Investigation of Hydrolysis Using Cellulase Enzyme Produced From Cow Rumen And Fermentation Method for Producing Ethanol from Nypa (Nypa fruticans Wurmb) Midrib

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Abstract. Nypa (Nypa fruticans Wurmb) has considered to have high potential for producing ethanol. This research was mainly focus on investigation to find: (1) influence of enzyme concentration and hydrolysis duration in producing reducing sugar from nypa midrib and (2) influence of Saccharomyces cerevisiae concentration and duration of fermentation process to convert the nypa midrib reducing sugar for producing ethanol. Experiments consisted of hydrolysis and fermentation processes. In hydrolysis process, two factors were tested, i.e. concentration of cellulase enzyme (5 ml, 10 ml, and 25 ml) and duration of hydrolysis process (12 hour, 24 hours, 36 hours, 48 hours, and 60 hours). Three replications were applied for each treatment. The best result of the hydrolysis was applied in fermentation experiments. Two factors, i.e. concentration of Saccharomyces cerevisiae (12.5% (v/v), 25% (v/v), and 37.5% (v/v)) and fermentation duration (4 days, 6 days, 8 days). Experiment results indicated that application of 5 ml of enzyme concentration and 12 hours duration of hydrolysis gave highest production of reducing sugar in hydrolysis process. Highest production of ethanol was gained by application of 37.5% concentration of S. cerevisae and 144 hours of fermentation process.

Keywords ethanol, nypa midrib, hydrolysis, fermentation, enzyme, cow rumen

1. Introduction
In 2025, it was predicted that need of energy for transportation sector will increase up to 1.554 million BOE (Billion Oil Equivalent). Consumption of energy for transportation was about 20% of total energy consumption. Main supply of energy consumption in the sector (97%) was fossil energy [1]. In anticipating possible problem in this sector, the Government of Indonesia issued a Regulation No 12 year 2015 which stated that since 1 April 2015 fuels mixed have to distribute by all companies which having fuels business permit.

Considering the above matters, Nypa (Nypa fruticans Wurmb) was considered as a potential alternative source for producing ethanol due to its abundant availability and carbohydrate content. In Indonesia, there are about 700,000 hectares of the Nypa plant growing area with population of 8,000 trees/hectare [3] and total number of the nypa was about 5,600 million trees [4].

Sugar content of Nypa sap is about 13.3% total sugar [5]. The sap is commonly used for producing sugar, but taste of the sugar is less preferred by consumers. This open opportunity to
produce ethanol from the nypa sap. In addition, high cellulose content of nypa fiber (42.22 % cellulose) is also potential for producing ethanol [6].

Utilization of nypa sap for producing ethanol has been concerned in most of research in Indonesia. Potential of other parts of nypa (which rich of cellulose) for producing ethanol should be intensively explored. Cellulose content is nearly half of all plant biomass; this gives a large potential source for producing biofuel in the near future. Moreover, biomass conversion into biofuels gives several advantages such as mitigation of greenhouse gas, neutrality of near carbon, reducing dependence on fossil fuels, and enhancing nations’ energy security [7]. However, cost for converting lignocellulosic materials into ethanol was considered infeasible for commercial scale [8].

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 lignin 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 considered 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].

Trisasiwi et al [26] reported that use of HCl in hydrolysis was more effective for producing reducing sugar than that of H2SO4. Moreover, 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.

In responding the above matters, this research was conducted to investigate effectivity of ethanol production from nypa midrib by hydrolysis process using cellulase enzyme produced from cow
rumen liquid and *Saccharomyces cerevisiae* in fermentation process. Investigation was mainly to find: (1) influence of enzyme concentration and hydrolysis duration in producing reducing sugar from nypa midrib and (2) influence of *Saccharomyces cerevisiae* concentration and duration of fermentation process to convert the nypa midrib reducing sugar for producing ethanol.

2. Materials and Methods

Materials

Fresh nypa midrib was originally 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 60 mesh of material size. Furthermore, the material was dried by exposing to sun for gaining samples with 12% moisture content.

Experimental design

A factorial experiment was arranged in a Randomized Complete Design. Two stages were involved in the experiment, i.e. hydrolysis and fermentation processes. In hydrolysis process, two factors were tested, i.e. concentration of cellulase enzyme (5 ml, 10 ml, and 25 ml) and duration of hydrolysis process (12 hour, 24 hours, 36 hours, 48 hours, and 60 hours). Three replications were applied for each treatment. The best result of the hydrolysis was applied in fermentation experiments. Two factors, i.e. concentration of *Saccharomyces cerevisiae* (12.5% (v/v), 25% (v/v), and 37.5% (v/v)) and fermentation duration (4 days, 6 days, 8 days).

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):

\[
RSC\% = \left( \frac{\text{sample absorbance} - \alpha}{b} \right) \times \left( \frac{\text{FP/sample weight}}{\text{sample weight}} \right) \times 100\%
\]

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 dichromate 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):

\[
EC\% = \left( \frac{\text{sample absorbance} - \alpha}{b} \right) \times \left( \frac{\text{FP/sample weight}}{\text{sample weight}} \right) \times 100\%
\]

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):

\[
\text{Yield of ethanol ()} = \frac{\text{ethanol content}}{\text{reducing sugar content}} \times 100\%
\]
3. Results and Discussion

Raw material characteristic
Nypa midrib powder used in the experiments has characteristic as presented in Table 1. Size of the powder was 60 mesh. A pretreatment was applied before the material used in experiments. Alkaline pretreatment was done using NaOH 3% for 90 minutes. It can be seen from the table that the salt content was high because growing area of the nypa is very close to South Indonesian Ocean.

| No | Composition           | Total (%) |
|----|-----------------------|-----------|
| 1  | Moisture content      | 12.22     |
| 2  | Reducing sugar content | 0.49      |
| 3  | Salt content          | 4.17      |
| 4  | Cellulose content     | 42.22     |

Hydrolysis
For hydrolysis purpose, cellulase enzyme was produced from cow rumen liquid. Table 2 and Figure 1 shows reducing sugar produced by enzymatic hydrolisis at various application of enzyme concentration and hydrolysis duration.

| Hydrolysis Duration (hours) | Reducing Sugar (% v/m) |
|-----------------------------|------------------------|
|                             | 5 ml | 10 ml | 25 ml |
| 12                          | 0.488| 0.478 | 0.483 |
| 24                          | 0.351| 0.346 | 0.386 |
| 36                          | 0.392| 0.422 | 0.341 |
| 48                          | 0.376| 0.432 | 0.402 |
| 60                          | 0.437| 0.381 | 0.412 |
Variance analysis on reducing sugar production in hydrolysis indicated that hydrolysis duration and concentration of enzyme did not significantly influence the reducing sugar yield. However, it can be seen from Table 2 and Figure 1 that 5 ml concentration of enzyme and 12 hours hydrolysis duration could produce highest reducing sugar (0.488% v/m). It was found that the enzyme activity was 0.048 U/ml.

Figure 1. Reducing sugar produced in hydrolysis process at various concentration of enzyme and hydrolysis duration.

Fermentation
Reducing sugar, obtained by hydrolysis using 5 ml concentration of enzyme and 12 hours hydrolysis duration, was used in fermentation experiments. Table 3 shows ethanol yielded in fermentation process at various concentration of *Saccharomyces cerevisae* and duration of fermentation.

| Reducing Sugar (% v/m) | Concentration of S. cerevisae (%) | Fermentation Duration (days) | Ethanol Production (% v/v) |
|------------------------|----------------------------------|------------------------------|---------------------------|
| 0.59                   | 12.5                             | 96                           | 0.497                     |
| 0.39                   | 144                              | -                            | -                         |
| 0.22                   | 192                              | -                            | -                         |
| 0.60                   | 25                               | 96                           | -                         |
| 0.32                   | 144                              | 0.220                        |
| 0.22                   | 192                              | 0.247                        |
| 0.69                   | 37.5                             | 96                           | 0.74                      |
| 0.38                   | 144                              | 0.75                         |
| 0.25                   | 192                              | -                            | -                         |

Table 3 indicated that production of ethanol was significantly detected in some application of S. cerevisae concentration and fermentation durations. The undetected ethanol production in some fermentation process was strongly presumed do to insufficient availability of reducing sugar or activity of the bacteria in converting the reducing sugar. According to the results presented in the table, highest production of ethanol was gained by application of 37.5% concentration of S. cerevisae and 144 hours of fermentation process.
4. Conclusions
Based on analysis of experiment results, conclusions could be drawn as follows: Application of 5 ml of enzyme concentration and 12 hours duration of hydrolysis gave highest production of reducing sugar in hydrolysis process. Highest production of ethanol was gained by application of 37.5% concentration of S. cerevisiae and 144 hours of fermentation process.

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