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# EFFECT OF DILUTE ACID AND ALKALINE PRETREATMENTS ON ENZYMATIC SACCHARFICATION OF PALM TREE TRUNK WASTE FOR BIOETHANOL PRODUCTION

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**Abstract**

Increasing energy demand, rising prices of crude oil, and security issues regarding oil supply are the aspects that underlay research about development of alternative energy. Arenga pinnata (or palm trees) is one of lignocellulosic biomass which is non-food plant, high cellulose content, and abundant in Indonesia which recognized as having great potential for bioethanol production. However lignins present can inhibit the action of enzyme at saccharification process, so that pretreatment plays an important role to degrade lignin and increase the number of cellulose which will convert to ethanol. The effect of pretreatment of the substrate using nitric acid ($HNO_3$), ammonium hydroxide ($NH_4OH$) each at 2%, 4%, 8%, and 10% (v/v) concentration, and steam pretreatment to reducing sugar and ethanol content were investigated. The pretreated samples was hydrolyzed using cellulase enzyme with substrate concentration 10% (w/v) for 24 h, 48 h, 72 h at 50°C and pH 5.0. Results from the enzymatic saccharification experiments showed that maximum reducing sugar and total sugar content of palm tree trunk waste pretreated using 10% (v/v) of $HNO_3$ are 5.320% and 5.834% was achieved respectively at 72 h of saccharification. While the maximum reducing sugar and total sugar content of palm tree trunk waste pretreated using 10% (v/v) of $NH_4OH$ are 2.892% and 3.556% respectively at 72 h of saccharification. Steam pretreatment shown the maximum reducing sugar and total sugar

respectively 1.140% and 1.315% at 72 h of saccharification. It shown that pretreatment using nitric acid ($HNO_3$) give the maximum results of reducing sugar and total sugar compared to others. The SSF process was carried out at 37°C, pH 4.8, and 100 rpm for 120 h using Saccharomyces cerevisiae and cellulase enzyme with 10% (v/v) of yeast concentration and 10% (w/v) of substrate concentration. The result shows that the highest ethanol content can be produced by a high concentration of substrate where the highest ethanol content was 2.648% by using $HNO_3$ 10% (v/v) compared to $NH_4OH$ 10% (v/v) that produced 0.869% and steam pretreatment that produce 0.102% ethanol content. This demonstrates the utility of palm tree trunk wastes in ethanol production.

**Keywords:**

Bioethanol; lignocellulose; substrate concentration; dilute acid pretreatment; alkaline pretreatment; SSF

## 1. Introduction

Increasing population trigger an increase in consumption of fossil fuel over the world. According to energy information, total petroleum consumption in the world on 2010 is currently 88.216 thousand barrels per day and it is increase to 91.253 thousand barrels per day by the year 2013 [1]. In 2014, the world energy consumption from fossil fuels accounted 86.3%, whereas renewable energy accounted about 9.2% [2]. An increasing of fossil fuels consumption causes the depletion of reserves, global warming and climate change. Therefore required an alternative energy to fulfill the needs of fuel oil nad environmentally friendly [3].

The importance of alternative energy source has become even more necessary not only due to the continuous depletion of limited fossil fuel stock, but also for the safe and better environment. One of them is bioethanol from biomass, that becomes an alternative energy production [4].

Bioethanol is a promising renewable energy source, can reduce the negative impacts on the environment, and environmentally friendly [5]. Moreover, utilization of crops into energy production would affect the increasing of agricultural sector [6] especially in developing countries such Indonesia.

Conventionally bioethanol produced from starch materials and sucrose containing such as corn and sugar cane, which is known with first-generation technologies [7]. However, the production of bioethanol from sugar producing crops and starchy material is impacted on food supply [8]. Therefore required other raw materials as replacement. One of the most promising options to meet this challenge is the production of bioethanol from lignocellulose, such as agricultural residues (e.g. wheat straw, grains, corn stover, etc) and forestry residues (e.g. sawdust, etc) using second generation

technologies [9]. Moreover, lignocellulosic materials are abundant and can reducie environmental impacts by generate very low greenhouse gas emissions [10].

Lignocellulose composed from three major components, lignin, cellulose, and hemicellulose. Lignin serves to strengthen plants and rigidity in order to prevent swelling of lignocellulosic materials [11]. One of the most abundant lignocellulosic biomass is palm tree. [12] at their study obtained that sugar palm trunk consist of 46.4% lignin, 40.6% cellulose, and 61.1% holocellulose. In Klaten Indonesia, starch from palm tree trunk utilized as a raw materials of bihun production. The smooth palm tree trunk has been soaked in water to obtained the starch produced. The remain of unused substrate or in this case palm tree trunk waste is further will be processed into bioethanol. Palm tree trunk wastes are not fully utilized and found in many remote areas, so it can be use as raw materials of second-generation biomass to produce bioethanol.

Production of bioethanol from lignocellulose including three main processes, including pretreatment, saccharification, and fermentation. During the pretreatment process, lignin will be broken in order to make cellulose easily accessible to the enzymes that convert polymers into fermentable sugars[13]. Saccharification performed by added an enzyme to convert the cellulose results into glucose required for fermentation. The use of enzymes in the saccharification process is more beneficial than chemicals, due to highly specific and can work at mild process conditions[14]. Most cellulase enzymes are relatively unstable at high temperatures. The maximum activity for cellulases enzymes occurs at 40-50°C and a pH 4.5-5.0[15]. In this previous study obtained that the enzyme activity was decreased when the temperature increased above 65°C and almost completely lose activity at 80°C[16]. Then the glucose will be fermented using several yeast or microorganisms. The most commonly yeast used for bioethanol production is S. cerevisiae due to low optimum pH, high tolerance to ethanol and inhibitors, and does not require oxygenation[17]. But the main difficulty with SSF of cellulose is the different temperature optima required for saccharification (45-50°C) and fermentation (20-30°C). And in the end, the SSF process is operated at temperature 35-38°C since the saccharification and fermentation are carried out simultaneously[18].

Various processes have been developed for ethanol production such as a Simultaneous Saccharification and Fermentation (SSF) which requires lower energy among others [19]. And various reports on bioethanol production have mentioned that SSF method has greater yield in production of ethanol than SHF because SSF method are less inhibition during saccharification[20] (Hader Castaño Peláez, 2013).

This research focused on 3 processes, there are pretreatment, saccharification, and fermentation. In pretreatment process, the effect of steam pretreatment, $HNO_3$ 10% (v/v), and $NH_4OH$ 10% (v/v) to lignocellulosic content and morphological view were investigated. In saccharification process, the

effect of steam pretreatment, 2%, 4%, 8%, 10% (v/v) concentration of $HNO_3$ and $NH_4OH$ during 24 h, 48 h, and 72 h to reducing sugar and total sugar content were examined. The effect of steam pretreatment, 2%, 4%, 8%, 10% (v/v) concentration of $HNO_3$ and $NH_4OH$ to ethanol content were also investigated in fermentation process.

## 2. Materials and Methods

### 2.1. Preparation of Raw Material

#### 2.1.1. Palm Tree Trunk Waste

Palm tree trunk waste as the raw materials was obtained from home industry Klaten, Central Java in the form of wet coarse powder. The raw materials were oven-dried at temperature of 80°C until the constant weight and milled into flour in a size of ±40 mesh. Subsequently, it stored at room temperature until used.

#### 2.1.2. Enzyme and Yeast

The commercial cellulases enzyme (SQzyme CS) used for the saccharification of palm tree trunk wastes were purchased from Suntaq International Limited (Nanshan District, China). It is a concentrated liquid Acid Cellulase enzyme made from the strain of Trichoderma reesei with activity 20000 u/g that was used in saccharification and fermentation processes to produce sugar from palm tree trunk wastes. While, S. cerevisiae used for ethanol production in fermentation process was obtained from Gadjah Mada University, Yogyakarta Indonesia.

#### 2.1.3. Innoculum Preparation

Innoculum was prepared by transferring a loop of cells of S. cerevisiae into a growth medium in test tube containing (g/L): glucose, 20; peptone , 20; yeast extract, 10; and agar, 15, that have been sterilized in advance using autoclave at 121°C for 20 min to prevent contamination. Then, the growth medium were incubated at 30°C for 72 h. Growth of S. cerevisiae was characterized by the appearance of white patches on the growth medium. This indicated that S. cerevisiae was ready to be moved to pre-culture medium containing (g/L): $(NH_4)_2HPO_4$, 2; urea, 6.4; $KH_2PO_4$, 2; and $MgSO_4.7H_2O$, 1. The pre-growth culture was sterilized for 20 min at 121°C and was incubated at 150 rpm and 30°C for 48 h. Then, main-culture medium was prepared on a rotary shaker at 150 rpm and 30°C for 72 h, in a medium containing (g/L): treatmented substrate, 20; yeast extract, 10; peptone, 20; $(NH_4)_2HPO_4$, 2; urea, 6; $KH_2PO_4$, 2; and $MgSO_4.7H_2O$, 1. The pre-culture and main-culture medium were ready to used for next processes.

### 2.2. Experimental Methods

#### 2.2.1. Pretreatment of Palm Tree Trunk Waste

Steam pretreatment conducted by soaked a 25 g of dry palm tree trunk waste in distilled

water. Dilute nitric acid ($HNO_3$) and ammonium hydroxide ($NH_4OH$) were also used for pretreatment process. A 25 g of dry palm tree trunk waste were soaked in various concentrations of $HNO_3$ and $NH_4OH$ solution: 2%, 4%, 8%, and 10% (v/v) at a solid to liquid ratio of 1:20 (w/v) in 500 ml beaker glass. The mixture is then stirred to ensure that all of the palm tree trunk waste contacts with the treatment solution became a slurry. The slurry were then autoclaved at 121°C for 60 min. The residue was removed from the solutions using filter paper on the vacuum filtration unit and washed with distilled water to obtain neutral pH. The use of pretreated substrate after washing with water can reduce the concentration of inhibitory products [21]. The residue was then dried until constant weight and ready for used at the next processes.

**2.2.2. Enzymatic Saccharification**

A 10 g dry pretreated palm tree trunk waste was placed in a 250 ml of erlenmeyer flask and mixed well with 100 ml of citrate buffer at pH 5.0. The slurry was sterilized at 121°C for 20 min. A 2 g of cellulase enzyme with substrate concentration 10% (w/v) and cellulase activity 20000 u/g were added to the mixture, and incubated at 50°C , 150 rpm, for 24 h, 48 h, and 72 h. The reaction was stopped by boiling the samples for 5 min and was taken to determine the total sugar and reduction sugar content.

**2.2.3. Simultaneous Saccharification and Fermentation (SSF)**

Glucose obtained from saccharification process was converted into ethanol by fermentation using yeast S. cerevisiae. Fermentation medium were carried out in 250 ml erlenmeyer flask. The flask were added with 10 g dry pretreated palm tree trunk waste and citrate buffer pH 4.8. It must be was sterilized for 20 min at 121°C. 10 ml of pre-culture, 10 ml of main-culture, and 2 g of cellulase enzyme were added to the flask and incubated at 37°C. After 120 h incubation period, the fermented medium was distillated to determined the ethanol content.

**2.3. Analysis**

**2.3.1. Determination of Lignin**

3 ml of 72% $H_2SO_4$ was added into 0.3 g of dried sample in glass tube and kept at room temperature for 2 h while stirred every 30 min to provide complete hydrolysis. 84 ml of distilled water was added to the sample and autoclaved for an hour. Cooled at room temperature and filtered the sample. The residue was dried at 105°C and accounting for ash by burning the hydrolyzed sample at 575°C in a furnace to determined the acid insoluble lignin. While, the acid soluble lignin was determined by measured the absorbance of sample at 320 nm. The lignin content was summation of acid insoluble lignin and acid soluble lignin [22].

**2.3.2. Determination of Hemicellulose**

150 ml of 500 mol/$m^3$ NaOH was added to a 250 ml of erlenmeyer that consist of 1 g of the dried sample. Boiled for 3.5 h and cooled at room temperature. Then, washed until neutral pH and

filtered the mixture. Dried the residue at 105°C in an oven until constant weight. The hemicellulose content (%w/w) was determined by calculate the difference weight between the sample before and after this treatment [22].

### 2.3.3. Determination of Cellulose

By assuming that extractives, hemicellulose, lignin, ash, and cellulose are the only components, the cellulose content (%w/w) was calculated by difference [22].

### 2.3.4. Determination of Reducing Sugar

Reducing sugar content was measured using DNS method by [23]. 3 ml of dinitrosalicylic acid (DNS) reagent was added into 3 ml of sample in the test tube and heat the mixture at 90°C for 10 min. Add 1 ml of Rochelle salt and cooled to room temperature. The absorbance was measured at 575 nm. Blanks were prepared by substituing distilled water as sugar solution with the same procedure. The reducing sugar content was determined by reference to a standard curve.

### 2.3.5. Determination of Total Sugar

Total sugar content was measured using phenol sulfuric acid by [24] by adding 0.05 ml of 80% phenol and 5 ml of concentrated $H_2SO_4$ to 2 ml of sample solution in colorimetric tube. Allowed for 10 min, shake in a water bath at 25" to 30"C for 15 min before readings. The absorbance was measured at 490 mμ for hexoses and 480 mμ for pentoses and uronic acids. Blanks were prepared by substituing distilled water as sugar solution with the same procedure. The total sugar content was determined by reference to a standard curve.

### 2.3.6. Determination of Ethanol Content

A Shimadzu GC-8A (Shimadzu Corporation, Japan) was used to analyze ethanol content in the sample by direct injection Gas Chromatography (GC). The flow rates of $H_2$, air, and $N_2$ (carrier gas) were set at 30, 300, and 2 ml/min, respectively. The temperature of FID detector was 285°C and the injection volume was limited to 0.1 µL. 50 mg of internal standard solution was added to 0.5 ml of sample that was dispensed into 1 ml of sample vial. After mixing, 0.1 µL of the sample solution was injected directly into a GC with syringe. The calculation of ethanol content was also reported by[25].

### 2.3.7. Scanning Electron Microscopy (SEM) Image Observation

A JEOL JSM-6510 scanning electron microscope (JEOL Ltd. Tokyo, Japan) with 1000-fold magnification was used to observed any morphological changes in biomass sample (raw materials and treated biomass of palm tree trunk waste). The dried samples were placed on aluminum specimen mount, using conductive carbon tape. Sputter gold coating was performed to prevent charging. All the specimens were examined in SEM under vacuum condition at accelerating voltage of 5 kV [21].

## 3. Results and Discussion

### 3.1. *The Effect of Pretreatment to Lignocellulosic Content*

Pretreatment of lignocellulosic biomass is an important process to remove lignins and hemicellulose from biomass due to the presence makes the access of enzymes to cellulose fibers

difficult. Therefore, the reactivity of cellulose is sufficiently increased due to the decreasing in the amount of lignin and hemicellulose [26]. Pretreatment with dilute acid and alkaline were the most widely used method for lignocellulosic materials. Dilute acid pretreatment used cheap chemicals, mild operating conditions, and is simple to perform. While, alkaline pretreatment will increase the accessbility enzymes to cellulose by removes lignin and part of the hemicellulose in saccharification process[27].

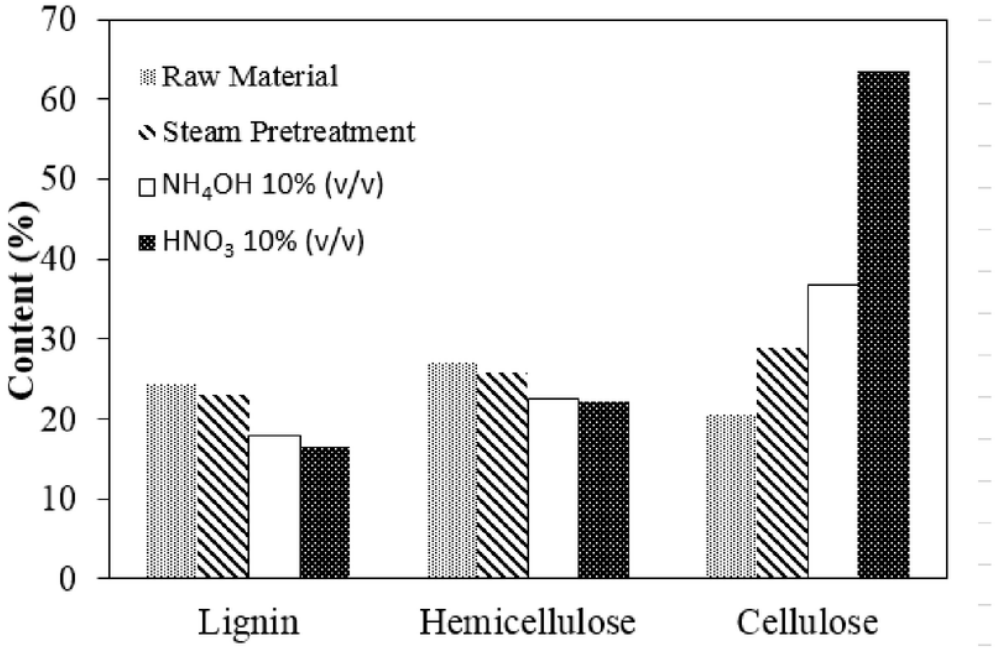


**Figure 1.** Lignocellulosic content of palm tree trunk waste before pretreatment and after pretreatment

Lignin and hemicellulose content were decreased after pretreatment process, while cellulose content was slightly increased (Fig. 1). Lignin content was decreased from 24.25% before pretreatment, to 17.85% and 16.50% after pretreatment using $NH_4OH$ 10% (v/v) and $HNO_3$ 10% (v/v) respectively. Hemicellulose was decreased from 26.98% before pretreatment, to 22.61% and 22.23% after pretreatment using $NH_4OH$ 10% (v/v) and $HNO_3$ 10% (v/v) respectively. While, the cellulose content slightly increased from 20.57% before pretreatment, to 36.70% and 63.53% after pretreatment using $NH_4OH$ 10% (v/v) and $HNO_3$ 10% (v/v) respectively. The dilute acid pretreatment will enhanced the digestibilty of cellulose by solubilize lignin and hemicellulose[28]. While, the mecanism of alkaline pretreatment is by saponification of intermolecular ester bonds crosslinking xylan hemicelluloses and other components (lignin and other hemicellulose) [28]. This indicates that lignin and hemicellulose content successfully removed and caused the increasing of enzyme to access cellulose [29].

### 3.2. *The Effect of Dilute Acid and Alkaline Concentration on Saccharification*

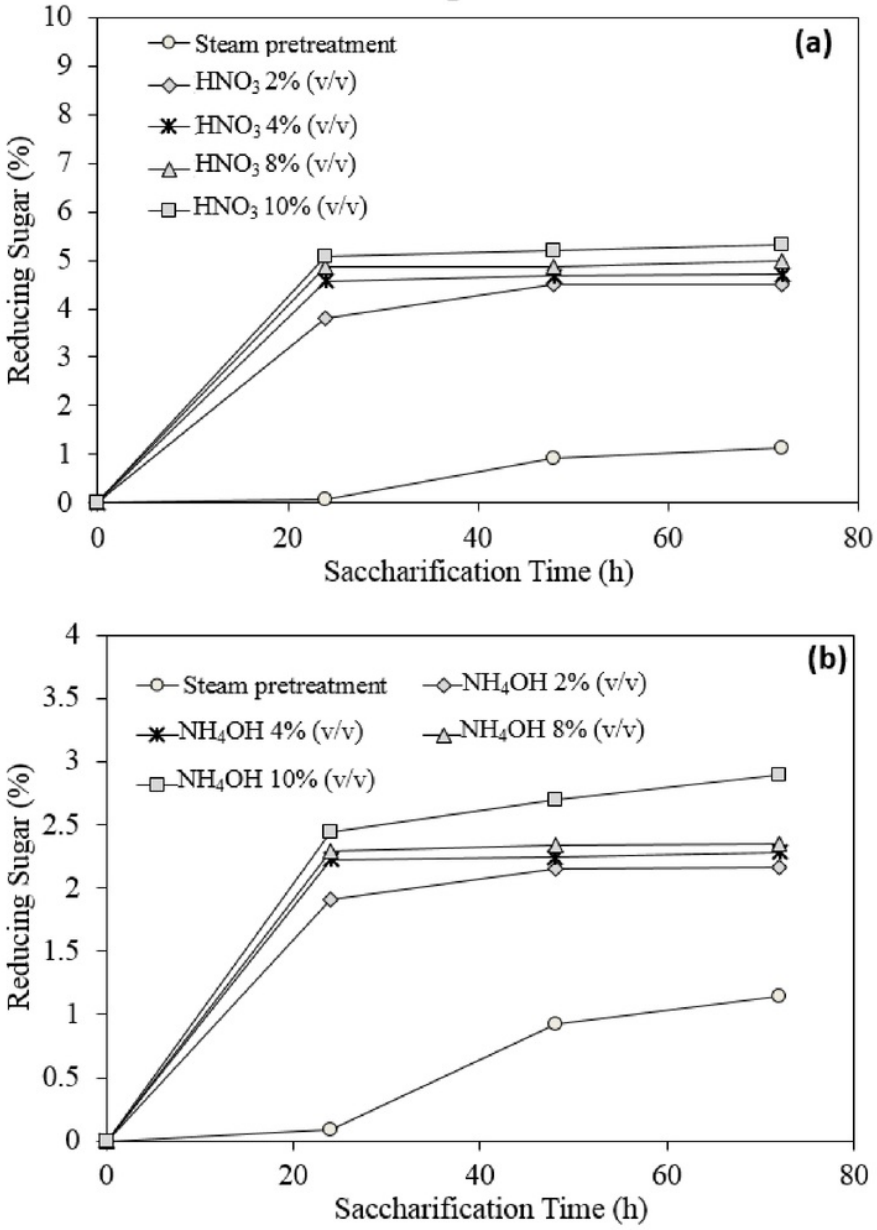


**Figure 2**. Reducing sugar for (a) $HNO_3$ pretreatment and (b) $NH_4OH$ pretreatment. Saccharification at cellulase activity 20000 u/g, pH 5.0, and 50°C.

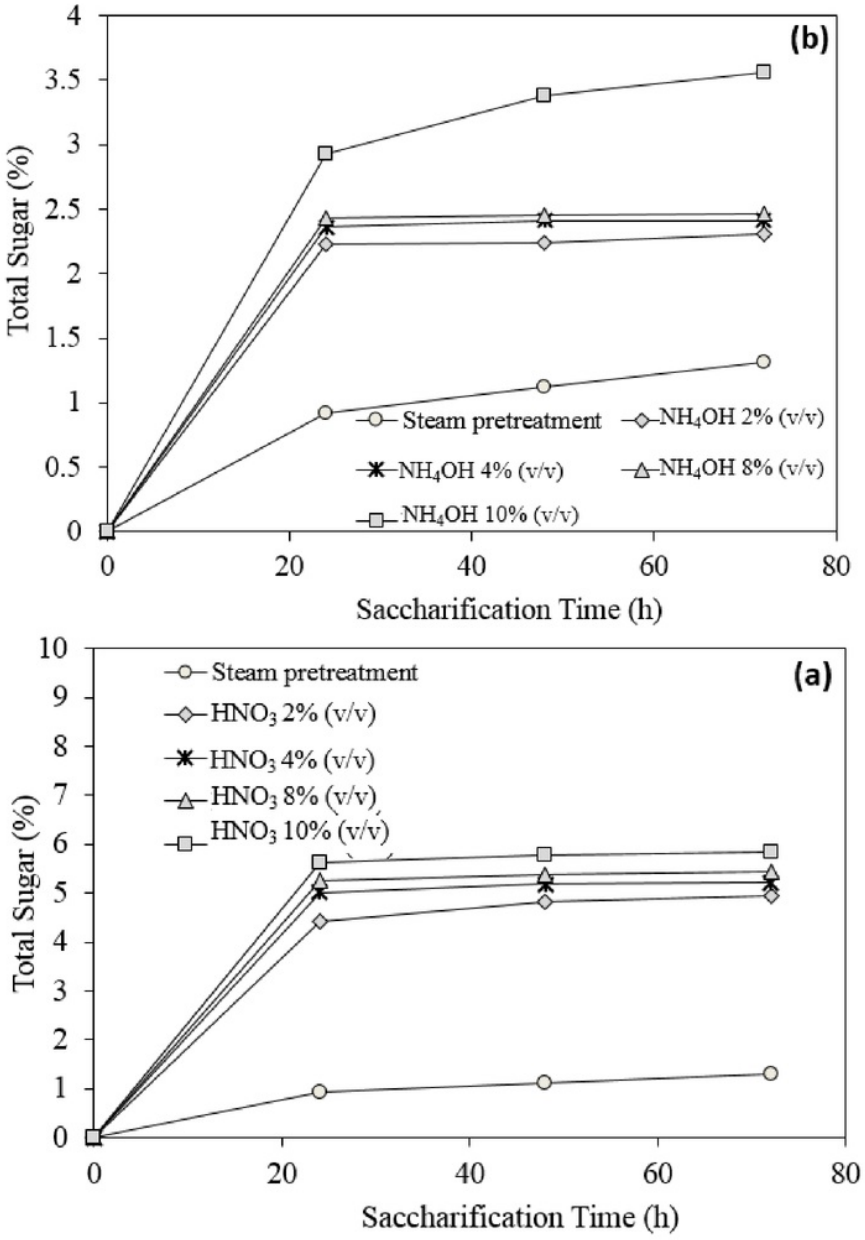


**Figure 3**. Total sugar for (a) $HNO_3$ pretreatment and (b) $NH_4OH$ pretreatment. Saccharification at cellulase activity 20000 u/g, pH 5.0, and 50°C.

From Fig. 2 and Fig. 3 can be seen that substrate concentration influenced the sugar content (reducing sugar and total sugar) in enzymatic saccharification process. The sugar content is increased as the increasing of substrate concentration, due to the random collisons between the substrate and the

active site of enzyme that happened more frequently[30]. By compared the result from the graphs, the highest sugar content was achieved from substrate with $HNO_3$ 10% (v/v) at 72 h of enzymatic saccharification with reducing sugar and total sugar were 5.320% and 5.834% respectively.

According to obtained data, saccharification rate was significantly higher for 72 h compared to 24 h and 48 h. This results are in line of the results obtained by[31] on pretreatment of rice straw by ionic liquid. They showed that prolongation of the saccharification time affect the saccharification results of glucose yield of pretreated straw with 75.4% and 90.9% for 24 h and 72 h, respectively, at 45°C using 20 FPU cellulose and 30 IU β-glucosidase per gram of substrate.

[21] also studied the effect of hydrolysis on Total Reducing Sugar (TRS) yield from sugarcane bagasse and spent citronella biomass. They found that TRS increased with an increase in the acid concentration and reaction time, where the highest TRS of citronella and bagasse obtained at 50°C, 48 h, using 10 FPU of cellulase are 226.99 mg/g and 282.85 mg/g respectively compared to hydrolysis at 50°C, 24 h, using 10 FPU of cellulase that only produced 204.56 mg/g TRS of citronella and 246.96 mg/g TRS of bagasse.

### 3.3. *The Effect of Dilute Acid and Alkaline Concentration on Fermentation*

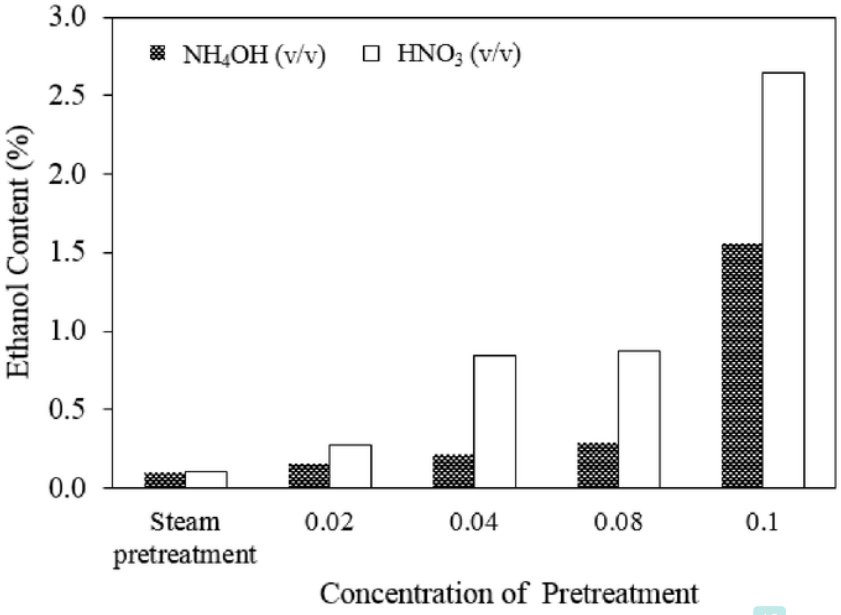


**Figure 4**. Ethanol content after SSF with *S. cerevisiae* and cellulase 20000 u/g at 37°C, pH 4.8, for 120 h.

According to [32] at 1-20% of biomasss concentration, the ethanol concentration is increased. But, it is getting lower at >20% of biomass concentration due to the absorption of liquid by biomass to

helped the enzymes work well. That is appropiate with Fig 4 that shown the ethanol content is increase slightly as the increasing of substrate concentration. From the experiment, the highest ethanol concentration obtained 2.6476% from $HNO_3$ 10% (v/v).

### 3.4. *Scanning Electron Microscope (SEM) Analysis*

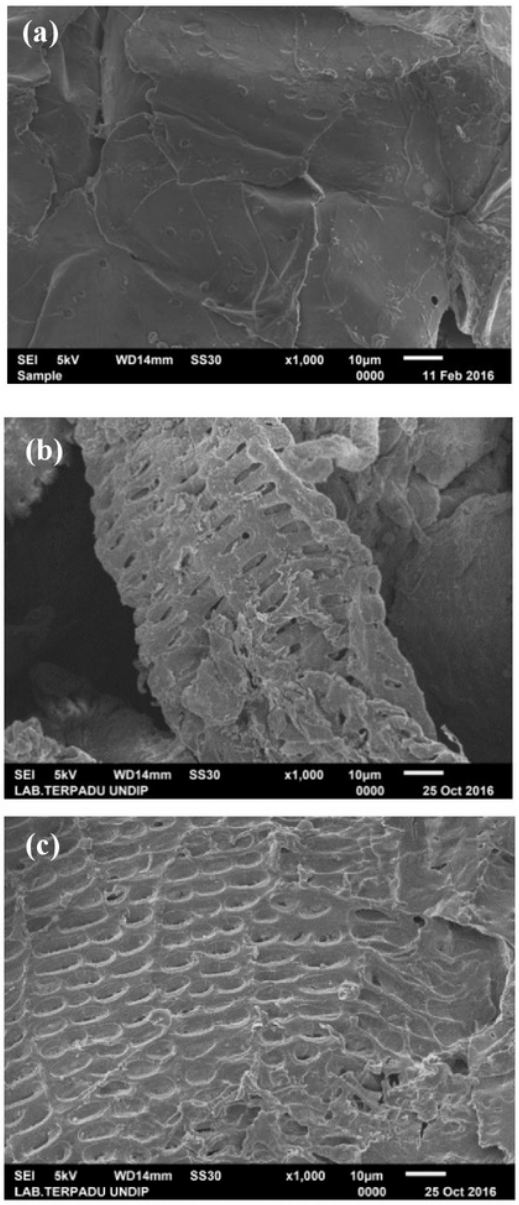


**Figure 5**. Morphological structure of palm tree trunk waste at magnification (1000x) (a) Raw material (b) Preteated with 10% (v/v) $HNO_3$, (c) Pretreated with 10% (v/v) $NH_4OH$. Scale bars represent 10 µm.

Fig 5a shows the morphology of palm tree trunk waste before pretreatment which is compact and has a smooth surface. The most apparent effect of $HNO_3$ prretreatment (Fig 5b) and $NH_4OH$ pretreatment (Fig 5c) is the pores that formed along the surface. Moreover, the lignins were removed substantially and the surface became very rough. The degradation of the cell wall proved that pretreatment process successfully break down the lignin and cellulose content of lignocellulose biomass[33].

## 4. Conclusions

The results of this study showed that palm tree trunk wastes contains a substantial amount of cellulose and is a potential raw material for bioethanol production which could be produced by high-concentration of substrate. Sugar content and ethanol content will be increase as the increasing of substrate concentration. The highest ethanol content was 2.6476% which achieved at concentration of 10% (v/v) of $HNO_3$. This study can serve as a step towards the optimization of pretreatment of palm tree trunk wastes and other lignocellulosic material for enhance ethanol content.


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PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6

PAGE 7

PAGE 8

PAGE 9

PAGE 10

PAGE 11

PAGE 12

PAGE 13

PAGE 14

PAGE 15

PAGE 16