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Ethanol from Oil Palm Empty Fruit Bunch via Dilute-Acid Hydrolysis and Fermentation by *Mucor indicus* and *Saccharomyces cerevisiae*

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Abstract: Oil Palm Empty Fruit Bunch (OPEFB) was hydrolyzed in a one-stage hydrolysis using dilute-sulfuric acid (0.2, 0.8%) at 170-230°C with a holding time of 5 and 15 min. The maximum yield of xylose was 135.94 g kg^{-1} OPEFB, obtained at 0.8% acid, 190°C and 5 min. The maximum yield of glucose was 62.70 g kg^{-1} OPEFB, obtained at 0.8% acid, 210°C and 5 min. Based on these results, two-stage hydrolysis was performed to produce hydrolyzates for the fermentation process. Hydrolyzate from the first stage was fermented by *Mucor indicus* while the hydrolyzate from the second stage was fermented by *Saccharomyces cerevisiae*. The corresponding ethanol yields were 0.45 and 0.46 g ethanol g^{-1} sugar consumed.

Key words: Oil palm empty fruit bunch, acid hydrolysis, ethanol, *Saccharomyces cerevisiae*, *Mucor indicus*

INTRODUCTION

With oil palm plantation and Crude Palm Oil (CPO) production reaching 6.8 million ha and 19.3 million metric ton in 2008, respectively, Indonesia is now the biggest palm oil producer in the world (DGPMA, 2008). At the same time, Indonesia accumulates a large amount of lignocellulosic wastes from the palm oil industry. The lignocellulosic wastes include the empty fruit bunch (OPEFB), the fronds and the trunks. Having negligible commercial value, these wastes become problematic. Not only do they cause serious disposal problems but they occupy a large area for storage.

If 1 ton of fresh fruit oil palm bunch produces nearly 234 kg of CPO and 217 kg of OPEFB (Lacrosse, 2004), about 17.9 million metric ton of OPEFB were accumulated in 2008. Traditionally, the empty bunch is burned for steam and power generation for factories and the ash is utilized as a potassium fertilizer. However, burning empty fruit palm oil bunch to solve the disposal problem causes another environmental problem, i.e., pollution as a result of incomplete combustion and very fine size of ash particles. Thus, utilization or conversion of these by-products to value-added products is of great importance. One process of growing interest in bioethanol production is producing ethanol from lignocellulosic wastes which are abundant from various sources of biomass. Bioethanol attracts interest because it can be used not only as a chemical for industry but also as an alternative fuel for vehicles. Lignocellulosic wastes from oil palm are potential raw materials for bioethanol production, especially due to their constant availability. The Oil Palm Empty Fruit Bunch (OPEFB) could contain cellulose (44.2%), hemicellulose (33.5%) and lignin (20.4%) (Azis *et al*., 2002). The cellulose and hemicellulose content of OPEFB can be hydrolyzed for example by chemical hydrolysis. Complete hydrolysis of cellulose results in glucose whereas the hemicellulose results in several pentoses and hexoses.

Among the chemical hydrolysis methods, dilute-acid hydrolysis is probably the most commonly applied. This method has been successfully developed to degrade lignocellulosic materials with considerable success (Taherzadeh *et al*., 1997). Due to the different structure of cellulose and hemicelluloses, dilute-acid hydrolysis is suggested to be carried out in 2 stages. In the 1st stage, hemicellulose is hydrolyzed under milder conditions whereas in the 2nd stage cellulose is hydrolyzed under harsher conditions (Galbe and Zacchi, 2002). A significant drawback of this method is the formation of inhibitors such as acetic acid, phenolic compounds, furfural and 5-Hydroxymethyl Furfural (HMF) which

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potentially decrease the ethanol production rate from the hydrolyzate. These inhibitors are formed by degradation of sugars from cellulose and hemicelluloses (Larsson *et al*., 1999). For industrial application, it is necessary to avoid the formation of inhibitors by choosing suitable hydrolysis conditions. In this research, dilute H_2SO_4 with a concentration <1% was used to hydrolyze OPEFB. Effects of some hydrolysis parameters which included concentration of acid used, temperature and reaction time are studied. The temperature was studied at 2 levels, i.e., low temperature (<200°C) and high temperature (>200°C). In addition to sugars (glucose and xylose), the hydrolyzate was also characterized with respect to furfural, HMF and acetic acid. The results from 2 different hydrolysis temperatures were used as a basis to determine the conditions to perform 2 stage hydrolysis. The hydrolyzates produced were then verified for ethanol production using *Saccharomyces cerevisiae* and *Mucor indicus* as the fermenting microorganisms.

MATERIALS AND METHODS

Oil Palm Empty Fruit Bunch (OPEFB): Oil palm empty fruit bunch used in the current rsearch was obtained from a palm oil company, Pagar Merbau, Medan, North Sumatra, Indonesia. Fresh OPEFB was shredded and sun-dried until the water content was around 10%. The size of dried OPEFB was reduced up to 2 mm length to obtain a small fraction of OPEFB. Based on the procedure described in the NREL chemical analysis and testing procedure (Ruiz and Ehrman, 1996), the OPEFB contained 35.8% glucan, 19.9% xylan, 28.5% acid-insoluble lignin and 3.6% acid-soluble lignin.

Hydrolysis: Cylindrical vessels made of a corrosion-resistant alloy (stainless steel 316 L) with a total volume of 140 mL (Swagelok®, Solon, Ohio, USA) were used as reaction vessels for the hydrolyses. The reactors were heated in an oil-bath equipped with a thermostat. As the heating medium, special oil that had a melting point of about 150°C was used. The oil-bath was equipped with a stirrer to homogenize the temperature within the tank.

Prior to hydrolysis experiments, 5 g of OPEFB was soaked in the tube reactors in 50 mL of 0.2 and 0.8% sulfuric acid solution. The tube reactors were then immersed in the oil-bath tank for 5 and 15 min and at various temperatures (170, 190, 210 and 230°C). After the hydrolysis reaction was completed, the reactors were cooled until room temperature. The liquor (the hydrolyzate) was drained and samples were taken to be analyzed for glucose, xylose, furfural, HMF and acetic acid. Total 2 stage acid hydrolysis of OPEFB was performed based on the selected conditions of one-stage acid hydrolysis of OPEFB. After the first stage of hydrolysis was completed and the liquor was drained, the solid residues remained in the reactors. Then, sulfuric acid was added and the solid residue was hydrolyzed again. The hydrolyzates from both 1st and 2nd stages were collected and stored at 4°C before being used in the fermentation process. Some hydrolyzate was taken as a sample to be analyzed for xylose, glucose, acetic acid, furfural and HMF. The samples were stored at -20°C before analysis.

Microorganisms: The yeast used in this research was *Saccharomyces cerevisiae* ATCC 96581 obtained from LGC standards (Sweden). The fungus used in this research was *Mucor indicus* CCUG 22424, obtained from Culture Collection University of Gothenburg (Sweden). A spore suspension was prepared by adding 5 mL of sterile distilled water to the slant and shaking it vigorously, resulting in 10-12×106 spores mL^{-1}. One mL of the suspension was used for inoculation of the medium to initiate fungal growth.

Cultivation conditions: One loop full of culture was grown in a 50 mL cotton-plugged Erlenmeyer flask on a shaker of 80 rpm at 30°C for 24 h. The liquid volume was 10 mL. The growth medium was a defined medium including 50 g glucose L^{-1} as previously reported (Taherzadeh *et al*., 1996). Then, inoculum culture was added into 100 mL of a medium containing glucose prepared using the same formula as before (Taherzadeh *et al*., 1996) in a 300 mL cotton-plugged Erlenmeyer flask. It was further incubated on a shaker of 80 rpm at 30°C for another 24 h. The final volume of inoculum culture was 110 mL. Fermentation by *S. cerevisiae* was performed under anaerobic conditions. The medium was hydrolyzate produced from the 2nd stage of the hydrolysis process. It was first adjusted to 5.5 by addition of 10% of NaOH. It was then sterilized at 121°C for 15 min. The fermentation was carried out in a 300 mL loop trap Erlenmeyer flask. The loop trap contains glycerol to avoid air diffusing into the flask but the gas outlet can still pass. Pure nitrogen was sparged through a sterile filter in order to maintain the anaerobic conditions.

The flask was incubated for 72 h at 30°C and shaken at 80 rpm. Off-line pH-control was done by addition of 10% of NaOH, keeping the pH around 5.5. The total liquid volume was 155 mL including 135 mL hydrolyzate, 5 mL inoculum and 15 mL of a solution containing mineral solution. Fermentation by *M. indicus* was performed under aerobic conditions using hydrolyzate produced from the first stage of the hydrolysis process. Fermentation was initially carried out by cultivating the fungus in the synthetic medium in a volume of 50 mL for 24 h. The synthetic medium contained in g L^{-1} $(NH4)_2SO_4$ 7.5, $MgSO_4 \cdot 7H_2O$ 0.75, KH_2PO_4 3.5, $CaCl_2 \cdot 2H_2O$ 1.0, yeast

extract 5.0 and glucose 5.0 as the carbon source. Then, 100 mL of hydrolyzate was added into the culture and incubated for another 7 days. Fermentation was conducted at 30°C on a shaker of 150 rpm. Off-line pH-control was done by addition of 10% of NaOH, keeping the pH around 5.5. Aerobic experimentation was carried out in a cotton-plugged erlenmeyer flask. Liquid samples were withdrawn at certain times to be analyzed by HPLC.

Analyses: The liquid samples were analyzed by HPLC (Waters, Milford, USA) which was equipped with a RI detector (Waters 410) and a UV absorbance detector (Waters 486).

Ethanol, HMF, furfural and acetic acid were analyzed using a hydrogen-based ion-exchange column (Aminex HPX-87H, Bio-Rad, USA) at 60°C with 0.6 mL min^{-1} eluent of 5 mm sulfuric acid. Glucose and xylose were analyzed using a lead-based ion-exchange column (Aminex HPX-87P, Bio-Rad) at 85°C with ultra-pure water as eluent at 0.5 mL min^{-1}. Concentrations of glucose, xylose, ethanol and acetic acid were determined by RI chromatograms while furfural and HMF were determined by UV chromatograms at 210 nm.

RESULTS AND DISCUSSION

One-stage hydrolysis: Oil palm empty fruit bunch was hydrolyzed in one-stage hydrolysis using 0.2 and 0.8% sulfuric acid at 170, 190, 210 and 230°C for 5 and 15 min. Table 1 shows that higher yields of glucose and xylose were obtained at 0.8% acid compared to the yields at 0.2% acid. At acid concentration of 0.8%, temperature had a significant effect on the hydrolysis of OPEFB. The maximum yields of glucose and xylose occurred at temperature of 210 and 190°C, respectively. The maximum yields of glucose and xylose were 15.8 and 59.9% of the theoretical yields, respectively. Theoretical glucose and xylose were calculated from the total content of xylan and glucan in the OPEFB. In addition, the maximum yields of furfural and acetic acid occurred at temperature 210°C whereas the maximum yield of HMF occurred at temperature 230°C. Increasing retention time of hydrolysis from 5-15 min resulted in decreasing the yields of glucose at 210°C and xylose at 190°C. On the other hand, the yields of furfural and HMF were significantly increased. The reason for this could be that longer retention time makes the sugars degrade to form furfural and HMF.

This also probably means that 5 min hydrolysis is actually enough to depolymerize cellulose at their corresponding temperature. On the other hand, since the concentration of acetic acid was higher at retention time of 15 min than at 5 min, it indicates that the hemicellulose part has been degraded within 15 min. Thus, the optimum retention time can be expected to occur between 5 and 15 min.

Two-stage hydrolysis: Based on the results from one-stage hydrolysis for sugar and with a goal to minimize the formation of by-products, two-stage hydrolysis was carried out using 0.8% acid concentration in the following conditions: first-stage hydrolysis was performed at 170°C for 15 min and the second-stage hydrolysis was performed at 210°C for 5 min.

At the end of the first stage, the hydrolyzate was drained and the solid residue remained in the tube reactor. The acid was added and the solid residue was hydrolyzed again according to the conditions at the 2nd stage of hydrolysis. The compositions of hydrolyzates from the 1st and 2nd stages are shown in Table 2.

Table 1: The yields of glucose, xylose, HMF, furfural and acetic acid at different hydrolysis temperature, retention time using acid concentration of 0.2 and 0.8%

Temp. (°C)	Retention time (min)	$Y_{glucose}$	Y_{xylose}	Y_{HMF}	$Y_{furfural}$	$Y_{acetic\ acid}$
(0.2% acid sulfuric acid)						
170	5	0.00	0.00	1.21	0.00	11.07
170	15	0.00	1.99	0.00	0.65	9.31
190	5	0.00	0.00	0.00	0.00	8.24
190	15	1.70	2.58	0.31	2.99	13.00
210	5	0.00	7.18	0.76	1.66	9.63
210	15	15.71	8.57	0.14	17.32	28.69
230	5	0.76	0.00	0.82	23.11	34.65
230	15	0.38	0.00	3.90	17.22	34.97
(0.8% acid sulfuric acid)						
170	5	7.76	53.20	0.00	1.02	12.08
170	15	7.81	89.76	0.58	6.65	17.54
190	5	12.12	135.94	0.86	10.47	23.67
190	15	25.32	105.65	4.97	53.49	34.18
210	5	62.70	94.42	13.69	27.44	8.98
210	15	54.67	0.00	25.95	84.21	36.48
230	5	52.54	0.00	28.16	64.12	29.51
230	15	4.50	0.00	5.99	40.10	24.72

Yield = (g kg^{-1} OPEFB)

Table 2: The concentrations of hydrolysis products from the first and the second stage

Condition			Concentration (g L^{-1})			
Acid (%)	Temperature (°C)	Time (min)	Glucose	Xylose	Furfural	HMF
0.8	170	15	0.00	7.08	0.02	0.03
0.8	210	5	5.33	0.00	1.42	1.10

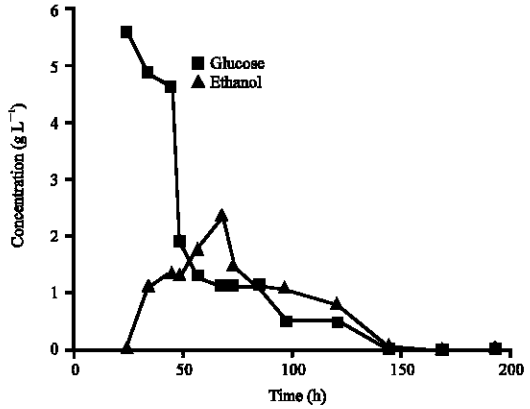


Fig. 1: The profile of glucose and ethanol during fermentation of the hydrolyzate from the first stage under aerobic condition using *Mucor indicus*

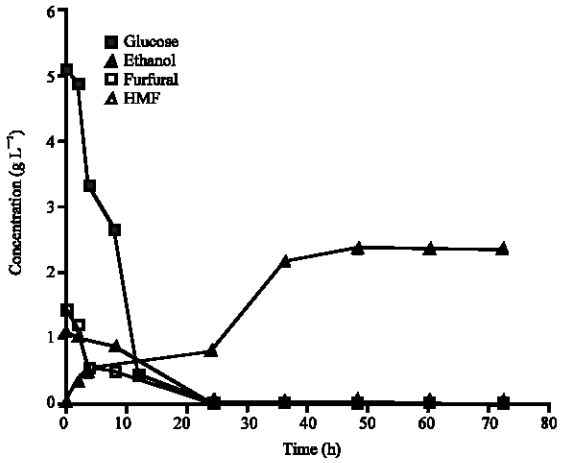


Fig. 2: The profile of glucose, ethanol, HMF, and furfural during fermentation of the hydrolyzate from the second stage under anaerobic condition using *S. cerevisiae*

Fermentation of hydrolyzate from the first stage by *M. indicus*: Since, the sugar within the hydrolyzate from the first stage was only xylose, the fermentation was carried by a xylose-assimilating microorganism, i.e., *M. indicus*. The parameters measured during fermentation were xylose consumption, ethanol production and consumption of HMF and furfural (Fig. 1). It is shown that *Mucor indicus* was able to assimilate xylose and produce ethanol. Ethanol concentration significatly increased after 24 h and reached its maximum value at 68 h, i.e., 2.3 g L^{-1}. This value corresponds to a yield of 0.45 g ethanol g^{-1} sugar consumed.

At low xylose concentration, ethanol concentration decreased until ethanol was depleted in the medium at 144 h. Meanwhile, furfural and HMF which were already present in very small amounts in the hydrolyzate were almost undetected in the medium. Thus, furfural and HMF are not a big concern in this fermentation.

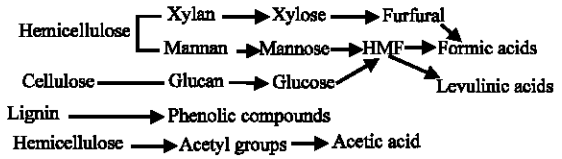
Fermentation of hydrolyzate from the 2nd stage by *S. cerevisiae*: In order to ferment the hydrolyzate from the 2nd stage, a hexose-assimilating microorganism, S. cerevisiae was used. Figure 2 shows that *S. cerevisiae* completely consumed glucose within 24 h. Ethanol was produced upto 2.4 g L^{-1}, corresponding to a yield of 0.46 g ethanol g^{-1} sugar consumed. Ethanol concentration remained constant until the end of fermentation time (upto 72 h). It can be concluded that <2 days is actually enough to produce ethanol by *S. cerevisiae* under anaerobic conditions. Furthermore, furfural and HMF which were present in the hydrolyzate were consumed or converted. It took 24 h for furfural and HMF to be completely consumed. Being available in plentiful amounts and having the character of a non-edible material, OPEFB is a potential and desirable raw material for ethanol production in Indonesia.

If we calculate theoretical ethanol production based on the composition of glucan and xylan in OPEFB as we used in this research 1 kg OPEFB will contain 358 g glucan and 199 g xylan. Theoretically, 202 g (256 mL) and 115 g (199 mL) of ethanol can be produced from glucan and xylan, respectively. However, taking into consideration the best yields of hydrolysis achieved in this research, i.e., 15.7% for glucan and 59.9% for xylan and the yields of ethanol from the fermentation process, i.e., 0.46 g ethanol g^{-1} glucose by *S. cerevisiae* and 0.45 g ethanol g^{-1} xylose by *M. indicus*, 1 kg OPEFB can be converted to 89 g or 112 mL ethanol in total.

Therefore, with 17.9 million tone of OPEFB accumulated in 2008, Indonesia can potentially produce about 2 billion L of ethanol. Nevertheless, any improvement especially in the hydrolysis process will increase the yield of ethanol from OPEFB which theoretically could be produced upto 7 billion L according

to the same principles of the previous calculation. With this amount, bioethanol could contribute to the solution of fossil fuel shortage in Indonesia. The Indonesian government predicts that national oil consumption will reach 36.5 billion L in 2010 of which about 20% is imported. As a result, the government has set up a program to replace >5% of fossil fuel with renewable fuels in 2025.

During dilute-acid hydrolysis, lignocellulosic material is degraded into its sugar components and other by-products according to the chemical reactions:



In this research, the maximum yield of glucose occurred at higher temperature (210°C) than that of xylose (190°C). Glucose is a monomer of cellulose and xylose is one of the monomer sugars from hemicelluloses. Cellulose exists in crystalline form and its structure is quite complicated which makes it resistant to chemical attacks. Meanwhile, hemicelluloses exist in amorphous form and are more easily degraded by chemicals to their monomer components (Sjostrom, 1993; Delmer and Amor, 1995). Thus, it can be understood that glucose needed higher temperature to be degraded than xylose. The yield of xylose obtained in this research is in the range of hydrolysis results from different feedstock, i.e., 55-83.3% (Garrote *et al*. 2002; Pessoa, 1997; Karimi *et al*. 2006).

However, this result is lower than the result from the concentrated acid hydrolysis which could reach a xylose yield of about 90% (Rahman *et al*., 2006).

In addition to sugars, furfural and HMF are formed as the by-products and are known as the most important inhibitors during fermentation of lignocellulosic hydrolyzates (Larsson *et al*., 1999). Therefore, low concentrations of furfural and HMF are expected. The results in this current research show that the yields of furfural and HMF were increased as the concentration of acid and the temperature increased (Table 1). The concentrations of furfural and HMF were negligible in the hydrolyzate from the first stage.

However, this was not the case with their concentrations in the hydrolyzate from the second stage (Table 2). Despite the presence of furfural and HMF, the hydrolyzate was equally fermentable by *S. cerevisiae* as it was by *M. indicus* for the hydrolyzate from the 1st stage. One possible explanation for this is that *S. cerevisiae* converted furfural into furfuryl alcohol and furoic acid by enzyme alcohol dehydrogenase and by enzyme aldehyde dehydrogenase, respectively (Taherzadeh *et al*., 1999). Like furfural, HMF was converted by *S. cerevisiae* into its corresponding alcohol, 5-Hydroxymethyl-Furfural alcohol (Taherzadeh *et al*., 2000). The high ethanol yield and the ability of the yeast to consume furfural and HMF suggest that the concentrations of those inhibitors were relatively low and at tolerable levels for *S. cerevisiae* used in this research.

CONCLUSION

In this study, dilute-acid hydrolysis can be applied to produce simple sugars from OPEFB. The hydrolyzates produced from the first and second stages were all fermentable by their corresponding microorganisms regardless of the presence of inhibitors such as furfural and HMF. The ethanol yields were at reasonable levels, i.e., 0.45 and 0.46 g ethanol g^{-1} sugar consumed. Based on these facts and considering the potentially huge amount of ethanol that can be produced from OPEFB, it is desirable to produce ethanol from OPEFB. However, further improvement is still needed for maximum results especially in the hydrolysis process.

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