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The Effectiveness of Bio-Delignification Empty Fruit Bunch of Palm Oil by *Aspergillus fumigatus* : Targeting for Bio-Hydrogen Production

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Abstract

As the world`s No. 1 producer of oil palm, Indonesia yields a huge amount of palm oil empty fruit bunch (EFB) as the biomass wastes. These EFB wastes are considered as a potential source of biofuel. Three fungal species isolated from different sources of indigenous Indonesian fungi (wood, bamboo, and oil palm empty fruit bunch) were evaluated for their capability to improve hydrolytic enzymes to biodegrade EFB and release sugars. The most potential fungus was obtained from EFB and identified as *Aspergillus fumigatus*. Bio-delignification using *A. fumigatus* was very effective for semisolid- or solid-fermentation in the

short duration of 7 days. The lignin was reduced up to 4.19 % without significant reduction of cellulose and hemicellulose contents. This duration was considered optimal to maintain a high amount of cellulose-hemi cellulose in EFB and to allow the partial degradation of the lignin for accessible cellulase. The effectiveness of the cellulase to access EFB was examined by enzymatic saccharification. The result showed that the reducing sugar of enzymatic saccharification on biodelignified EFB reached 6.64 g/L, which was 40% higher than that for un-biodelignified EFB. The optimal EFB saccharification was 27 FPU/g EFB with the highest reducing sugar of 8.60 g/L. Furthermore, the resulting sugar, as an intermediate product, can be used to produce hydrogen by *E. aerogenes*.

Keywords: Bio-delignification, Empty Fruit Bunch (EFB) of Palm Oil, *A. fumigatus,* Bio-Hydrogen, Production.

INTRODUCTION

World's requirement for fuel is increasing at high rate meanwhile the fossil fuel is decreasing on much higher rate. Many innovations were achieved for energy carriers from lignocellulosic biomass. [1,2]. The usage of biomass as raw material to produce fuel is environmental friendly and cost effective since its carbon cycle will slow down greenhouse effect as well. Bio-Hydrogen (H_2) is considered as a clean energy carrier which has a great potential to be an alternative fuel. Utilization of Hydrogen as fuel in fuel cell, no pollutant generated, only water. In comparison with fossil fuel, Hydrogen has a higher energy yield [3].

EFB of palm oil is one of the most potential lignocelluosic biomass [4]. Global palm oil production is dominated by Indonesia and Malaysia. These two countries together account for around 85 to 90 percent of total global palm oil production. Indonesia is currently the largest producer and exporter of palm oil worldwide [5]. Lignin, cellulose and hemicelluloses are composer of lignocellulosic biomass. Lignin will limit the

enzymatic digestibility to hemicellulose and cellulose because lignin structure is very complex and bond tightly, irregular, random and its major constituent are aromatic compounds. The aromatic group found in lignin, which are interconnected with aliphatic chains, consisting of 2-3 carbons. As a result of the complexities lignin components of lignocellulose is difficult to be broken. This is due to the crystal structure of the lignin is more crystalline than the cellulose and hemicellulose.Therefore, lignocellulose should be pretreated to break out the crystalline structure of lignocellulose so that cellulose and hemicellulose can be degraded by cellulase [6]. Lignocellulosic biomass is a complex raw material, at 50–80 % dry basis (db), contains cellulose and hemi cellulose for about 45 % and 25 % respectively. Those polysaccharides bound together by lignin by nearly 30 % db. Lignin is partly covalent associated to hemicellulose. This position preventing hydrolytic enzymes and acids from accessing some regions of the holo-cellulose [7].
Lignin removal process is very costly. Delignification by alkaline 0.5% KOH was effective to convert 91.8 % of carbohydrate in raw biomass [8]. Pretreatment EFB by soaking with NaOH in the range of 0.1 M to 0.5 M at temperature for 140^{o}C for 15 minutes did not provide positive influence on EFB conversion, the yield was found only around 3.18 to 5.69 % [9]. The physicochemical method requires high temperature 140-160^{o}C, and the chemical methods are corrosive in nature and demand neutralization. This such method offers low yield of carbohydrates and generate inhibitors for further microbial processes [10]. Under acidic conditions, hydrolysis of hemicelluloses undergo dehydration with formation of furfural and HMF which also inhibit the enzymatic or microbial processes [11].Therefore, biological treatment for delignification that could help in reducing the chemical use and pollution risk is needed urgently.[12].

In this study, indigenous Indonesia’s fungal species, were used to improve the efficiency of cellulase accessibility to hydrolyze cellulose, without totally removing lignin from EFB. Therefore, this work was done to proof the effect of saccharification in EFB.

MATERIALS AND METHODS

Microorganism

Some fungi were isolated from 3 different lignocelluosic biomass: EFB, wood and bamboo. The EFB was taken from Bengkulu province, in Sumatera; while wood and bamboo were taken from around PUSPIPTEK Laboratory in Banten, Java. All fungi were inoculated in potatoes dextrose agar (PDA)..

Materials

EFB was obtain from various palm plantations in Sumatera and west Java. This EBF was dried naturally and cut into small pieces0.3 -0.5 cm.

Potassium phosphate buffers were used at pH 6 in order to keep pH condition [13, 14].

Composition analysis of EFB by Chesson methods [15]. *Acremonium cellulolyticus* Cellulase was obtained from Laboratory of Biotechnology - Shizuoka University, from Meiji Seika Kaisha, Ltd. (Tokyo).

METHODS

Delignification was done in 50 mL liquid working volume containing 1.10^{-4}M of $CuSO_4.5H_2O$ and $MnCl_2.4H_2O$ as metal activator, in phosphate buffer at pH 6.0 in 37^oC.

Biodelignification was done using 20; 60; 100; 140; g EFB / L .[16, 17, 18,19].

Saccharification of delignified EFB was conducted bycellulose. The reducing sugar (RS) was monitored during saccharification process. Optimation of saccharification was done byvarying amount of cellulase at 9; 18 ; 27 and 36 FPU/g EFB.

The concentration of reducing sugars in the medium was determined by the dinitrosalicylic acid (DNS) method.

The total cellulase activity was determined by the standard IUPAC, Measurements of Cellulase Activities [20,21]

Identification of Fungi

A potential fungal were subjected to genotypic identification. Identification was

carried out at Center of Excellence Biological Resources-Genome Indigenous Studies (IBR-GS CoE), University of Indonesia.

RESULT AND DISCUSSION

Screening and isolation of fungi

The three fungal types were designated as F1 (obtained from wood), F2 (obtained from bamboo), and F3 (obtained from EFB). Figure 1 showed the three fungal types observed under the microscope with 1,000 times of magnification.

Three isolated fungi obtained in this work is shown in Figure 1. F1, a fungal strain obtained from wood exhibited the following colony characteristics white, flat and cottony. F2 obtained from bamboo, showed various color (white, cream and yellow), and the light spore turned to dark brown after three days. F3 isolated from EFB showed white and cottony, the mycelium turned white to green and the hyphae are hyaline.

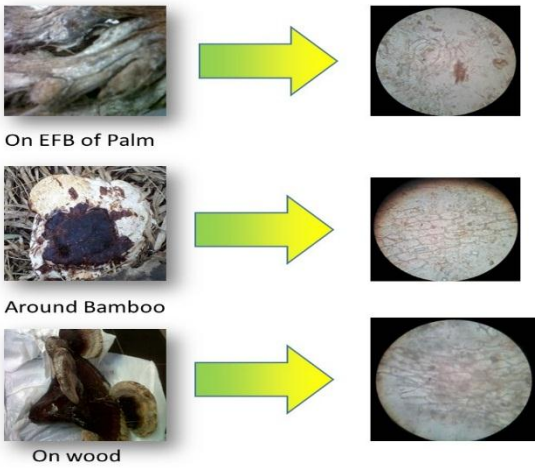

Figure 1: Three types of fungi isolated from different sources (wood, bamboo, and EFB of palm oil), designated as F1, F2, and F3. The left site is observation by microscope with magnification 1.000 times.

Delignification

In the previous work, delignification process of lignocellulosic biomass were done from 2-8 weeks [22]. In this work, in order to increase accessibility of cellulase and breakdown lignin, these three fungi were applied on EFB for 0-8 days

During the experiment, reducing sugar (RS) was monitored to check the sugar content. Figure 2 showed the RS concentration during delignification. We assumed that the sugar was taken and digested by all of the three fungi strains and shown by the level of reducing sugar (RS)and compare to the control (without fungal).

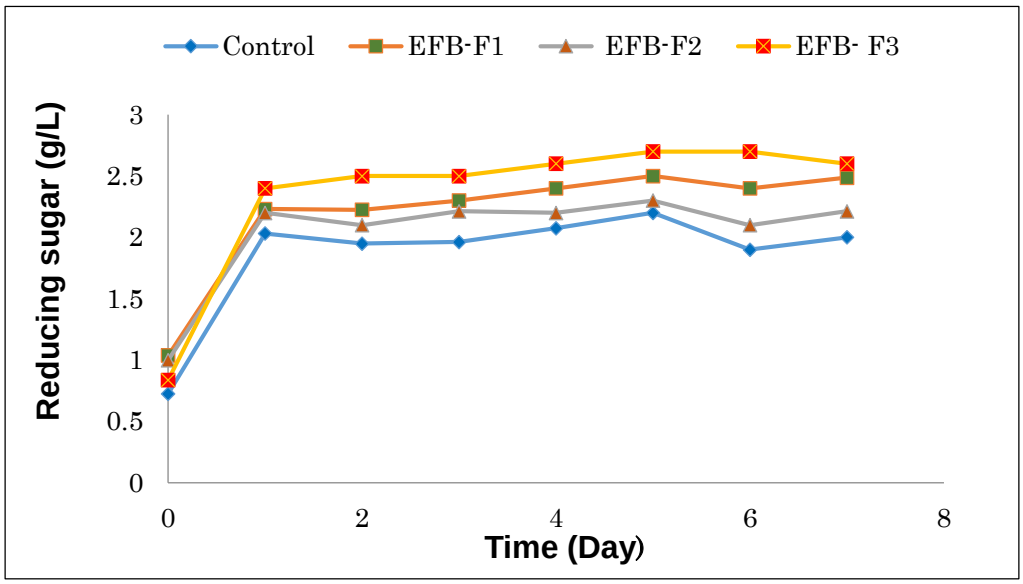

Figure 2: Reducing Sugar content during delignification of EFB for 7 days

Qualitative test was performed to determine whether or not the cellulase produced by the fungi. Observation was done Potato Dextrose Agar (PDA) media. The media changed from clear to cloudy. The cellulolytic index (CI = 1.1) of the F3 strain was the smallest compound with two others F1 and F2 (2.2 and 1.4) as shown in Figure 3.Those three fungi potentially delignify EFB. However among them, F3 is most potential because of the lowest cellulase production indicated with the smallest cellulolytic index on the PDA medium (Table 1) The formation of a clear zone surrounding the fungal colony indicated cellulose degradation by hydrolysis [23].

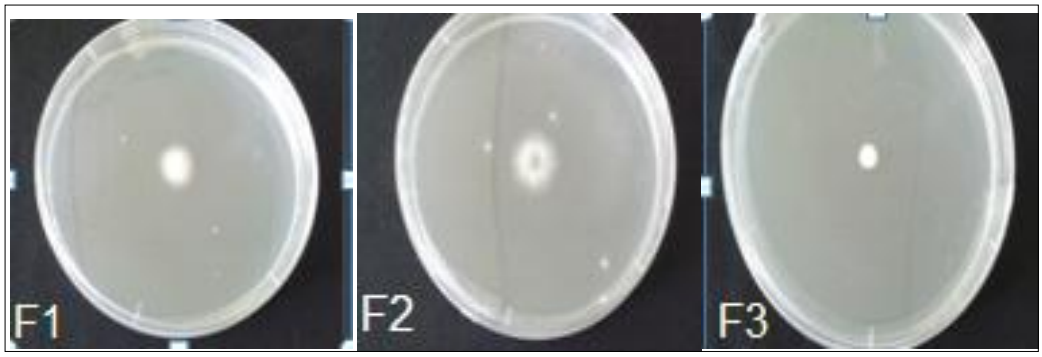

Figure 3. Three isolates fungi (F1, F2, and F3) were cultivated in the PDAmedia containing cellulose.

Table 1. Cellulolytics Index of three isolate fungi (F1, F2, and F3) were cultivated in the PDA media containing cellulose.

No.	Fungi	Clear Zone Diameter	Colony Diameter	Cellulolitycs Index
1	F1	2.2	1	2.2
2	F2	2.4	1.7	1.4
3	F3	0.9	0.8	1.1

Saccharification of delignified EFB

The delignified EFB was subjected to enzymatic hydrolysis by cellulase to confirm the correlation between delignification and the effectiveness of EFB hydrolysis. As shown in Table 2, the reducing sugar of F1, F2, and F3 reached 4.029, 3.482, and 4.111 g/L respectively which were higher than that in the control (3.162 g/L). The extended saccharification did not increase the concentration of reducing sugar, we suspected that the sugar produced inhibits the saccharification.

Table 2. Reducing sugar of saccharification delignified EFB in 3 and 7 days delignification.

Delignified EFB (5 g)	**Duration of delignification**			
	3 days		**7 days**	
	Reducing Sugar			
	(g/L)	**%**	**(g/L)**	**%**
Control	3.162		4.764	
EFB-F1	4.029	27.41%	5.637	18.32%
EFB-F2	3.482	10.13%	5.323	11.73%
EFB-F3	4.111	29.99%	6.635	39.27%

To observe the effect of delignification, saccharification has been conducted in delignified EFB which using these three fungus (F1, F2, F3). Extended duration of delignification up to 7 days showed better saccharification. Tabel 2 showed improvement in the reducing sugar of EFB delignified by F3 which reached 6.635 g/L. This value is the higher (1,87 g/L) than the control. The reducing sugar of F1 and F3 were increased by 0.87g/L and 0,56 g/L compared to the control.

For comparison, pretreatment process by *A. fumigatus* (F3), wild type, with other microorganisms offers the possibility of delignification in short time with less decrease of cellulose and hemicellulose as shown in Table 3. The percentage of lignin degraded by *A. fumigatus* compared with other microorganism reported previous. However the delignification presented was consider very high and short of time (7 days). This strain is was used in EFB in order to open access for the enzymes in further saccharification and fermentation processes, such as the production of hydrogen from EFB.

Table 3. The comparison of Bio-delignification and The Reduction of Cellulose and Hemi-Cellulose

Microorganism	**Biomass**	**Duration**	**Percentage degradation of raw material (%)**			**Ref.**
			Lignin	**Cellulose**	**hemicellulose**	
Mycelial INBI 2-26	Cut straw	23 days	29.8	51.4	72	27
Pycnoporus cinnabarinus	*Prosopis juliflora*	15 days	11.89	3.32		28
Pycnoporus cinnabarinus	*Lantana camara*	15 days	8.36	4.87		28
Pleurotus ostreatus	Sugarcane Bagasse	5 days	5.53			29
B. adusta	Canola	7 days	2.61 (-0.37)	+0.04 (+1.72)	4.8 (-0.79)	30
B. adusta	Canola	21 days	17.89 (-2.54)	+4.98 (+2.11)	3.23 (+0.53)	30
C. caperata	Canola	21 days	38.87 (-5.52)	+9.69 (+4.11)	0.06 (+0.01)	30
F. gilva	Canola	21 days	10.14 (-1.44)	+3.30 (+1.40)	+6.77 (+1.11)	30
Pleurotus tuberregium	Canola	21 days	19.79 (-2.81)	+8.21 (+3.48)	2.07 (-0.34)	30
P. pulmonarius	Canola	21 days	29.23 (-4.15)	+5.59 (+2.37)	2.2 (-0.36)	30
A. fumigatus	EFB of Palm oil	7 days	(-4.19)		(-2.78)	This work

*In the bracket () shows the decreased of lignin, cellulose and hemicellulose from the raw material.

Identification of fungus

The fungus (F3) isolated from EFB as the most potential for delignification was subjected to species identification. Genotypic identification of F3 confirmed that the similarity of 99% to *Aspergillus fumigatus*. *A. fumigatus* is a member of the class Ascomycetes [24]. In particular, members of the genus *Aspergillus* are known as the producers of laccase [25]. Indicated by qualitative analysis using guaiacol (data not shown). The ability to produce laccase is an important property possessed by F3 in this work, because this enzyme involved in degradation of lignin and removal of phenolic compounds which is potentially toxic during lignin degradation [26].

Further investigation delignification of *A. fumigatus*

We conducted further delignification of EFB by *A. fumigatus* (F3) to find out the lignin breakdown and cellulose-hemicellulose by laccase and cellulase respectivelly. As shown in Figure 4. The amount of lignin was about 27.50% for 14 days, the decreased was 5.55% in comparison with the control (33.05%).

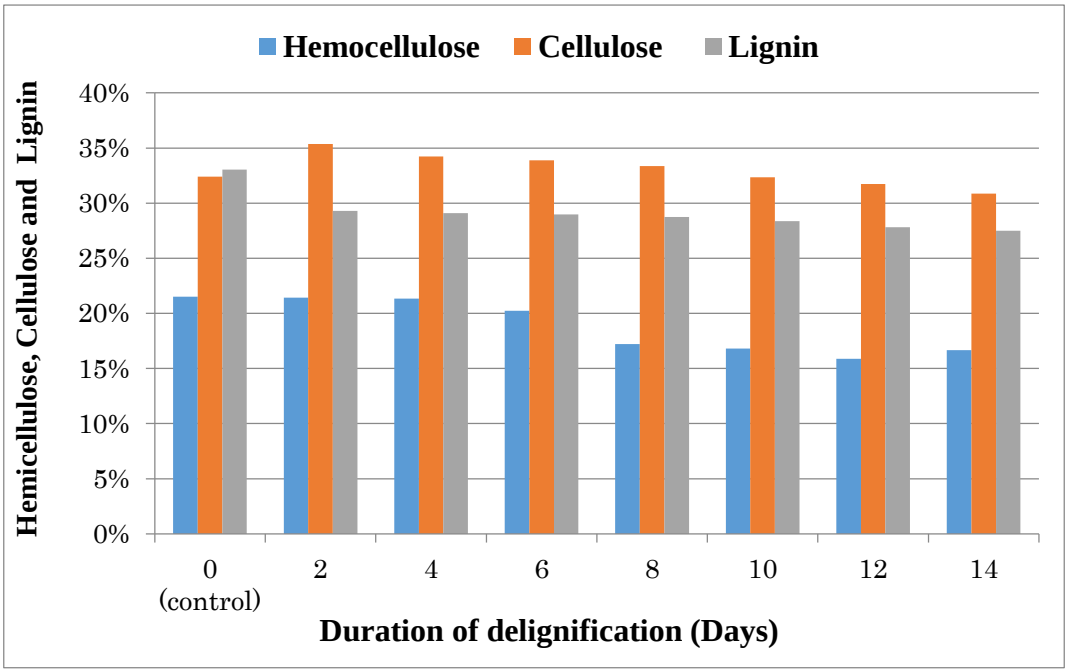

Figure 4. Delignification of EFB by *A.fumigatus* : degradation of lignin, cellulose and hemicelluloses over days.

The amount of lignin decreased to 28, 97%) on 6 day duration of delignification. On 2-8 days duration of delignification, the amount of cellulose increased but on 10 day, cellulose decreased to 0.06 %. Probably, beside lignin, cellulase and hemicellulase was consumed by *A. fumigatus* also produce cellulase, although in that time the activity of cellulase was very low. Decreasing of the cellulose and hemicelluloses accompanied by the increase of lignin. Therefore, in order to minimize loss of cellulose-hemicellulose proposed to conduct biodelignication between 6 and 8 days. The 7 days of delignification, concentration of cellulose was 33.62 % or increased 1,21 %.

The subsequent experiment was to figure out the amount of EFB in liquid medium as shown in Figure 5. *A. fumigatus* (F3) grew very slowly when it was cultivated in small amount of EFB (20 gr/L , 60 gr/L) medium containing $CuSO_4$ and $MnCl_2$. The forming fungal mycelia tend to grow on medium surface. The decrease in lignin content is very small or the same as the control for the duration of 7 days of biodelignification. The delignification was effective in a small amount of the liquid medium, the substrate was not submerged, at decrease of lignin was 100 g EFB/L and 140 g EFB/L (26,65% and 25,45% respectively).

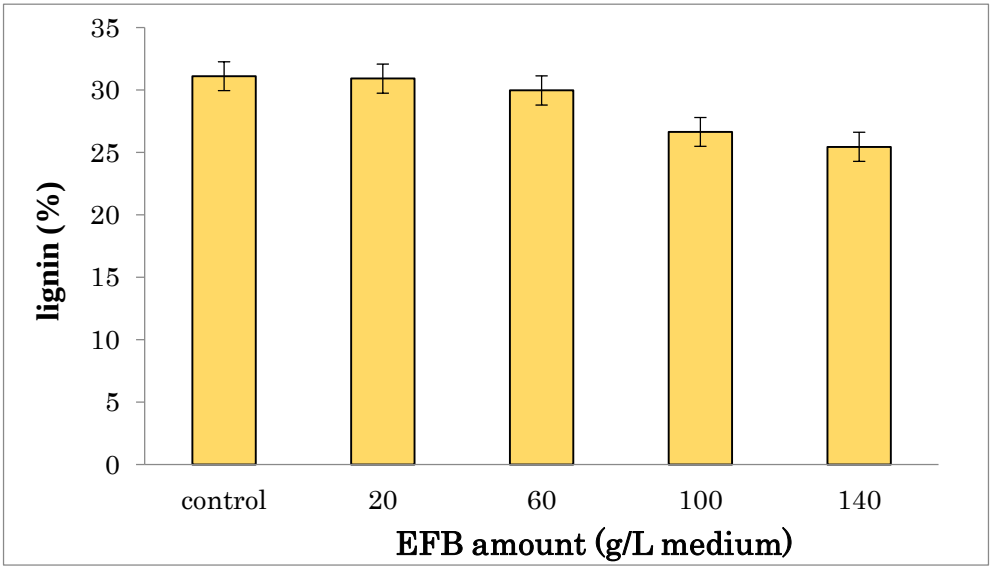

Figure 5. Delignification of EFB by *A. Fumigatus*: over EFB content at 50 mL liquid medium

Improvement of the saccharification has been conducted by increasing the cellulase amount as shown in Figure 6. The concentration of cellulase was increased from 9 FPU to 36 FPU. The concentration of cellulase was increased from 9 to 36 FPU cellulase/gram of delignified EFB. There was no significant difference between 27 and 36 FPUcellulase/g of delignified EFB (8.60 and 8.65g/L, respectively). Therefore, saccharification of treated EFB is enough with 27 FPU cellulase/g of delignified EFB. Due to the high price of cellulase (27 FPU cellulase/g of delignified EFB), this concentration enzyme was enough to increase the reducing sugar up to 8.60g/L compare with that of control.

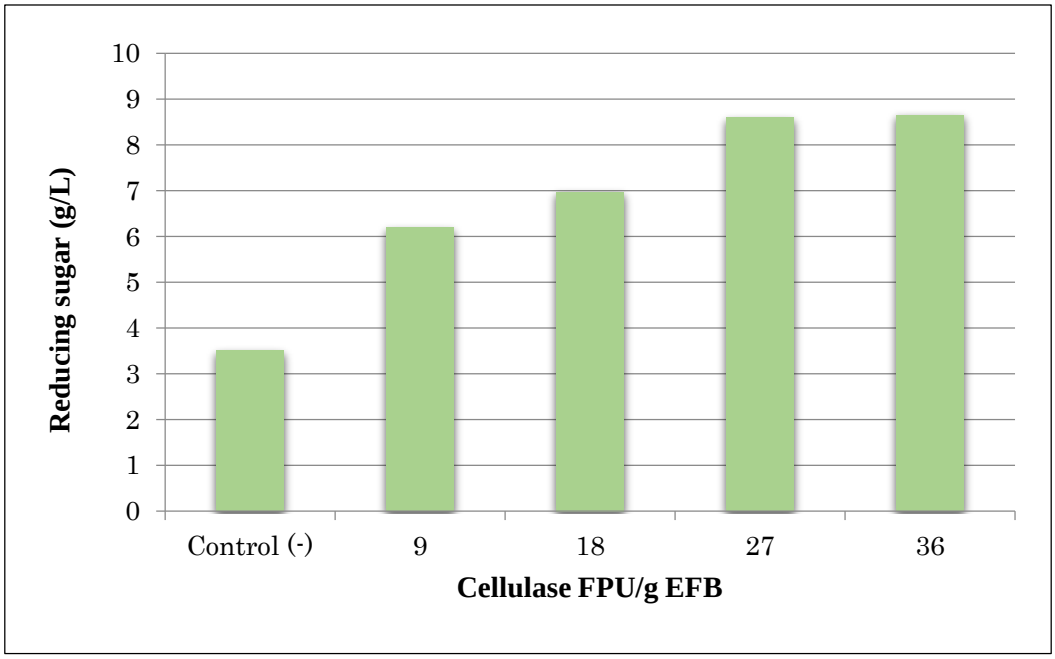

Figure 6. Saccharification of EFB at various concentration of cellulase

CONCLUSION

A.fumigatus (F3) isolated from EFB in this work effectively degrades partially lignin present in EFB. Therefore, this strain improves the accessibility of cellulose to saccharifyEFB. Moreover, degradation of cellulose and hemi cellulose was very low,

withdecomposition percentage of 1.58% and 5.94%, Because this work intended to degrade lignin partially in order to increase accessibility of cellulase, we suggested that biodelignification should be done less than 8 days to minimize the loss of cellulose-hemicellulose which will be used for next microbial processes such as saccharification and fermentation for bio-hydrogen production. *A. fumigatus* (F3) exhibited the best performance for delignification in comparison with other isolate from different environment, wood and bamboo. In addition, increasingcellulase in the saccharification of delignified EFB showed the improvement in the effectiveness of reduced lignin. Using cellulaseat 27 FPU /g EFBis8,6 g/L considered as the most optimal because the higher cellulose concentration 36 FPU/g EFB did not improve the amount of reducing sugar significantly.

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