production of paperboards and papers and feedstock of textile, food, methane, lactic acid, construction, reinforcing fiber, biomass energy like bio-ethanol (Tsuda 1998, Scurlock *et al*. 1998, Lee *et al*. 2001, Kobayashi 2004, Vu *et al*. 2004). Bamboo produces cellulose 2-6 times more than pine does. Its biomass increased by 10-30% daily, while wood biomass increased by 2.5% daily. Bamboo can be harvested after 4 years, while we have to wait for 8-20 years to harvest fast growing trees (Herliyana *et al.* 2005). Based on fiber morphology and physical and chemical properties, betung and yellow bamboos were better raw materials than tali, andong, ampel and black bamboo to be utilized for pulp and paper (Fatriasari & Hermiati 2008).

Basically, the lignocellulose materials are complex polymer which consisting of homo-polymer of cellulose, branch polymer of hemicelluloses and hetero-polymer of lignin. Biological pretreatment is one of the kind approach pretreatment used to convert the materials to any application. White rot fungi are the most effective basidiomycetes of this pretreatment (Sun & Cheng 2002, Zhang *et al*. 2007) to remove lignin from plant cell (Haakala *et al*. 2005, Akhtar *et al*. 1998). These fungi secrete the lignolytic enzymes to penetrate (Messner 1994) and to distribute the enzyme on the substrate (Kirk *et al*. 1980). However, many of these fungi have the ability to not only depolymerize and metabolize lignin but also to degrade cellulose and hemicelluloses (Blanchette 1991). Base on this reason, the selection of white rot fungi that have high delignification selectivity is important step.

Biological pretreatment using white rot fungi on the lignocellulose materials are widely range utilization like bio-energy, feed, enzymatic hydrolysis, biobleaching, pulp and paper, enzim production etc (Isroi *et al.* 2011). Liquid inoculums of single culture of white rot fungi (*Trametes versicolor*, *Phanerochaete chrysosporium* and *Pleurotus ostreatus*) were applied on non wood materials like betung bamboo for biopulping process (Akhtar *et al*. 1998, Fatriasari *et al.* 2007, 2009, 2011), bagasse (Fitria *et al*. 2007, Anita *et al*. 2011a,b, Hossain & Anantharaman 2006), rice straw (Ermawar *et al*. 2006), oil palm empty fruit bunch, bamboo for bio-ethanol process as well. Investigation of the effect of white rot fungi pretreatment on the pulp and paper properties, hydrolysis performance etc had been reported in the previous research. This bamboo was also pretreated by mixed culture of white rot fungi. This study had been reported in our parallel study, in which the pretreatment using mixed culture of white rot fungi could improved the anatomical characteristic especially on the fiber morphology and its derived value. This research was conducted to complete investigation on the anatomical characteristic changes after single culture of white rot fungi pretreatment on betung bamboo.

Materials and Methods

Sample preparation

Fresh, barkless 2 years old bamboo betung (*Dendrocalamus asper*), from Nanggewer, Cibinong was cut using a hammermil to obtain ± 1.6 cm bamboo chips in length. The chips were then stored in a refrigerator to avoid microorganism contaminant. They were then kept for 24 hours at room temperature continued by sterilization in an autoclave for 45 minutes at 121 ^{0}C before fungi application.

Inoculum stock preparation

T. versicolor, *P. chrysosporium* and *P. ostreatus* inoculum cultured on Malt Extract Agar (MEA) *Slant* (10.65 g MEA was diluted in 300 ml aquadest) for 7-14 days. Five ml of the *JIS Broth* medium was injected in each slants, and the fungi was then scratched by ose. The suspension was then poured into 95 ml of JIS Broth medium (3 g KH_2PO_4, 2 g $MgSO_4.7H_2O$, 25 g glucose, 5 g pepton, and 10 g *malt extract* were added into 1 l aquadest) and incubated stationery for 7-8 days. A 10 g of *corn steep liquor* was then poured into 100 ml of inoculum. The inoculum was then homogenized by a high speed waring blender 2 twice each for 20 seconds. The solution obtained was used as inoculum stock.

The oven dried weight (ODW) of 250 g bamboo chips was put into heat resistant plastic bag. A 25 ml of each liquid inoculum stock was injected into bamboo chips and then were incubated in a room temperature (29-30 °C) for 30 and 45 days. After finishing incubation period, part of samples was treated maceration process to determine the anatomical characteristic.

Fiber and vessel dimension measuring

To measure the fiber and vessel dimension, the bamboo chips were separated with maceration process based on Schutze method (Sas 1961). Fiber and vessel dimension were measured under microscope observation. Fiber dimension was included fiber lengths, lumen and fiber diameters, and cell wall thicknesses, while the vessel dimension was included length and diameter vessel. The fiber length, fiber diameter and cell wall thickness measurement was conducted 30 and 15 times, respectively to find out the average value. Fiber derived value was calculated based on fiber dimension data consisted runkel ratio, felting power, muhlsteph ratio, coefficient of rigidity, and flexibility ratio. The macroscopic and microscopic characteristics were also observed using optical microscope. Macroscopic structure was investigated on the cross section of samples.

Results and Discussion

Fiber dimension and its derived value is one of important lignocellulose properties used to predict the pulp properties. After biological pretreatment using single culture of white rot fungi, the treated fiber was lower than untreated sample. The fungi hypae penetration in to cell wall structure of bamboo caused lignolytic enzyme secretion to support lignin degradation activity. Based on LPHH criteria, these fiber qualities were included in grade I (the fiber length up to 2 mm). The fiber length of bamboo pretreated by *T. versicolor* (TV) for 45 days showed the highest data, meanwhile the *P. chrysosporium* (PC) pretreatment for 30 days affected the lowest fiber length (Table 1). This all fiber length dimension after white rot fungi pretreatment is included in the long fiber. The long fiber also affects paper strength, pulp handling, and the surface smoothness of paper. The fiber and lumen diameters of fungi pretreatment were wider; meanwhile the cell wall thickness of bamboo was thinner. The lignin degradation affected the fiber dimension changes. This fiber will support the better formation of fiber bond in the paper forming process by providing the broader surface area in interfiber bonding formation. The improvement of fiber dimension will be predicted to increase the pulp and paper properties. This changes pattern of fiber dimension was also reported in mixed culture WRF pretreatment on betung bamboo. White rot fungi not only produce a whole set off enzymes for the lignin degradation but also can act as a transporter for these enzymes into the wood flakes and change psychological conditions required for enzymatic reaction (Islam *et al*. 2007). *T. versicolor* produces manganese peroxidase (MnP) and laccase, hemicellulase, and cellulase (Yang *et al*. 2007). These enzymes use low molecular weight mediator to attack lignin (Perez *et al*. 2002). *P. ostreatus* and *T. versicolor* produce 3 lignolytics i.e. laccase, lignin peroxidase (LiP), and manganase peroxidase (MnP). Lignin peroxidase (LiP), has the structural difference from manganase peroxidase (MnP) in the ability to oxidize chemical bonds in lignin (Lobos *et al*. 2001). On the other hand, MnP degrade lignin indirectly by providing H_2O_2 for reaction of lignin peroxidase (Hossain & Anantharaman 2006). These enzyme was discovered in *P. chrysosporium* was MnP (Kirk & Chang 1990). LiP demonstrated the ability to oxidize the lignin bond. In contrast, MnP degrades lignin indirectly by providing H_2O_2 for precusors (Kirk & Chang 1980, Hossain & Anantharaman 2006).

In general, pretreatment using *P. ostreatus* and *P. chrysosporium* for 30 days provided the highest and lowest fiber dimension value properties, respectively. Anatomical structure of bamboo consists of fiber and vessel with each proportion whereas high fiber proportion of bamboo is preferable to be utilized for any application effectively. The vessel length of vessel was shorter than the fiber's, but the vessel diameter was since in versa. The best vessel dimension value was showed by pretreatment of *P. chrysoporium* for 30 days.

The pulp and paper properties are also influenced by fiber derived value. This parameter most affects the physical properties of paper sheet. Runkel ratio is the ratio of cell wall thickness to lumen diameter, whereas this value (below to one) is very good for the paper. The fiber derived value after fungi pretreatment was shown in Table 3. All treatments caused positive effect in decreasing of runkel ratio, coefficient of rigidity and flexibility ratio. The significant decreasing in runkel ratio was affected by both of the thinner cell wall and wider cell diameter. This condition accelerated to form pulp sheet which had the high tensile index. Bamboo pretreated by single culture of WRF showed a good fiber derived value and the pulp quality was included grade I based on FAO and LPHH criteria with the total score of 525. There was no difference in this score compared to our previous study on mixed culture fungi.

Table 1 The Fiber dimension value

Pretreatment	Single Culture						Untreated*
Incubation days	30 days			45 days			0 days
Fungal Species	TV	PC	PO	TV	PC	PO	
Fiber Length (mm)	4.1842	3.2515	3.9554	3.8676	4.0811	3.6413	4.693
Value	6	1	4	3	5	2	
Fiber Diameter (mm)	0.02994	0.02990	0.03131	0.02970	0.02978	0.03176	0.0250
Value	4	3	5	1	2	6	
Lumen diameter (mm)	0.0256	0.0258	0.0267	0.0250	0.0253	0.0272	0.007
Value	3	4	5	1	2	6	
cell wall thickness (mm)	0.00217	0.00207	0.00232	0.00234	0.00236	0.00230	0.009
value	2	1	4	5	6	3	
subtotal	15	9	18	10	15	17	0
Rank	3	5	1	4	3	2	

Source: Fatriasari & Hermiati (2008)

Table 2 Vessel dimension value

Single culture pretreatment						
Incubation days	30 days			45 days		
Fungal Species	TV	PC	PO	TV	PC	PO
Vessel length (mm)	0.9365	0.9456	0.8681	0.9159	0.9603	0.9359
Value	4	5	1	2	6	3
Vessel diameter (mm)	0.1714	0.1973	0.1837	0.2489	0.1695	0.18656
Value	2	5	3	6	1	4

Table 3 The Fiber derived value

Parameter	Incubation days	Requirement		*Single culture*								
				PC	Pulp quality (Score)		TV	Pulp quality (Score)		PO	Pulp quality (Score)	
		FAO*	LPHH		FAO	LPHH		FAO	LPHH		FAO	LPHH
Fiber length	30 days	>2.00 mm	2.200µ	3.2515±0,558	I (100)	I (100)	4.1842±0.8207	I (100)	I (100)	3.9554±0.6242	I (100)	I (100)
	45 days			4.0811±0,7975	I (100)	I (100)	3.8676±0.5928	I (100)	I (100)	3.6413±0.9014	I (100)	I (100)
Runkel ratio	30 days	<0.25	0.25	0.168±0.060	I (100)	I (100)	0.172±0.036	I (100)	I (100)	0.1777±0.050	I (100)	I (100)
	45 days			0.187±0.064	I (100)	I (100)	0.194±0.058	I (100)	I (100)	0.174±0.044	I (100)	I (100)
Felting Power	30 days	> 90	90	108.433±26.70	I (100)	I (100)	156.887±31.718	I (100)	I (100)	117.169±18.433	I (100)	I (100)
	45 days			129.198±18.946	I (100)	I (100)	131.256±31.144	I (100)	I (100)	113.828±38.855	I (100)	I (100)
Muhlstep ratio	30 days	>60	80	207.2±50.804	III (25)	IV(25)	216.8±34.976	III(25)	IV(25)	232±46.623	III(25)	IV(25)
	45 days			226.4±52.281	III (25)	IV(25)	233.6±42.029	III(25)	IV(25)	230.4±41.619	III(25)	IV(25)
Flexibility ratio	30 days	>0.8	0.8	0.858±0.022	I (100)	I (100)	0.854±0.026	I (100)	I (100)	0.850±0.035	I (100)	I (100)
	45 days			0.845±0.044	I (100)	I (100)	0.839±0.039	I (100)	I (100)	0.853±0.031	I (100)	I (100)
Coeff. of rigidity	30 days	<0.1	0.1	0.071±0.022	I (100)	I (100)	0.073±0.013	I (100)	I (100)	0.075±0.018	I (100)	I (100)
	45 days			0.078±0.022	I (100)	I (100)	0.080±0.020	I (100)	I (100)	0.073±0.016	I (100)	I (100)
Total Score	30 days				525	525		525	525		525	525
	45 days				525	525		525	525		525	525

Source : *Syafii & Siregar 2006

A sharp increasing of muhlsteph ratio happened in all of fiber pretreated with single culture of WRF. This trend was also found in mixed culture of WRF pretreatment. The longer incubation time tended to increase this value in all kind of fungi pretreatment. The lower muhlsteph ratio resulted the lower folding power of pulp sheet that caused the dissatisfactory paper quality (Rhamdani 1994).

Bamboo was dominated by pharencyma recapitulated on Table 5 (the fiber proportion based on 1.000.000 mm^{-2} area). The average of fiber. intercellular channel and metaxylem. and pharencyma proportion were 31.85%. 1.44%. and 65.18% respectively. The high pharencyma proportion tended to produce the lower pulp yield caused by the only fiber that can be converted to be cellulose to form paper sheet.

This below figure showed the macroscopic and microscopic images of bamboo under single culture of WRF treatment. The image indicated a degradation pattern caused by fungi activity in substrate (A-F).

The fungi secreted the hyphae to penetrate on to cell wall via surface of bamboo and degrade lignin polymer. Macroscopic vascular bundle of untreated bamboo can be seen in Image H. This bundle has function as food and water channel. In comparison with mixed culture of WRF fungi pretreatment. The degradation intensity of substrate showed the similar level.

SEM images were reported by Fatriasari *et. al* (2010) showed that the degradation activity of WRF on the substrate. Other study on microscopic level of the fungal growth patterns of *P. chrysosporium* and *C. subvermispora* in aspen wood chips showed that *P. chrysosporium* grew well on both across the chip surfaces and throughout the cell wall.The degradation of cell wall was possible occured. The fungi might be decolonized the xylem in all planes. first via vessels and then via ray parencyma. The decay captured the nutrient that was reserved within ray parechyma and then colonize it to ditribute the fungal mycelium throughout the sapwood (Akhtar *et al*. 1998).

Table 4 Recapitulation of fiber derived value scoring

White rot fungi treatment		Incubation periods (days)	Fiber derived value					Sub total	Rank
			Runkell ratio	Felting power	Muhlstep ratio	Flexibility ratio	Coeff. of rigidity		
Single culture	PO	30	4	**3**	2	3	2	14	5
		45	3	2	3	4	4	16	4
	PC	30	2	1	6	6	6	21	2
		45	5	4	4	2	3	18	3
	TV	30	1	6	5	5	5	22	1
		45	6	5	1	1	1	14	5

Table 5 Fiber proportion per mm^{-2}

No	Base Area	Fiber Proportion		Interseluler Channel and Metaxylem Proportion		Pharenchyma Proportion	
		Value	%	Value	%	Value	%
1	1.000.000	32 .393.65	32.44	-	-	675 606.35	67.56
2		248 555.69	24.86	28 935.51	2.89	722 508.80	72.25
3		365 810.88	36.58	48 174.06	4.82	586 015.06	58.60
4		275 724.20	27.57	-	-	724 275.80	63.27
5		367 253.13	36.73	-	-	632 746.87	63.27
6		329 013.47	32.90	9 550.39	0.96	661 436.14	66.14
Average		318 458.50	31.85	14 443.33	1.44	667 098.17	65.18

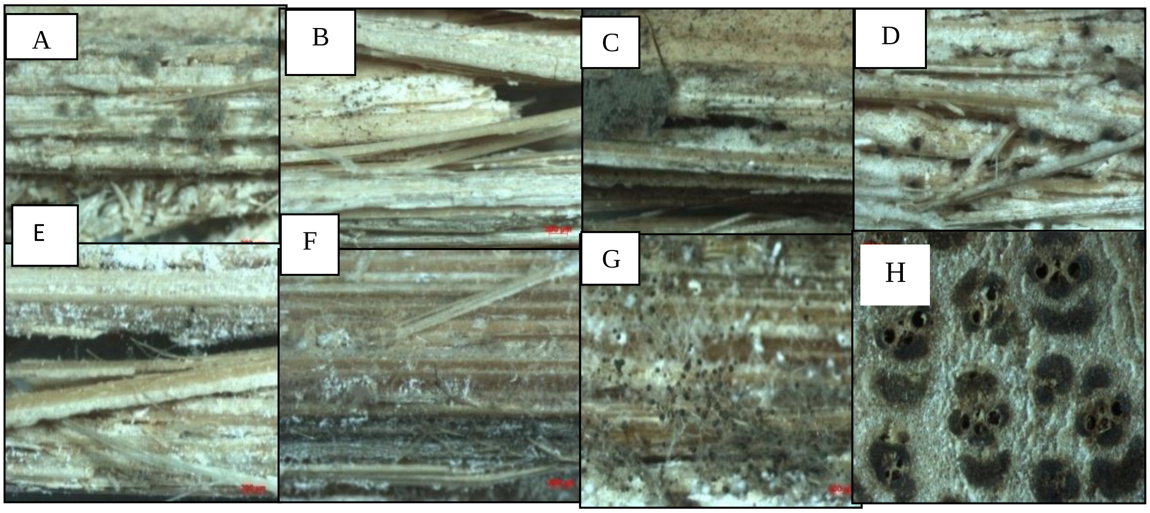

Figure 1 Macroscopic image of bamboo pretreated by *P. chrysosporium* (PC 30 days (A). PC 45 days (B). *P. ostreatus* (PO 30 days (C). PO 45 days (D)). *T. versicolor* (TV 30 days (E). TV 45 days (F)). untreated bamboo (G). vascular bundle (H)

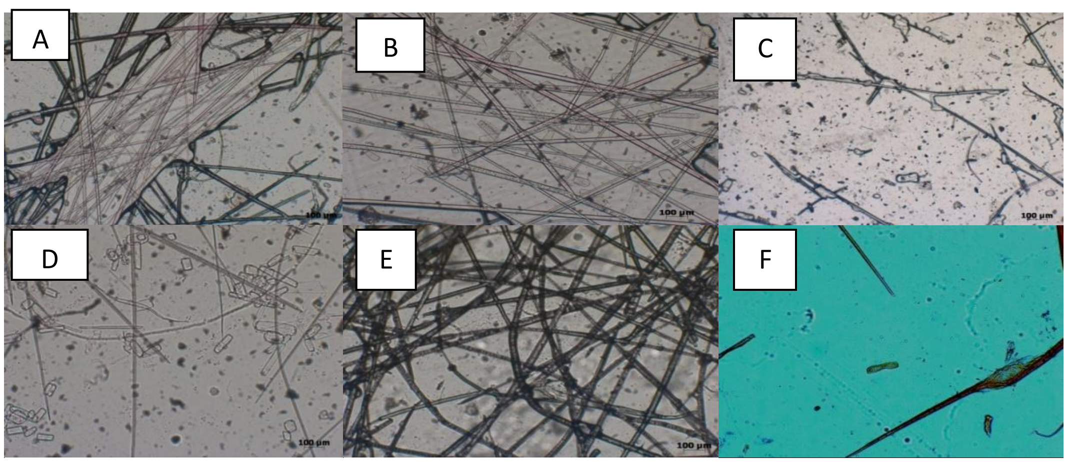

Figure 2 Microscopic image of bamboo pretreated by *P. chrysosporium* (PC 30 days (A), PC 45 days (B), *P. ostreatus* (PO 30 days (C), PO 45 days (D)), *T. versicolor* (TV 30 days (E), TV 45 days (F))

The maceration process was done to separate the bundle fiber of cell wall to be single fiber (Figure 2). Pharenchyma cell had shorter fiber with wider diameter (image D). The fungi degradation affected the damage in single fiber imaged in bamboo pretreated by *T. versicolor* for 45 days (F). However on single culture fungi treatment gave no significant difference among mixed culture treatment on the fiber separation process.

Conclusion

The single culture of white rot fungi pretreatment on betung bamboo improved the fiber morphology and its derived value. There was the decreasing of fiber length, cell wall thicknesses, while the lumen and fiber diameter were since in versa. All pretreatment provided better fiber derived value, except muhlsteph ratio. Bamboos had a higher pharencyma proportion that tended to produce the lower pulp yield. There is no significant difference degradation pattern caused by fungi activity in all substrates based on the macroscopic images. Bamboo pretreated by *T.versicolor* for 30 days showed the best fiber dimension and fiber derived value and treated bamboos were predicted in producing pulp with superior grade quality (grade I).

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