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SUGAR PRODUCTION BY DIGESTING OF OIL PALM EMPTY FRUIT BUNCH USING EXTRACELLULAR ENZYMES FROM *Aspergillus niger* AND *Trichoderma reesei* FOR ETHANOL PRODUCTION

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ABSTRACT

Extracellular enzymes which obtained from 4 days cultivation *Aspergillus niger* and *Trichoderma reesei* on solid state fermentation of oil palm empty fruit bunch (OPEFB) were used for lignocellulosic-rich OPEFB digestion. The enzymes were concentrated using 70% saturated ammonium sulphate, dialysed against 20mM acetate buffer at pH 5 and adjusted one tenth (v/v) from the initial volume with the same buffer. The concentrated enzymes were then used in hydrolysation of powdered OPEFB. Amount of 10.65 mg/ ml and 11.47 mg/ml sugars were produced when each concetrated enzyme *A. niger* and *T. reesei* mixed with2%OPEFB. These hydrolysation were done on 100 ml total volume, incubated at 37°C with 100 rpm shaken for 36 hours. Further, both hydrolyzates results were sterilised and fermented anaerobically using *Saccharomycess cerevisiae* at concentration 0.5mg/ml cells and incubated in 30°C for 24 hours. Colorimetric analysis using QuantiChrom Kit DIET-500 at OD 580nm gave results the alcohol production were 0.86% and 0.92% which were similar with Gas Chromatograph analysis that of 0.83% and 0.93%, respectively.

Keywords: extracellular enzymes, hydrolysation, fermentation

INTRODUCTION

It is forecasted, in year 2000–2020 Indonesia is the largest producer and exporter of oil palm in the international market (Sumathi *et al.*, 2008). A huge amount of lignocellulosic material oil palm empty fruit bunch (OPEFB) is generated during production of oil palm from the fruit and remained as agriculture waste product (Baharuddin *et al.*, 2013; Huzairi *et al.*, 2013). Abundant of OPEFB is accumulated in the field, and considered to be difficult materials to digest (Purwandari *et al.*, 2013) which consists of three main polymeric components, i.e., cellulose (46.7%), hemicellulose (17.9%),and lignin (4.2%) (Quintero *et al.*, 2011). Thus, OPEFB is a common waste problem in oil palm plantations. Unadventurously, they are burned resulting in air pollution and massive residues on landfills. To solve the disadvantages, several of processing methods i.e., biologically (Huzairi *et al.*, 2013; Zhang *et al.*, 2013) mechanical (Baharuddin *et al.*, 2013; Shamsudin *et al.*, 2011), chemical (Kim and Ho 2013; Rahman *et al.*, 2007) have been investigated which shift the structural and chemical compositions of OPEFB lignocellulose to sugars (Ye *et al.*, 2014) and was converted to renewable energy bioethanol (Cui *et al.*, 2014; Hoon *et al.*, 2011). Further, researchs related to OPEFB utilization, have also been developed to produce biogas (Mohamed *et al.*, 2013; O-thong *et al.*, 2013; She *et al.*, 2013), enzymes (Ariffin *et al.*, 2008; Ottenheim *et al.*, 2014), sugar and oligosaccharides derivates (Ling A *et al.*, 2014; Ottenheim *et al.*, 2014; Rahman *et al.*, 2006) and other products. However, in fact, little attention is put forward on OPEFB utilisation in terms of economic point of view. In this paper, microbial utilization of OPEFB to produce sugar using crude extract from *A.niger* and *T. reesei*, and conversion of its hydrolysates to ethanol will be reported.

MATERIALS AND METHODS

Extracellular Enzymes Production

Source extracellular enzymes were obtained from solid culture of one kilogram sterilized OPEFB in a ten liter flask which each culture was inoculated with *A. niger* and *T. reesei*. The enzymes were harvested after optimum incubation at 30°C for 5 days. Enzymes extraction

was done by adding 1000 ml water containing 1% NaCl, shaken at room temperature for 9 hours and filtered using paper filter on funnel Buchner. To remove remaining cells from the filtrate, centrifugation at 4000 rpm for 20 minutes was performed. The supernatant which containing enzymes were then concentrated by ammonium sulfate at 70% saturation, dissolved on 30 ml acetate buffer pH5. The remaining ammonium sulphate was removed through dialysis on mPES MicroKros Filter Modules C02-E010-05-S 10 KDa against the same buffer for 24 hours, and then enzyme solution brought to one tenth (v/v) from the initial volume using buffer above. The two concentrated enzymes were stored at 4ºC until needed for OPEFB hydrolysis.

Enzymatic Hydrolysis OPEFB

OPEFB hydrolysis was done by using concentrated extracellular enzymes containing 2% powdered OPEFB. Two grams of powdered OPEFB was suspended into 100 ml concentrated crude enzyme, incubated at 37ºC, shaken 100 rpm, 36 hours. Enzyme reaction was stopped by boiling at 100ºC for 10 minutes. The resulting supernatant as hydrolysate was recovered and separated from remaining OPEFB substrate by filtration on paper filter and then centrifuged at 4000 rpm for 10 minutes. Hydrolysate was stored at 4ºC for next sugar analysis. This experiment was carried out twice.

Sugar Analysis of OPEFB Hydrolysate

Total sugar of OPEFB hydrolysate was estimated by phenol-sulfuric colorimetric method (Dubois, *et al.*, 1956). The reducing sugar of hydrolysate was also measured using Somogy (Somogyi *et al.*, 1926) and Nelson (Nelson *et al.*, 1944) methods. Sugar components of hydrolysate were analysed by Gas Chromatograph (GC) Thermo Scientific Trace 1310 equipped withTG-225MS 15m x 0.25mm x 0.25µm column. GC was setup at 190°C (5 minute hold) to 250°C at 8°C/ min (5 minute hold) and carrier gas hydrogen adjusted at flow rate 45cm/sec. One milliliter OPEFB hydrolysate sample was transmethylated as alditol acetate (Arai and Murao 1978; Spiro *et al.*, 1972). An amount 0.5µl of sample was injected to GC for analysis. The hydrolysate was also analyzed by using TLC in silica gel plate (Merck, 60 F254). A mixture (v/v) of butanol : ethanol : chloroform : amonia (4 : 7.5 : 4 : 8) was used as solvent system and 0.1% sulfuric acid containing 0.1% vanilin was employed for detection.

Anaerobic Fermentation and Analysis of Ethanol Concentration

In anaerobic fermentation to produce ethanol, yeast cells *S. cerevisiae* was used. The yeast was pre-cultured aerobically in 100 ml medium pH 6 in 1 liter shaker flasks, containing yeast-extract (0.3%), malt extract (0.3%), pepton (0.5%) and glucose (1%). The culture was incubated at 25°C under shaker set at 120 rpm for 24 hours. Active yeast cells were harvested by centrifugation at 4000 rpm for 5 minutes. The pellet was suspended into sterilized water with the concentration of cells 25 mg cells/ ml and further used for source inoculum in anaerobic fermentation of OPEFB hydrolysate. Fermentation was done in 50 ml medium using mini fermenter at 30ºC, inoculated with 25 mg cells (≈1ml) of source yeast prepared above. The ethanol concentration was analysed using QuantiChrom Kit DIET-500 colorimetric method at OD 580nm. Ethanol concentration was also analysed using GC above equipped with Trace GOLD TG-1301MS GC column with sample volume 0.5µl. The GC machine was setup at 250°C with carrier gas helium at flow rate 35cm/sec. The remaining sugar component after fermentantion was analysed with the same method as described earlier at Sugar Analysis of OPEFB Hydrolysate.

RESULTS AND DISCUSSION

Optimization and Production of Extracellular Enzymes

Extracellular enzyme produced by *A. niger* and *T. reesei* in OPEFB medium were done without any nutrient added that has been confirmed. For

preliminary step, the optimization of enzyme extracellularproductionfromthetwospecieswere done in small scale using 10 gr OPEFB medium. Evidence revealed that in 3 days cultivation, *A. niger* and *T. reesei* grow well in OPEFB medium. Much mycelium with their black spores covered whole of OPEFB medium. Additionally, OPEFB was also contain crude protein nearly 0.3% on dry-basis which was needed as a nitrogen source for fermentation. Mean, *A. niger* and *T. reesei* certainly utilized carbon and nitrogen from OPEFB for their growth. Further, we observed that in 5 days cultivation, small amount liquid phase was produced, indicating hydrolysation process happen. Accordingly, some extracellular enzymes were released by both *A. niger* either *T. reesei* during solid state fermentation. The extracellular enzyme was then harvested by 1% NaCl extraction. The enzyme activity against 1% OPEFB alkali extract substrate was measured daily based on reducing sugar produced. Thus, incubation days of cultivation to produce extracellular enzyme released optimally can be represented as maximum of enzyme activity to produce reducing sugar against OPEFB alkali extract substrate. The activity levels of extracellular enzymes were, therefore, determined for incubation periods of 1 to 7 days as shown at Figure 1.

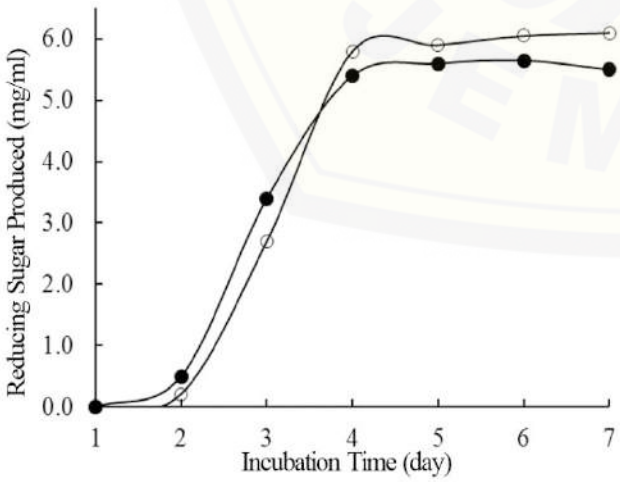

Fig 1. Optimizing of extracellular enzymes production of *A. niger* (֍) and *T. reesei* (⌘) on OPEFB.

As stated in Figure 1, extracellular enzymes of *A. niger* and *T. reesei* has highest activity to hydrolysed OPEFB alkali extract substrate when incubation time was done in 4 days. Both isolates produced reducing sugars 5.4 mg/ml and 5.8 mg/ml, respectively. Based on this result, the enzyme production was scaled up in 1 kg of OPEFB medium. The enzymes activity were also examined and gave similar results compare with previous small scale enzymes production, where the enzymes hydrolysed OPEFB alkali extract and produced 5.1 mg/ml and 5.3 mg/ml reducing sugars. It was confirmed already that genus *Aspergillus* (Alam *et al.*, 2011; Ottenheim *et al.*, 2014) and *Trichoderma* (Shafawati *et al.*, 2013; Wang Z *et al.*, 2014) release some extracellular enzymes which decomposed cellulose, hemicellulose, and lignocellulose of OPEFB.

OPEFB Hydrolysis and Sugar Analysis

Hydrolysis was carried out in 100 ml concentrated enzymes containing 2% of powdered OPEFB and incubated at 37°C with 100 rpm shaken for 36 hours. The hydrolysis process by the two enzymes were measured every 4 hours. They produced optimum of reducing sugar production in between 32 and 36 hours incubation. The longer incubation time, the more higher the degree of hydrolysis. And thereafter 40 hours the hydrolysis is still in progress but not significant increasing in reducing sugar production and could presumably attain similar degree of hydrolysis at very much later time. Analysis revealed that enzymes from *A. niger* and *T. reesei* released 10.65 mg/ml and 11.47 mg/ ml reducing sugars after 36 hours hydrolysis. In addition, OPEFB consist of 66.5% total sugar (or 13.3 mg/ml when the suspension contain 2% OPEFB)measuredbyphenol-sulfuriccolorimetric method (20)so that it can be concluded that concentrated enzyme from *A. niger* and *T. reesei* digested OPEFB reach 81.7% and 89.2% degree of hydrolysis, respectively. To improve the degree of hydrolysis, optimization of hydrolysation must be done, e.g., pH, temperature and period of incubation time. In another words to make

the process more effective, suitable pretreatment and enzymatic reaction parameters need to be optimized (Hassan *et al.,* 2013).

As shown at Table 1, phenol sulfuric acid analysis showed that the total sugar of OPEFB hydrolysates were 10.86 mg/ml and 11.87 mg/ ml respectively when hydrolysis of OPEFB were done by concentrated enzymes from *A. niger* and *T. reesei*. However, GC analysis revealed that OPEFB hydrolysates contain much sugar as monosaccharides, that of10.65 mg/ml and 11.47 mg/ml. Means, OPEFB which hydrolysed by both concentrated enzymes from *A.niger* and *T. reesei* produced sugar rich hydrolysates as monosaccharides but poor in oligosaccharides. The monosaccharides concentration in hydrolysates were 98.1% and 96.6%. TLC analysis displayed that the hydrolysis product was primarily monosaccharide glucose spot respectively (figure not shown). The ability of both extracellular enzymes from *A. niger* and *T. reesei* to release monosaccharides from complex polysaccharides due to that species could produce broad spectrum of extracellular enzymes. The capability of crude enzyme to hydrolyze OPEFB and produce glucose and xylose as monosaccharide could be due to the fact that some genus *Aspergillus* and *Trichoderma* produce of various enzymes such as cellusases, glucoamylase and xylanase (Chen *et al.,* 2014; Chandra *et al.,* 2009; Krijgsheld *et al.,* 2013; Perrone *et al.,* 2007).

Analysis of Anaerobic Fermentation

Fermentation to produce ethanol was done using OPEFB hydrolysate as medium without any adjusting of either pH or sugar concentration. Further, both hydrolyzates results were sterilised and fermented anaerobically using *S. cerevisiae* at concentration 0.5mg/ml cells and incubated in 30ºC for 24 hours. Colorimetric analysis using QuantiChrom Kit DIET-500 at OD 580nm gave results the alcohol production were 0.86% and 0.92% which were similar with Gas Chromatograph analysis that of 0.83% and 0.93%. The remaining sugar in hydrolysate after fermentation as monosaccharides was also analysed by GC. After 24 hours fermentation, glucose concentration were very low with concentration value 0.12 mg/ml and 0.18 mg/ml. But, the xylose almost nearly same comparing with initial concentration, that of 0.53 mg/ml and 0.71 mg/ml, correspondingly. This evidence demonstrated that *S. cerevisiae* difficult ferments monosaccharide xylose from OPEFB hydrolysate, similar result as reported by Sudiyani *et. al*. 2013. But in contrast *S. cerevisiae* definitely utilized and converted glucose to ethanol. By these results, improvement hydrolysis OPEFB must be done which expected to increase the yield of sugar as monosachharides in OPEFB. Adjusting and optimizing environment factors in hydrolysis and as well as parameters in fermentation such as sugar concentration, pH of medium and temperature are also needed (Ferreira *et al.,* 2009; Shill *et al.,* 2012; Viell *et al.,* 2013).

Tabel 1. Sugar Analysis of OPEFB Hydrolysates

Hydrolysate analysis	Sugar concentration (mg/ml) in OPEFB hydrolysis using concentrated enzyme *A. niger* (A) and *T. reesei* (B)
	A
Gas Chromatograph Analysis (Monosaccharides component as alditol acetates)	
- Glucose	8.81
- Xylose	1.11
- Others	0.73
Total monosaccharides	**10.65**
Phenol Sulfuric Acid Analysis	
Total Sugar	**10.86**

CONCLUSION

In this study, bioconversion of potential cheap material of OPEFB to ethanol was successfully done. And it is necessary to develop more efficient method with the target to improve the ethanol yield as well as for profitability reason process. Enzymatic hydrolysis and fermentation process must be evaluated in order obtain high ethanol yield. Xylose as pentose sugar has not been consumed by *S. cereviseae*. Therefore, assessment of other pentose-consuming microorganisms with the aim to completely ferment the sugars released in OPEFB hydrolysates must be investigated.

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