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Biological Pretreatment of Oil Palm Frond Fiber Using White-Rot Fungi for Enzymatic Saccharification

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Abstract

Oil palm frond is one type of lignocellulosic biomass abundantly and daily available in Indonesia. It contains cellulose which can be converted to glucose, and further processed to produce different kinds of value –added products. The aim of this research is to study the effects of biological pretreatment of oil palm frond (OPF) fiber using *Phanerochaete chrysosporium* and *Trametes versicolor* on the enzymatic saccharification of the biomass. The OPF fiber (40-60 mesh sizes) was inoculated with cultures of the two fungi and incubated at 27 °C for 4 weeks. The samples were taken after 1, 2, 3, and 4 weeks of incubation. Chemical components of the biomass after pretreatment were analyzed. The saccharification of the pretreated samples using cellulase and β-glucosidase was performed in a water bath shaker at 50 °C for 48 hours. The concentration of reducing sugar increased with increasing of incubation time, either in those pretreated with culture of *P. chrysosporium* or with *T. versicolor*. Pretreatment of OPF fiber using single culture of *T. versicolor* for 4 weeks gave the highest reducing sugar yield (12.61% of dry biomass).

Abstrak

***Pretreatment* Biologis Serat Pelepah Kelapa Sawit Menggunakan Jamur Pelapuk Putih untuk Sakarifikasi Enzimatis**. Pelepah kelapa sawit (PKS) merupakan salah satu biomassa lignoselulosa yang tersedia cukup melimpah di Indonesia. Serat PKS mengandung selulosa yang dapat dikonversi menjadi glukosa, dan selanjutnya dapat diproses lebih lanjut untuk menghasilkan berbagai produk yang mempunyai nilai tambah. Tujuan penelitian ini adalah untuk mempelajari pengaruh *pretreatment* biologis pada serat PKS menggunakan *Phanerochaete chrysosporium* dan *Trametes versicolor* terhadap hasil sakarifikasinya. Serat PKS (40-60 mesh) diinokulasi dengan kedua kultur jamur dan diinkubasi pada suhu 27 °C selama 4 minggu. Contoh diambil setelah masa inkubasi selama 1, 2, 3, dan 4 minggu dan dianalisis kandungan komponen kimianya. Sakarifikasi PKS yang telah diberi *pretreatment* menggunakan selulase 20 FPU/g substrat dan β-glukosidase 14 CBU/g substrat dilakukan dalam *water bath shaker* pada suhu 50 °C selama 48 jam. Konsentrasi gula pereduksi meningkat dengan semakin lamanya waktu inkubasi, baik pada contoh dengan perlakuan *P. chrysosporium* maupun dengan *T. versicolor*. Pretreatment serat PKS menggunakan kultur tunggal *T. versicolor* selama 4 minggu menghasilkan rendemen gula pereduksi tertinggi (12,61% dari berat kering biomassa).

Keywords: enzymatic saccharification, oil palm frond, reducing sugar, white-rot fungi

1. Introduction

Oil palm frond (OPF) is one type of lignocellulosic biomass abundantly available in Indonesia. It is available daily since it can be obtained during pruning when harvesting the fresh fruit bunch. Besides that, the OPF can also be obtained during replanting of the oil palm trees. As much as 10.40 t/ha of dry OPF generated per year during the pruning [1]. Thus, with oil palm plantation area of about 20 million ha, there is 208 million t/year of OPF produce in Indonesia. This amount of biomass has the potential to be utilized as raw material of some value added products. There are some studies regarding the use of OPF, as a mixture for ruminants feed [2], for pulp [3], and for making compost [4]. Another potential use of OPF is for production of bioethanol. The fresh OPF can be directly pressed, and the juice obtained contains sugar; therefore, it can be directly fermented to ethanol [5]. Besides that, the frond fiber can be used as cellulose feedstock for

Makara Seri Teknologi, 2013, 17(1): 39-43
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bioethanol. The OPF fiber contains 15.5% lignin, 41.7% cellulose, and 16.4% hemicellulose [5], while oil palm empty fruit bunch (OP EFB) fiber contains more lignin (27.6-32.5%) and comparable amount of cellulose (41.3-46.5%) [6]. Thus, based on the main chemical components of the fiber, OPF fiber is more potential than OP EFB fiber as bioethanol feedstock. The less lignin in the fiber makes it easier for the pretreatment process.

There are only a few reports on the use of OPF fiber for production of bioethanol. To the best of our knowledge, there are only two reports on the use of oil palm frond fiber for bioethanol production with emphasis on the effects of hot compressed water pretreatment [7-8]. These methods of pretreatment have resulted in quite high glucose yield, based on the potential glucose in OPF. Another interesting report is the microscopic studies of oil palm frond after various kinds of pretreatments, that are chemical (acid and alkaline), liquid hot water (autoclaving at 121 °C for 15 minutes), and microbial pretreatment using *Aspergillus niger* [9]. This study suggests that the most effective pretreatment is liquid hot water. Biodegradation of lignin in OPF by white rot fungi is reported by Namoolnoy *et al*. [10]; however, the species of the isolates of white rot fungi used is not clear. White rot fungi are known as an effective lignin degrading microorganisms, so that they can help in the pretreatment of lignocellulosic feedstock for bioethanol production. The fungi produce three types of ligninolytic enzymes, lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase. The process takes quite a long time; however, it uses less energy and is more environmentally friendlier than physical, mechanical or chemical processes. The species *Phanerochaete chrysosporium* and *Trametes versicolor* are the most commonly used white rot fungi in lignin biodegradation studies, because these two fungi have good ligninolytic properties, can grow fast, and are easy to handle in the culture [11]. This research is aimed at studying the effects of two species of white rot fungi, *P. chrysosporium* and *T. versicolor* for OPF pretreatment on the sugar obtained after saccharification.

2. Methods

The OPF was obtained from Kertajaya Plantation, PT Perkebunan Nusantara VIII, Banten, Indonesia, while the *P. chrysosporium* and *T. versicolor* cultures were from Research Center for Biology, Indonesian Institute of Sciences (LIPI), Cibinong, Bogor, Indonesia. The OPF was chopped, dried and ground to produce particles of 40-60 mesh sizes, and then, the chemical components of the biomass were analyzed [12].

The pretreatment was conducted by adding 60 mL of JIS (Japan Industrial Standard) broth (3 g KH_2PO_4, 2 g $MgSO_4.7H_20$, 25 g glucose, 5 g peptone, and 10 g malt extract in 1 L mixture solution) into 30 g of biomass. The mixture was sterilized for 30 minutes. The *P. chrysosporium* and *T. versicolor* were cultured in malt extract agar (MEA) slant for 7 days, and then they were transferred into 100 mL JIS broth, and incubated for 10 days at room temperature. The inocula were homogenized using warring blender at high speed for 2x20 seconds. The cultures (10%) were put in the medium containing OPF fibers, and they were incubated at 27 °C up to 4 weeks. A control sample of OPF (Control 2), which was exposed to sterilization but not pretreated with fungi was also prepared and incubated for 4 weeks. The fungi pretreated samples were taken after 1, 2, 3, and 4 weeks of incubation and sterilized for 30 minutes to destroy the fungi, while the control sample was taken only after 4 weeks of incubation and it was not exposed to further sterilization. The samples were then washed with distilled water to remove the fungi. The control sample was also subjected to the same washing treatment. The residues of these samples were used as substrates in the saccharification using Novozyme cellulase (enzyme activity 70 FPU/g) and β-glucosidase (enzyme activity 240 CBU/g). The cellulase and β-glucosidase loadings were 20 FPU/g dried substrate and 14 CBU per g dried substrate, respectively. The saccharification was performed at 50 °C for 48 hours in a water bath shaker, following the Laboratory Analytical Procedure for Enzymatic Saccharification of Lignocellulosic Biomass provided by National Renewable Energy Laboratory (NREL) [13]. The reducing sugar concentration in the hydrolysate and the reducing sugar yield were determined after the analysis of reducing sugar using Nelson-Somogyi method [14]. The yield of reducing sugar was calculated based on the dry weight of saccharification substrate and based on the dry weight of the initial OPF biomass used. The original sample of OPF (Control 1) and the pretreatment control of OPF (Control 2) were also subjected to the same saccharification process as the fungi pretreated samples. The pretreatment and saccharification experiments were conducted in triplicates.

3. Results and Discussion

Oil palm frond used in this study contained 9.91% of ethanol-benzene extract, 16.77% of lignin, 36.33% of α-cellulose, and 30.34% of hemicellulose. This was comparable with the chemical components reported by other researchers [3,5,7-8], except for the hemicellulose content which was higher than that in the three reports [3,5,7], but almost the same as that reported by Goh *et al*. [8]. The change of these chemical components in OPF after pretreatment using *P. chrysosporium* and *T. versicolor* is presented in Table 1.

The ethanol benzene extract in the fungal pretreated samples were lower than that in the original OPF sample. The OPF contains sugar, such as fructose,

Table 1. Chemical Compositions of OPF Fiber Before and After Pretreatment

Components	Treatment	Incubation (weeks)				
		0	1	2	3	4
Ethanol benzene extract (%)	Control 1*)	9.91 ± 0.49	-	-	-	-
	Control 2**)	-	-	-	-	2.49 ± 0.11
	P. chrysosporium	-	2.91 ± 0.27	4.59 ± 1.58	3.79 ± 0.5	6.38 ± 1.38
	T. versicolor	-	3.07 ± 0.70	4.59 ± 0.31	3.76 ± 0.81	7.25 ± 1.30
Lignin (%)	Control 1*)	16.77 ± 0.30	-	-	-	-
	Control 2**)	-	-	-	-	28.18 ± 0.38
	P. chrysosporium	-	27.71 ± 0.73	27.75 ± 2.10	26.71 ± 1.35	24.08 ± 2.21
	T. versicolor	-	28.35 ± 0.35	27.71 ± 0.31	27.18 ± 0.38	25.79 ± 0.73
α-cellulose (%)	Control 1*)	36.33 ± 4.10	-	-	-	-
	Control 2**)	-	-	-	-	37.26 ± 0.81
	P. chrysosporium	-	35.18 ± 0.46	41.13 ± 0.42	42.39 ± 2.36	42.15 ± 1.14
	T. versicolor	-	35.61 ± 0.46	35.24 ± 0.37	36.13 ± 1.53	33.84 ± 0.93
Hemicellulose (%)	Control 1*)	30.34 ± 4.54	-	-	-	-
	Control 2**)	-	-	-	-	30.06 ± 1.31
	P. chrysosporium	-	29.21 ± 0.92	22.69 ± 0.63	23.77 ± 1.45	22.49 ± 1.82
	T. versicolor	-	31.05 ± 0.30	28.84 ± 0.27	29.72 ± 1.30	29.21 ± 0.43

*) Control 1 is original sample of OPF
**) Control 2 is pretreatment control of OPF (sterilized OPF without fungi treatment), incubated for 4 weeks

glucose and sucrose [5]. Some of these sugar types, which might be counted as ethanol-benzene extract, were removed when the OPF was washed after fungal pretreatment. The decrease of ethanol-benzene extract was also noted in the pretreatment control sample (Control 2), which was exposed to sterilization but not inoculated with the fungi. In this control sample, which was also incubated for 4 weeks, the ethanol-benzene extract was 2.49%. The ethanol benzene extract in the OPF increased again with increasing of incubation time. This might be due to the formation of some extractives by the fungi during the pretreatment. The ethanol-benzene extract content in the OPF pretreated with either *P. chrysosporium* or *T. versicolor* was almost the same, and so was the pattern throughout all the incubation times.

The lignin contents in the fungal pretreated samples were higher than that in the original OPF sample. The increase of lignin content was also noted in the control sample (Control 2), which was exposed to sterilization, but not inoculated with the fungi. In this control sample (Control 2), which was also incubated for 4 weeks, the lignin content is 28.18%. So far, we could not know what might cause this phenomenon. However, we noted that there is also an increase of lignin content in the OPF pretreated with hot compressed water [8]. In their report, the lignin content in the raw OPF is 19.23%, while those in the pretreated samples are 19.68-51.78%. Unfortunately, there is also no explanation for this phenomenon. It seems that the increase of lignin content in the pretreated samples is due to the decrease of other chemical components in the samples, for example, due to the decrease of hemicellulose [7] or in our case is due to the decrease of ethanol-benzene extract and hemicellulose. The lignin content in the fungi pretreated samples did not decrease until 3 weeks of incubation, but it decreased after 4 weeks of incubation using each fungus. This result is in good correlation with our previous studies on the use of some white rot fungi for pretreatment of rice straw [15] or sugarcane bagasse [16], which show that the optimum incubation time is 4 weeks.

The α-cellulose content in the samples pretreated with *P. chrysosporium* for 2-4 weeks were higher (42.39% the highest) than that in the original OPF sample, while the hemicellulose content were lower (22.49% the lowest) than that in the original OPF sample. However, the α-cellulose and hemicellulose content in the sample pretreated with *T. versicolor*, 34-36% and 28-31%, respectively, were almost the same as those in the control sample (Control 2), 37.26% and 30.06%, respectively, and in the original OPF fiber. The pretreatment process is expected could reduce lignin and hemicellulose content in the lignocellulosic biomass. The fungi pretreatment in this experiment shows that *P. chrysosporium* seems to be more effective than *T.*

Makara Seri Teknologi, 2013, 17(1): 39-43
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versicolor in removing hemicellulose from the OPF fiber, so that it is expected that the result of saccharification of the samples pretreated with *P. chrysosporium* are better than those pretreated with *T. versicolor*. Besides that, the α-cellulose content was also higher in the samples pretreated with *P. chrysosporium* than that pretreated with *T. versicolor*. Unfortunately, the results of the saccharification show that the reducing sugar concentration obtained from the samples pretreated with *T. versicolor* were higher than that pretreated with *P. chrysosporium* (Fig. 1). The reducing sugar yields obtained from the OPF fiber pretreated with *T. versicolor* were also higher (15.12% of dry substrate or 12.61% of dry biomass) than those pretreated with *P. chrysosporium* (12.37% of dry substrate or 10.44% of dry biomass) (Fig 2A and 2B). So far, we could not understand what might cause this phenomenon. The yield of reducing sugar obtained in this experiment was lower than that obtained from hot compressed water pretreatment, which can resulted in 92% of theoretical glucose (with the cellulose content of 45%, it was approximately equal with 41% of dry biomass) [7].

It is also interesting to note that saccharification of the original sample of OPF fiber produced reducing sugar. It means that the OPF fiber might contain some free polysaccharides or oligosaccharides which could be directly hydrolyzed by the cellulase and β-glucosidase enzymes. Analysis of cold-water extract of the original OPF shows that the OPF fiber contains 9.36% cold water extract. The fresh OPF also contains some simple sugars, such as glucose, sucrose and fructose [5]. These reducing sugars in the original samples of OPF fiber might also contribute to the reducing sugar content of the saccharified original OPF fiber samples (Control 1). The oligosaccharides and simple sugars contained in the OPF fiber were probably washed away when the sterilized samples (Control 1) and the fungi pretreated samples were washed by distilled water after the pretreatment, or they were consumed by the white rot

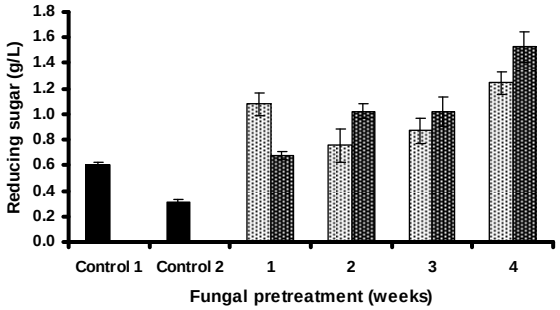

Fig 1. Reducing Sugar Concentration in the Hydrolysate After Saccharification of OPF Fiber Pretreated with *P. chrysosporium* (▒) and *T. versicolor* (▓). Control 1 is Original Sample of OPF Fiber. Control 2 is Pretreatment Control of OPF (Sterilized OPF Fiber without Fungi Treatment), Incubated for 4 Weeks

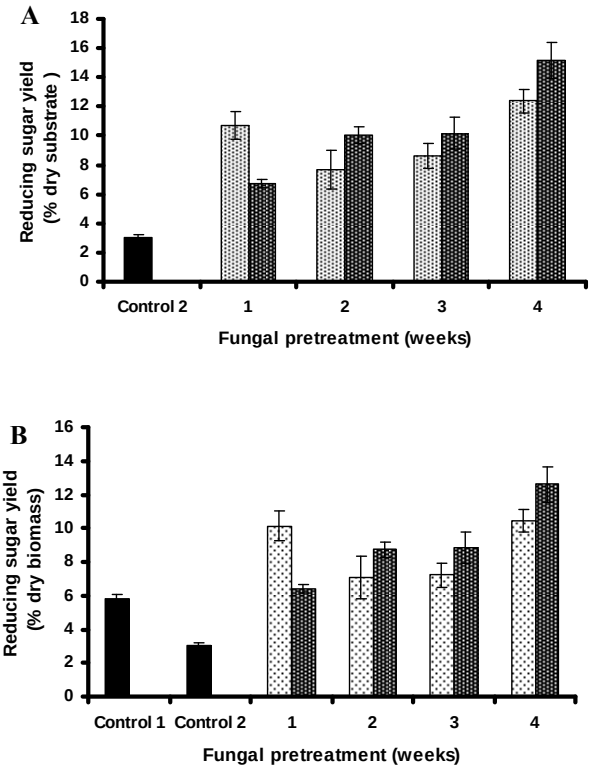

Fig 2. Reducing Sugar Yield Obtained from the Saccharification of OPF Fiber Pretreated with *P. chrysosporium* (▒) and *T. versicolor* (▓), based on Dry Saccharification Substrate (A) and based on Initial Dry OPF Fiber Biomass (B). Control 1 is Original Sample of OPF Fiber, Control 2 is Pretreatment Control of OPF (Sterilized OPF Fiber without Fungi Treatment), Incubated for 4 Weeks

fungi, so that the concentration and yield of reducing sugars were decreased in the Control 2 (sterilized OPF fiber without fungi treatment). The concentration and yield of reducing sugar started to increase again when the samples were pretreated with white rot fungi, which means that the reducing sugars obtained after saccharification were resulted from the enzymatic hydrolysis of the cellulose and hemicellulose contained in the OPF fiber after the lignin that bound the cellulose and hemicellulose was degraded by the white rot fungi.

4. Conclusions

Pretreatment of OPF fiber with *P. chrysosporium* and *T. versicolor* decrease the ethanol-benzene extract and lignin content in the fiber. *P. chrysosporium* could decrease the hemicellulose content and increase the cellulose content in the fiber, while *T. versicolor* could not. On the other hand, results of the saccahrification show that higher reducing sugar yield was obtained from the OPF fiber pretreated with *T. versicolor*. Since the main objective of pretreatment is to obtain as much sugar as possible, it can be concluded that pretreatment

of OPF fiber with *T. versicolor* is better than that with *P. chrysosporium*, with the highest reducing sugar yield (12.61% of dry biomass) after incubation for 4 weeks.

In order to better understand the mechanism of the pretreatment, it is important to analyze the components in the washed away solution after pretreatment and to do the saccharification without washing the substrate after fungi pretreatment.

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