Investigation of Acid-Hydrolysis and Fermentation Method for Producing Ethanol from Nypa (Nypa fruticans Wurmb) Midrib

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Abstact- Nypa (*Nypa fruticans* Wurmb) has high potential for producing ethanol. This research was made to find: (1) suitable type and concentration of acid for hydrolyzing nypa midrib, (2) duration of hydrolysis to gain optimum yield of reducing sugar from nypa midrib, (3) suitable type and concentration of starter for fermenting process, and (4) duration of fermentation for optimally producing ethanol. Experiments consisted of hydrolysis and fermentation processes. Two factors were evaluated in hydrolysis process, i.e. type of acid (H_2SO_4 and HCl) by applying 3 concentration levels (1 M, 2 M, 3M) and duration of hydrolysis process (1 hour, 2 hours, 3 hours, 4, hours 5 hours). The best result of hydrolysis experiments was then used in fermentation experiments. The fermentation experiments involved two factors, i.e. type of starters (*Saccharomyces cerevisiae* and *Zymomonas mobilis*) with three levels of concentration (5 % (v/v), 7.5 % (v/v), 10 % (v/v)) and fermentation duration (2 days, 4 days, 6 days). Result of the experiments indicated that use of HCl in hydrolysis was more effective for producing reducing sugar than that of H_2SO_4. The best combination was found by application of 5 hours hydrolysis duration and use of 3M HCl whereas the yield of reducing sugar was 2.76% (28.86 mg/ml). The 28.86 mg/ml of reducing sugar could be fermented to produce 4.00 mg/ml ethanol with alcohol content 3.80% by using 10% (v/v) *Saccharomyces cerevisiae* as starter in 6 days fermentation process.

Keywords ethanol, nypa midrib, hydrolysis, fermentation

1. Introduction

Consumption of energy for transportation sector in Indonesia was predicted to increase up to 1.554 million BOE (Billion Oil Equivalent) in 2025. This sector consumes 20% of total energy consumption. Most of consumed energy in the sector (97%) was supplied by fossil energy [1]. This fact was a factor that encourage the Government of Indonesia to issue a Regulation No 12 year 2015 concerning provision, utilization, and commerce of biofuels stated that since 1 April 2015 all companies which having fuels business permit have to distribute fuels mixed with 15% biofuels [2].

In Indonesia, a potential alternative source for producing ethanol is nypa (*Nypa fruticans* Wurmb) because of its abundant availability and carbohydrate content. The Nypa plant grows and occupies about 700.000 hectares land with estimated plant population was about 8.000 trees/hectare [3]. Total population of the nypa plant was about 5.600 million trees [4]. Nypa sap contains 13.3 % total sugar [5], while nypa fiber has about 42.22 % cellulose [6]. Communities in Indonesia coastal areas used nypa sap for processing sugar, but the sugar obtained has a taste of slightly salty and less preferred by consumers, so that production of nypa sugar is not well developed. This open opportunity to produce ethanol from the nypa.

Most of research in Indonesia was mainly focused on utilization of nypa sap for producing ethanol. Utilization of other parts of nypa (which rich of cellulose) for producing ethanol has not yet intensively explored. In fact, cellulose makes up nearly half of all plant biomass. Therefore, cellulosic (or lignocellulosic) ethanol is considered as the largest potential source of biofuel in the near future. In addition, conversion of the biomass into biofuels has several advantages including greenhouse gas mitigation, near carbon neutrality, lesser dependence on fossil fuels, and improvement in nations' energy security [7]. However, Kumar *et al*. [8] mentioned that processing cost for converting lignocellulosic materials into ethanol has not yet feasible for commercial scale.

The structure of lignocellulosic biomass, such as nypa midrib, is very complex in nature and is not suitable to be used directly in a fermentation process for producing ethanol. Therefore, pretreatment is necessary to be applied for increasing efficiency of fermentation process. Identifying and developing cost-effective pretreatment methods of lignocellulosic biomass is a major challenge. The pretreatment is addressed to change the biomass structure by removing lignin and hemicellulose, reduce cellulose crystalline, increase porosity and increase the internal surface area into a microscopic size. Most pretreatments require some sort of size reduction to achieve better efficiency in terms of high sugar yield [9, 10].

It is presumed that different lignocellulosic materials could give different reducing sugar yield in hydrolysis process. Focusing on decomposition of nypa frond using two-step-hot-compressed water hydrolysis (230ºC/10 MPa/15 min in the first stage and 270ºC/10 MPa/30 min in the second stage), Phaiboonsilpa *et al*. [11] reported that the nypa frond were hydrolyzed to hemicelluloses, cellulose, and ligin to an extent of 107.4%, 83.6%, and lignin 90.7% respectively. While Mussatto at al. [12] mentioned that temperature is a critical factor for producing reducing sugar from hemicellulose and cellulose. They suggested hydrolysis with temperature below 160ºC for producing high yield of reducing sugar. Encouraging results of those researchs are considered unapplicable for rural application in Indonesia. Therefore, application temperature of 100ºC in hydrolysis was investigated in the present research.

In addition to temperature application, acid treatment was also cosidered to be applied in the present research. Some previous research indicated that acid treatment in hydrolysis gave considerable contribution in producing high yield of reducing sugar from hemicellulose and cellulose. Manzoor *et al*. [13] reported that after pretreatment with sulfuric acid, enzymatic hydrolysis enhanced and the achieved yield of sugar was around 90%. The main objective of the acid pretreatment is to degrade the hemicellulose of the bagasse, and this increases the surface area of the materials. This will then be more suitable for hydrolysis. Use of acids was also investigated by Lavarack [14]. It was reported that hydrochloric acid was less active for the degradation of lignocellulosic materials (sugarcane feedstock) when compared to sulfuric acid.

In fermentation process, incomplete utilization of all the sugars including hexoses (C6; glucose, galactose, and mannose) and pentose (C5 sugars; xylose and arabinose) is another factor for high cost of the lignocellulosic materials. However, a lot more progress has been made in modifying various microbes including yeast (e.g., *Saccharomyces cerevisiae*, *Scheffersomyces (Pichia) stipites*, *Kluyveromyces marxianus)* and bacteria (e.g., *Zymomonas mobilis*, *Escherichia coli*, *Klebsiella oxytoca*) to make them capable of fermenting both hexoses and pentose at comparatively high yields [14, 16, 17, 18, 19, 20, 21, 22]. Research efforts in making microbes capable of fermenting pentose can be found in several reviews [18, 20, 23, 24, 25].

Considering the potential and abundant availability of nypa cellulose in Indonesia and to seek possibility of ethanol production in rural community level, this research was conducted to investigate effectivity of ethanol production from nypa midrib by using HCl and H_2SO_4 in hydrolysis process at 100ºC temperature and two starters (*Saccharomyces cerevisiae* and *Zymomonas mobilis*) in fermentation process. Investigation was mainly focused to find: (1) suitable acid and its concentration for hydrolyzing nypa midrib, (2) duration of hydrolysis to gain optimum yield of reducing sugar from the nypa midrib, (3) suitable starter and its concentration for fermenting the nypa midrib hydrolyzate, and (4) duration of fermentation for optimally producing ethanol.

2. Materials and Methods

2.1. Materials

Fresh nypa midrib was taken from 6 years old nypa plant growing at Karangbenda village, Adipala district, Cilacap regency, Indonesia (7°40'00.9"S 109°10'24.3"E). The whole nypa midrib was chopped and grinded to provide size of material 30 mesh. Afterwards, the material was sun dried to provide samples with 12% moisture content.

2.2. Experimental design

A factorial experiment was setup in a Randomized Complete Design. An experiment consisted of two stage processes, i.e. hydrolysis and fermentation processes. Two factors were tested in hydrolysis process, i.e. type of acid (H_2SO_4 and HCl by applying 3 concentration levels, i.e. 1M, 2M, and 3M) and duration of hydrolysis process (1 hour, 2 hours, 3 hours, 4, hours, and 5 hours). Samples were prepared to provide 3 replications for each treatment. Temperature applied in hydrolysis process was 100ºC. The best result of the hydrolysis experiments was then used in fermentation experiments. The fermentation experiments tested two factors, i.e. type of starters (*Saccharomyces cerevisiae* and *Zymomonas mobilis*) with three levels of concentration, i.e. 5 % (v/v), 7.5 % (v/v), and 10 % (v/v), and three level of fermentation duration (2 days, 4 days, and 6 days).

2.3. Variables and Measurements

Measurements of observed variables were conducted as follows:

a. *Reducing sugar content*. Sample solution was prepared using Nelson-Somogyi method and measurement was conducted using spectrophotometer at 540 nm wavelength. Reducing Sugar Content (RSC) was calculated using the following equation (1):

$$RSC\% = \frac{sample\ absorbance - a}{b} x \frac{FP}{sample\ weight} x100\%$$

Where FP is dilution factor, while a and b are regression constants. Value a and b were determined by regression equation of standard glucose solution curve.

b. *Ethanol content*. Ethanol content was measured using spectrophotometer method. Preparation of sample was conducted as follows: a total of 1 ml of solution gained in fermentation was inserted in the edge of the Conway cup, and the other edge of the cup that was given 1 ml of saturated solution of potassium carbonate. At the center of the cup was given a solution of potassium bichromate sulfuric acid. Afterwards, the cup was sealed and shaken gently so that the two solutions were well mixed. The solution was then allowed to settle for 1 hour. Solution at the center of the cup was taken using a pipette and placed in 10 ml of cooked pumpkin. Remaining solution was rinsed in a petri dish with distilled water, then it put in a flask and distilled water added up to the mark. Solution in the flask was measured its absorbance at a wavelength of 480 nm using a spectrophotometer. Charts for describing relationship between alcohol concentration and absorbance were made in order to obtain regression equations for calculating ethanol content in the sample. The ethanol content (EC) was calculated using the following equation (2):

$$EC\% = \frac{sample\ absorbance - a}{b} x \frac{FP}{sample\ weight} x100\%$$

Where FP is dilution factor, while a and b are regression constants. Sample absorbance was measured using spectrophotometer at 480 nm wavelength. Value a and b were determined by regression equation of standard glucose solution curve.

c. *Yield of ethanol*. Yield of ethanol was calculated based on ratio of ethanol content and reducing sugar content. The calculation was done using the following equation (3):

$$Yield\ of\ ethanol\ (\%) = \frac{ethanol\ content}{reducing\ sugar\ content}$$

3. Results and Discussion

3.1. Hydrolysis

3.1.1. Use of H_2SO_4

Results of hydrolysis process can be seen in Figure 1 and Figure 2. Variance analysis on reducing sugar content produced in hydrolysis process indicated that concentration of H_2SO_4 significantly influenced the yield of reducing sugar content. Moreover, the variance analysis indicated that the hydrolysis duration did not significantly influence the yield of reducing sugars. However, interaction of the hydrolysis duration and the concentration of H_2SO_4 significantly influenced the reducing sugar yield; this was indicated by the variance analysis which pointed out a strong interaction between the two factors. This indication can also be clearly observed in Figure 2 whereas increasing trend were showed by the yield of hydrolysis using 1 M and 2 M H_2SO_4, while an opposite trend was showed in hydrolysis using 3 M H_2SO_4.

Figure 1 shows that application of higher H_2SO_4 concentration in an hour hydrolysis process tended to increase the reducing sugar yield. The highest reducing sugar yield was 1.28% which was produced in an hour hydrolysis using 3M H_2SO_4. However, longer duration of hydrolysis caused a decrease trend of the reducing sugar yield with the higher concentration of H_2SO_4. This trend was also observed in hydrolysis of mercerizes sisal by Lacerda *et.al*. [26] and it was found that longer duration of hydrolysis and higher concentration of acid led to more intense glucose decomposition.

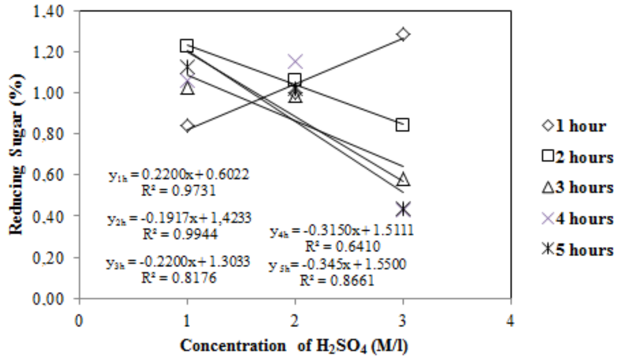

Figure 1. Reducing sugar yield in hydrolysis using various concentration of H_2SO_4.

3.1.2 Use of HCL

Results of hydrolysis process using various concentration of HCl can be seen in Figure 3 and Figure 4. It was indicated by variance analysis that extraction reducing sugar from nypa midrib cellulose was strongly influenced by the duration of hydrolysis process and the acid concentration. The highest yield of reducing sugar was 2.76% which was produced in 5 hours hydrolysis with 3 M HCl.

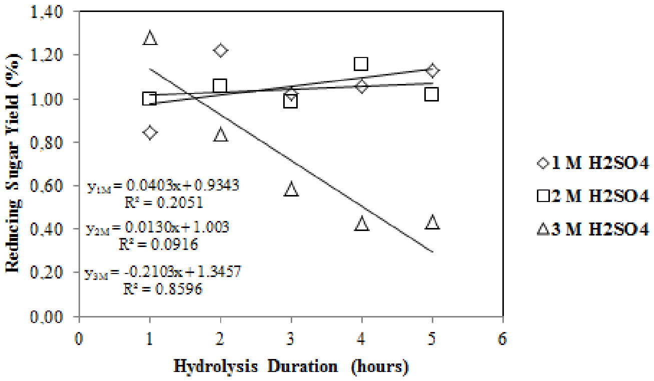

Figure 2. Reducing sugar yield in various duration of hydrolysis using H_2SO_4.

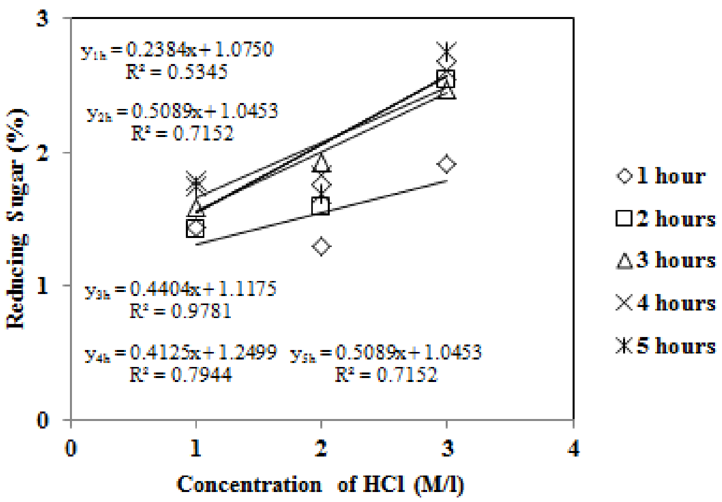

Figure 3. Reducing sugar yield in hydrolysis using various concentration of HCl.

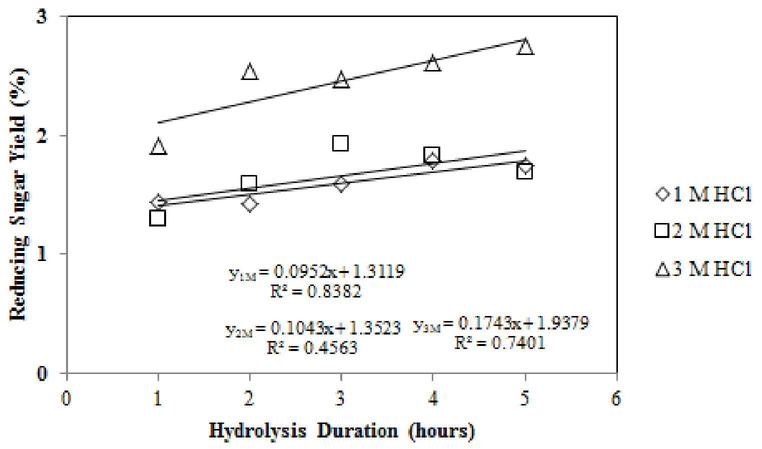

Figure 4. Reducing sugar yield in various duration of hydrolysis using HCl.

3.2. Fermentation

3.2.1. Fermentation of H_2SO_4 hydrolyzate

Two batch of hydrolyzate, obtained from the 1 hour hydrolysis of nypa midrib powder using 3M H_2SO_4, was used in fermentation to produced ethanol; the hydrolyzate contained 0.13% of reducing sugar. The first batch was supplemented with *Saccharomyces cerevisiae* as starter in fermentation process, while the second batch was supplemented with *Zymomonas mobilis*. After 6 days fermentation process, there was no indication of ethanol production in all samples. It is presumed that availability of reducing sugar in samples were not sufficient for producing ethanol. Further investigation should be conducted to find minimum availability of reducing sugar at fermentation condition in this research; this could be useful information as there is no previous research found to confirm that result.

3.2.2. *Fermentation of HCl hydrolyzate*

Fermentation processes were applied by using two batch of hydrolyzate derived from the 5 hours hydrolysis of nypa midget powder using 3M HCl; the hydrolyzate contained 2.8% of reducing sugar. *Saccharomyces cerevisiae* was supplemented as starter in the first batch, while *Zymomonas mobilis* was applied in the second batch. Observation results shows that indication of ethanol production was significantly detected in one sample during 6 days fermentation process. Yield of ethanol found in the sample was 3.8 %. In previous research by Trisasiwi *et al.* [5] on nypa sap fermentation using *Saccharomyces cerevisiae*, it was found that fermentation of 13.3% total sugar can produced ethanol yield of 7.5%.

3.3. General Discussion

The results discussed above indicates that production of cellulosic ethanol from nypa midrib is possible. Referring to the results, further investigation should be made in order to find better combination of treatments for improving reducing sugar yield. In addition, crytalline alteration during hydrolysis should also be investigated to get insights behaviour of the nypa midrib in the subjected treatments.

4. Conclusions

Based on analysis of the experimental results, it can be concluded that:

1. Use of HCl in hydrolysis was more effective for producing reducing sugar than that of H_2SO_4.
2. The best combination of treatment for hydrolysis process using H_2SO_4 was gained by applying one hour duration of hydrolysis and used of 3M H_2SO_4; the yield of reducing sugar was 1.28%. In hydrolysisprocess using HCl, the best combination was application of 5 hours duration of hydrolysis and used of 3M HCl whereas the yield of reducing sugar was 2.76%.
3. The 2.76% of reducing sugar could be fermented to produce ethanol 3.80% by using 10% (v/v) *Saccharomyces cerevisiae* as starter in 6 days fermentation process.

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