Pretreatment of Starch-Free Sugar Palm Trunk (*Arenga pinnata*) to Enhance Saccharification in Bioethanol Production

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Abstract. Starch-Free Sugar Palm Trunk (*Arenga pinnata*) can be utilized to produce bioethanol because of their high lignocellulosic contents. Production of bioethanol from lignocellulosic materials consist of pre-treatment, saccharification and fermentation processes. In this work, conversion of starch-free sugar palm trunk (*Arenga pinnata*) to fermentable sugar and bioethanol was carried out through g pretreatment, saccharification and fermentation processes. The pretreatment was carried out by addition of 1% (v/v) HNO₃ and NH₄OH for 30 min and 60 min, respectively. The saccharification was carried out at enzyme cellulase loadings of 10 and 20 FPU/g and substrate loadings of 10 and 20 g for NH₄OH pretreated samples. Fermentation was carried out using two methods i.e. separated hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) techniques. The results showed that pretreatment using NH₄OH was more effective than HNO₃ for 60 minutes. Furthermore, the results also presented the reduction of the lignin content of 9.44% and the increase of cellulose content to 18.56% for 1% (v/v) NH₄OH 60 min of pretreatment. The increase of enzyme cellulase (20 FPU/g substrate) and substrate loading (20 g) could produce more reducing sugar (17.423 g/L and 19.233 g/L) than that at 10 FPU/g substrate and 10 g substrate (11.423 g/L and 17.423 g/L), respectively. The comparison of SHF and SSF showed that SHF process yielded higher ethanol (8.11 g/L) as compared to SSF (3.95 g/L) and nontreatment process (0.507 g/L) for 72 h..

1 Introduction

World oil reserves continue to decrease, it is estimated that global oil production will decrease from 25 million to 5 million barrels in 2050 [1]. Bioethanol is one of the renewable energy that is used to reduce the dependence on petroleum fuels and environmental impacts [2]. Bioethanol fuel production from starch sources have been widely applied especially in USA and Brazil. The current biofuel production has been moved towards non-food raw materials, such as forestry or agricultural residues. Sugar palm trees (*Arenga pinnata*) or known locally as Aren Palm is one of the potential sources which have been identified for bioethanol production [3]. To increase the use of the potential sugar palm plants, a large scale sugar palm plantations which is as wide as 800,000 to 4,000,000 ha have been developed in Indonesia [4]. The sugar sap from its flower stalks is the most prominent of aren's products. Besides, the sugar palm tree also generates edible fruits, starch from trunk and fibres for building material and household utensils [4], [5]. Flour (starch) is obtained from the extraction of

the central part of the stem after the palm tree is no longer producing sap [6]. Starch free sugar palm trunk (SFSPT) is a solid waste derived from sugar palm starch extraction. Dry SFSPT is comprised of lignin (37% w/w), cellulose and hemicellulose (49.5%). Lignocellulose from industrial waste palm flour, SFSPT, (*Arenga pinnata*) can be used to manufacture of bioethanol therefore the environmental damage can be reduced. Bioethanol is produced by fermentation of the fermentable sugars generated by enzymatic hydrolysis of cellulose and hemicellulose which are structural carbohydrates. However, lignin is a complex aromatic polymer that forms a cover surrounding the carbohydrate fractions that are limiting the accessibility of carbohydrates to hydrolytic enzymes. Therefore, delignification is important to improve enzymatic digestibility of lignocellulose [7].

Pretreatment is necessary to degrade lignin in bamboo culms to enable bioethanol production as the bamboo lignin network prevents enzymatic saccharification and fermentation [8]. Tutt et al. [9], reported the effect of pretreatment with a dilute solution

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of sulfuric acid, hydrochloric acid, nitric acid and potassium hydroxide, followed by enzymatic hydrolysis of pretreated wheat straw and subsequent fermentation to ethanol. The highest glucose concentration of 316.7 g/kg biomass and the lowest (221.3 g/kg) were achieved by pretreatment with nitric acid and hydrochloric acid, respectively. The best ethanol yield of 104.3 g/kg was achieved by samples pretreated with KOH (rinsed). In another study by Jeong and Lee [10] pretreatment of yellow poplar biomass with oxalic acid which showed the structural was changed for pretreated biomass. The increasing severity of pretreatment resulted an gradual increased of biomass degradation rate which caused the increased crystallinity value and the surface area of the pretreated biomass. Effect of nitric acid on pretreatment for ethanol production of rice straw has been studied by Kim et al. [11], pretreatment conditions were HNO₃ concentration (0.2–1.0%), temperature (120–160°C) and reaction time (1–20) min. A maximum xylose yield of 86.5% and an enzymatic digestibility of 83.0% were achieved at a condition of 0.65% HNO₃, 158.8°C and 5.86 min. Pretreatment of sorghum fibers with ammonium hydroxide followed by enzyme hydrolysis of pretreated fiber and subsequent ethanol production have been evaluated. The treatments removed 44% lignin and 35% hemicellulose at a ratio of 1:0.14:8 sorghum fibers, ammonia, and water, 160°C for 1 h under 140–160 psi pressure. Ethanol yields were 25 g/100 g dry biomass and 10 g ethanol/100 g dry biomass for treated and untreated biomass, respectively [12].

The objective of this study was to evaluate the effect of pretreatments of starch free sugar palm trunk (SFSPT) using dilute nitric acid and ammonium hydroxide on the hydrolysis and subsequent fermentation using Simultaneous Saccharification and Fermentation (SSF) and Separated Hydrolysis and Fermentation (SHF) methods to produce ethanol. The comparison of fermentation by SHF and SSF were also evaluated.

2 Materials and Methods

2.1 Feedstock Materials

Lignocellulose biomass (*Arenga pinnata*) was taken from solid waste of the palm starch production house located in Klaten, Central Java Indonesia. Sugar palm trunk, usually brought to the plant in pieces about four feet high. Furthermore, palm trunks are milled using a milling machine to get trunks powder. To extract the palm starch from palm trunk powder by pouring the powder with water continuously and subsequent filtered to separate starch and solid waste containing cellulose. The solid waste or biomass fibre of starch-free sugar palm trunk (SFSPT) was dried in the sun to dry (+2-3 days) then it was milled to small particles by a grinding with machine and sieved to obtain a size 40 mesh and subsequent stored in a dry place prior to use. The starch-free sugar palm trunk (SFSPT) was composed of 23.96% cellulose, 26.82% hemicelluloses, and 18.57% lignin based on its dry weight [13].

2.2 Pretreatment

Starch-free sugar palm trunk (SFSPT) substrate added with alkaline solution of 1% (v/v) NH₄OH or acid solution 1% (v/v) HNO₃ at concentration of 50g/500 ml solution was pretreated in an autoclave for 30 min or 60 min at temperature 120°C. The samples were filtered with whatman filter paper to separate solids and liquids, the the pretreated solids was washed repeatedly with distilled water until pH 7 (neutral). The pretreated solid (SFSPT) was dried using an oven at a temperature of 65°C to obtain a constant weight. The pretreated solids (SFSPT) are stored for further use as raw material for hydrolysis.

2.3 Enzymatic Hydrolysis

Non treatment and The pretreated solid SFSPT (NH₄OH 1% (v/v) for 60 min) was hydrolyzed using commercial cellulase enzyme (SQzyme CSP, China). Hydrolysis was conducted in a 500 mL erlenmeyer flask containing 200 mL of 50 mM sodium acetate buffer solution and loaded with 20 g substrate, 0.5 g yeast extract, 1 g peptone. The pH was adjusted to 4.8 using hydrochloric acid. The flasks were autoclaved at 121°C for 20 min. After the flasks was cooled to 30°C, the cellulase enzyme was loading at 10 FPU/g substrate or 20 FPU/g substrate. The hydrolysis was conducted in a shaker incubator for 72 h at 150 rpm and temperature 50°C. Samples were taken for analysis of reducing sugars.

2.4 Inoculum Preparation

2.4.1 Preculture of *Aspergillus Niger*

Isolates of *Aspergillus niger* was subculture in test tubes slant containing PDA (Potato Dextrose Agar) and incubated at 30°C for 72 h. To enrich amount of cell, so must be preculture. Making pre-cultur in 100 mL consists of 1 g (NH₄)₂SO₄; 0.3 g urea; 0.3 g KH₂PO₄; 0.05 g MgSO₄·7H₂O; 0.05 g CaCl₂, Autoclave 121°C for 20 min, shaker 120 rpm, incubation time 72 h.

2. 4.2 Preculture of *Saccharomyces cerevisiae*

Isolates of *Saccharomyces cerevisiae* was subculture in 100 mL and divided into 10 reaction tubes slant containing 2 g glucose; 2 g peptone; 1 g yeast extract, and 1.5 g agar. To increase the amount of cells that must be preculture, preculture made in 100 mL containing 0.2 g (NH₄)₂HPO₄; 0.64 g urea; 0.2 g KH₂PO₄; 0.1 g MgSO₄·7H₂O. Sterilization 121°C for 20 min, shaker 120 rpm, incubation time 72 h.

2.4.3 Main culture of *Aspergillus niger*

In order to be used *Aspergillus niger* should familiarized with the addition of the substrate. Making 2 main culture in 200 mL and 20 mL plus preculture consists of 4 g substrate which has been in treatment; 2 g yeast; 4 g peptone; 2 g (NH₄)₂HPO₄; 0.6 g urea; 0.6 g

KH_2PO_4 ; 0.1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Autoclave 121°C for 20 min, main culture incubated for 72 h and 120 rpm shaker.

2.4.4 Main culture of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae start to familiarized with the addition of the substrate, making main culture in 200 mL (+ 20 mL preculture) consisting of 4 g substrate which has been in treatment; 2 g yeast; 4 g peptone; 0.4 g $(\text{NH}_4)_2\text{HPO}_4$; 1.2 g urea; 0.4 g KH_2PO_4 ; 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Autoclave 121°C for 20 min, main culture was incubation for 72 h and 120 rpm shaker.

2.5 Simultaneous Saccharification and Fermentation (SSF) And Separated Saccharification and Fermentation (SHF)

Solid substrate without pretreatment and previously pretreated with 1% (v/v) NH_4OH autoclave for 60 min at 121°C and hydrolized with 20 FPU and 20 g substrate for 72 h was fermented with *Saccharomyces cerevisiae* alone for SHF method. For SSF *Saccharomyces cerevisiae* and *Aspergillus niger* was used. Biomass 50 g in 500 mL solution, mainculture *Saccharomyces cerevisiae* added 1 mL/g and mainculture *Aspergillus niger* 1 mL/ g. Fermentation for 72 h 30°C . Shaker for 72 h, after 72 h is done, then distillation to separate the liquid and the substrate. Ethanol was obtained stored at a temperature of 20°C .

3. Result and Discussion

3.1 Effect of Acids and Alkaline pretreatments on Lignin, Hemicellulose and Cellulose

Amount of Lignocellulosic biomass is very abundant and can be converted to energy using technology [14]. Lignocellulosic biomass contains lignin which envelops cellulose, lignin will inhibit the enzyme accessibility and can decrease the rate of hydrolysis [8]. Biomass palm trunks can not be directly fermented due to very rich content of lignin, so that necessary to delignification treatment with the addition of acids and alkaline. Delignification also affected by pretreatment time and temperature. Long treatment time will effect to high cellulose [15]. Breakdown of lignin very necessary to remove the cellulose contained.

In Fig 1, can be seen initial lignin content of the raw material, after treatment with HNO_3 and NH_4OH , the lignin content in raw materials has decreased, both with pretreatment using HNO_3 and NH_4OH . The maximum decrease of lignin obtained for pretreatment 1% (v/v) HNO_3 is 8.28% for 60 min treatment and 1% (v/v) NH_4OH is 9.44% for 60 min treatment. while the hemicellulose content varies in each treatment, and the content of cellulase has increased significantly. The maximum increase in the cellulose treatment 1% (v/v) HNO_3 is 17.33% and 1% (v/v) NH_4OH treatment to a maximum is 18.56% for 60 min treatment. This indicates

the acidic and alkaline pretreatment can damage lignin [16], so the content of cellulose can be out and will be converted into sugar and then fermented using *Saccharomyces cerevisiae* to produce ethanol.

Fig. 1. Lignin after pretreatment using (A) 1% (v/v) HNO_3 for Non-impregnation; Impregnation of 30 min and 60 min. (B) 1% (v/v) NH_4OH to Non-impregnation; Impregnation 30 min and 60 min.

3.2 Relations with reduced sugar content of ethanol after fermentation

When fermented, amount of reduced sugar content greatly affect amount ethanol produced. Levels of bioethanol produced with SSF treatment process using 1% (v/v) NH_4OH produce high levels greater than the levels of ethanol using 1% (v/v) HNO_3 pretreatment SSF. Characteristic of pretreatment alkaline are able to eliminate lignin without having a great effect to other components [17]. The highest ethanol is 3.91 g/L for 60 min 1% (v/v) NH_4OH treatment and 2.73 g/L for 1% (v/v) HNO_3 treatment. Pretreatment of acids and alkaline aim to improve the yield of sugar, for the next can be converted into ethanol using *S. cerevisiae*. Pretreatment with alkaline, when compared with the acid or enzymes may increase 8 times and 23 times the yield of fermentable sugars [18]. From the literature, pretreatment on wheat straw with dilute HNO_3 gave an ethanol yield of only 95.0 g.kg^{-1} and highest glucose yield (316.7 g.kg^{-1}) [9]. Saha et al. (2013) reported that NH_4OH is the best pretreatment on pteris and could produce 0.642 mg/ml reducing sugar and 0.333 mg/L ethanol with 0.075 gm of yeast at 50 h under the conditions pH 7, 35°C , substrate diameter $45\mu\text{m}$ - $63\mu\text{m}$,

substrate loading 0.25 gm in 100 ml working volume [19].

Fig. 2. Bioethanol after fermentation (SSF 72 h) 1% (v/v) HNO₃, 1% (v/v) NH₄OH and non-impregnation; impregnation for 30 min and 60 min with 20 FPU/g substrate enzyme loading and 20 g substrate.

3.3 Correlation between reducing sugar and ethanol in SHF and SSF processes

Enzyme loading for the hydrolysis of biomass palm trunks used 10 and 20 FPU/g celluclast. Raw materials used previously have been treated by NH₄OH 60 min. As seen in fig 3, the maximum yield achieved of glucose produced by the enzyme loading 20 FPU/g celluclast and 20 g substrate is 19.223 g/L, while the glucose produced with non treatment is 1.718 g/L. Increasing the amount of celluclast and hydrolysis time will be proportional to the increase in the amount of glucose produced. Ayeni et al. [20], reported the highest reducing sugar on shea tree sawdust in 4 day hydrolysis time, 50 FPU/g dry biomass, 45°C, pH 4.8, 40 g/L substrate concentration was 482.40 mg/g dry biomass. In variations of the use of enzymes cellulase SHF process will affect the content of reducing sugar produced, can be seen in graph A, where the variation of cellulase enzymes 20 FPU/g produces (17.423 g/L for 72 h), more reduced sugar content than 10 FPU/g variation (11.423 g/L for 72 h), while the non treatment produced 1.718 g/L for 72 h. To effect substrate amount of raw materials also affect the content of reducing sugar obtained when the hydrolysis process is carried out on raw materials. In the graph (B), shown by the increasing amount of substrate then reducing sugar obtained is also greater. The variation of 20 g substrate could produced 19.233 g/L than 10 g substrate was 17.423 g/L and non treatment was obtained 1.718 g/L for 72 h. In this stage of saccharification enzyme, modified cellulose into cellobiose and further into simple sugars such as glucose, saccharification process using enzymes celluclast, enzyme hydrolysis process takes place at pH 4.8 and the temperature of 45-50°C [21]. From Ganguly et al. [22], and Aveni et al. [20], the addition of biomass loading and hydrolysis time influences increase in reducing sugar, the maximum substrate loading to produces highest reducing sugar is 7% for 27.78 mg/g while the 40 g/L substrate

concentration could produce maximum sugar yield for 42.4%.

Fig. 3. (A) Graph reduction sugar versus time with 10 g substrate (1% (v/v) NH₄OH treatment for 60 min), the enzyme loading 10 FPU/g substrate and 20 FPU/g substrate, while (B) The relationship of reducing sugar versus time with substrate loading 10 g and 20 g (1% (v/v) NH₄OH treatment for 60 min, 20 FPU/g substrate enzyme loading).

In the use of this type of fermentation process between SHF and SSF, have some differences apart in terms of stages and the time required in the process. SHF process to produce bioethanol is higher, and less energy is required and the production cost can be minimized. SHF process for treatment and nontreatment used 20 FPU/g substrate enzyme loading and 20 g substrate (1% (v/v) NH₄OH impregnation for 60 min).

As It is presented by fig 5, the concentration of bioethanol using the process of SHF was higher as compared to one obtained by using the SSF. Fig 5 also shows that from the graph (A) and (B) it appears that the fermentation time for 72 h, concentrations of bioethanol on the two types of fermentation process has a very significant difference in the SHF obtained bioethanol concentration of 8.11 g/L, while the non treatment was obtained 0.57 g/L. The SSF process derived bioethanol concentration of 3.95 g/L, while the non treatment was obtained 0.57 g/L.

Fig. 4. (A) graph ethanol on fermentation time for the SHF with 1% (v/v) NH_4OH for 60 min, 20 FPU/g substrate cellulase and 20 g substrate biomass loading, (B) graph ethanol on the SSF fermentation process with 1% (v/v) NH_4OH for 60 min, 20 FPU/g substrate cellulase and 20 g substrate biomass loading.

Fig. 5. Graph ethanol GC

GC graph showing measurements of bioethanol for the SHF and SSF.

4. Conclusion

Lignocellulose from industrial waste palm flour, SFSPT, can produce an ethanol with several processes, pretreatment, saccharification and fermentation. Pretreatment SFSPT with NH_4OH was more effective than H_3PO_4 for 60 min impregnation. The more cellulase and substrate loading has a greater reducing sugar (17.3 g/L for 20 FPU/g and 19.2 g/L for 20 g substrate) for 72 hours saccharification. SHF process significantly produce higher ethanol than SSF process for 72 hours (8.11 g/L for SHF and 3.95 g/L for SSF) for 72 h fermentation.

Acknowledgements

The authors would like to thank the Directorate of Research and Community Service, Ministry of Education, Technology and Higher Education Indonesia (Kemenristekdikti) for funding this Research (PB Kompetensi 2017).

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