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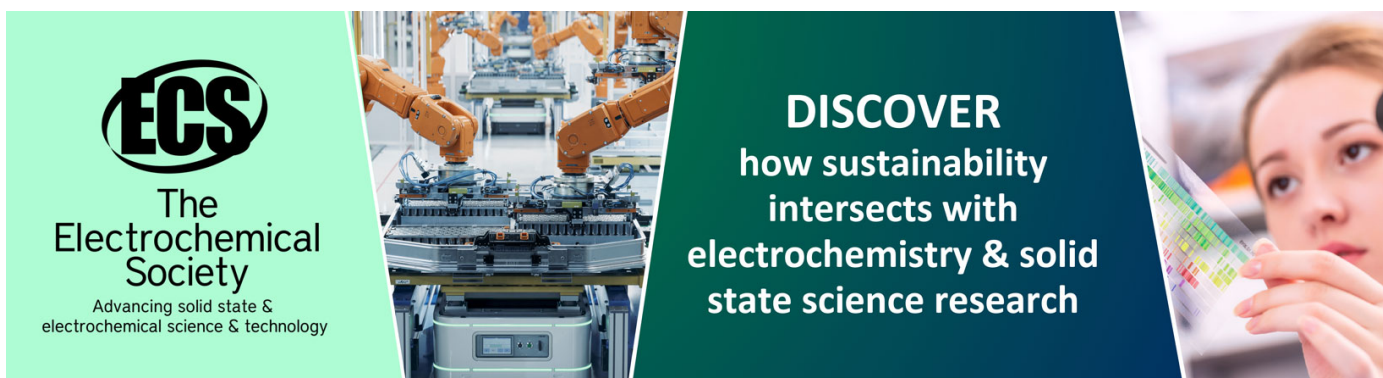
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Bioethanol Production From Banana Stem By Using Simultaneous Saccharification and Fermentation (SSF)

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Abstract. The rapid growth and development of industries in the world result in a greater energy needs. Some studies show that ethanol can be used as an alternative energy. However, bioethanol production from food raw materials such as sugar and starch has drawback that cause the food crisis. This aim of this study was to convert banana stem into bioethanol. Banana stem contained of 44.6% cellulose, 36.0% hemicellulose and 19.4% lignin. After banana stems were pretreated with acid (H₂SO₄) and alkaline (NaOH) at a concentration of 2% w/v at 121 °C for 30 minutes, then subsequently the simultaneous saccharification and fermentation (SSF) were carried out by using mixed cultures of *Aspergillus niger*, *Trichoderma reesei* and *Zymomonas mobilis* at various enzymes ratios of (1:1:1), (1:2:1), (1:2:2), (1:1:2) and various pH (4, 5 and 6) with SSF time for 144 hours and temperature of 30°C. The results show that acid pretreatment showed better results than the alkali pretreatment. After acid pretreatment and alkali pretreatment, lignin content of pretreated banana stem reduced to 15.92% and 16.34%, respectively, cellulose increased to 52.11% and 50.6% respectively, hemicellulose reduced to 28.45% and 28.83%, respectively. The SSF showed that pH 5 gave the highest bioethanol. The highest concentration of bioethanol (8.51 g/L) was achieved at the SSF process at pH 5 with a ratio *Aspergillus niger*, *Trichoderma reesei* and *Zymomonas mobilis* enzymes of (1:1:2).

1. Introduction

Energy is one of the most basic need in human life. The growing of transportation sector has contributed the rise of petroleum consumption that accounts for 60% of fossil fuels consumption which also caused the pollution generation to the environment. [1]. However, the limitation the fossil oil feedstock and the negative impact of the fossil oil environmentally, ecologically or economically have led to a search for renewable sources of energy globally in recent years [2]. Several studies have emphasis on the use of forestry and agricultural residues for the production of lignocellulosic material. Banana pseudo-stem is one of lignocellulosic material which has higher holocellulose content than straw and lower lignin content than wood and straw. Banana pseudo-stem has potential as bioethanol feedstocks because of its acceptable content of cellulose and low lignin content. Moreover, in Indonesia, banana pseudo-stems were found abundantly as natural resource like other subtropical and tropical regions [3]. In Indonesia, the planting area of banana is large which reached 88.728 ha in 2015 [4]. The banana pseudo-stem has the high cellulosic content 42.2 - 63 % that can be utilized for the production of fermentable sugars for bioethanol production [6].

Prior to the conversion of cellulose and hemicelluloses into monomeric sugars and the sugar into ethanol using a microorganism, the pretreatment step is required. The goal of the pretreatment is to remove the hemicellulose and lignin barrier around the cellulose fibres and to open the crystalline



structure of the cellulose, resulting the cellulosic fibres more accessible to enzymatic attack[7]. Cellulose, the major component of pretreated biomass is then undergo enzymatically saccharification using cellulolytic enzymes. The cellulose saccharification is further undergo enzymatic fermentation to produce ethanol. Cellulose saccharification and the hexose fermentation can be carried out either separately (SHF process) or simultaneously (SSF process). The simultaneous saccharification and fermentation processes combines enzymatic saccharification of cellulose with simultaneous fermentation of its main derived sugar (glucose) to ethanol [8]. For the complete hydrolysis of cellulose, cellulases enzymes is employed to breakdown of cellulose to glucose. *T. reesei* and *Aspergillus* strains are known to have ability to produce β -glucosidase, the cellulolytic enzyme. The β -glucosidase production from mixed culture of *A. Niger* BKMFM 1305 with *T. reesei* RUT C30 has been examined using waste paper lignocellulosic material as carbon source [9]. *Z. mobilis* was reported had good results for ethanol synthesis in varied environmental conditions, including temperature and phase of growth, concentration or type of carbon source [10]. The aims of the current study were to investigate the effect of acidic (H_2SO_4) or alkaline (NaOH) agents on banana stem pretreatment and ethanol concentration produced by simultaneous saccharification and fermentation (SSF). The effect of mixed culture ratios of *A. niger*, *T. reesei* and *Z. Mobilis* enzymes in SSF process on ethanol produced from acid pretreated banana stem were also studied.

2. Material and method

2.1. Preparation of raw materials and Pretreatment

Banana stem was obtained from local farmer in Central Java. The banana stems were chopped to small pieces approximately 3cm x 3 cm x 3cm x 0.5 cm and dried until the water content less than 10%. The dried banana stem was milled and sieved to 40 mesh size. Material was placed in sealed container and stored in a dry place at room temperature. The acid (H_2SO_4) and alkaline (NaOH) treatments were carried out by an autoclaving the 10% (w/v) banana stem dried solution at 121°C for 30 minutes using 2% (w/v) H_2SO_4 (p.a) or NaOH (p.a) solution. After pretreatment, the slurry was filtered to obtain the solid of pretreated banana stem contained a lignocellulosic material. Then the solid was repeated washed with distilled water until pH 7 and the solid was dried at 65°C until its constant weight.

2.2. Preparation of enzymes

Aspergillus niger and *Trichoderma reesei*, *Zymomonas mobilis* microbes were used in this study. Pure *A. Niger* 6018 and *T. reesei* 6012 were cultured on the Potato Dextrose Agar (PDA) at temperature of 30°C. Pure cultures of *Z. mobilis*-0056 ENCC was incubated on RM medium containing nutrients: 20 g/L glucose, 10 g/L yeast extract, 2 g/L KH_2PO_4 , 15 g/L agar. The culture time for *A. niger* 6018 and *T. reesei* 6012 was 3 days and 7 days, respectively then stored at 4°C. For enzymes production, pre-culture and main-culture of *A. niger* 6018 and *T. reesei* 6012 were prepared, respectively. The pre-culture medium (10 ml) for both *A. niger* 6018 and *T. reesei* 6012 contained similar composition of nutrients: 3 g/L urea, 10 g/L $(\text{NH}_4)_2\text{SO}_4$, 3 g/L KH_2PO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/L CaCl_2 . The pre-culture mediums were sterilized by autoclaving at 121°C and a pressure of 0.18 MPa for 20 minutes. The pre-cultures of *Z. mobilis* was incubated for 72 hours at 30°C using a shaker at speed of 120 rpm. The pre-culture media of *Z. mobilis* (10 ml) contains: 100 g/L glucose, 10 g/L yeast extract, 1 g/L $(\text{NH}_4)_2\text{SO}_4$, 1 g/L KH_2PO_4 , and 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was sterilized at a temperature of 121°C and a pressure of 0.18 MPa for 20 minutes. The main culture (100 ml) of both *A. niger* 6018 and *T. reesei* 6012 had similar composition of nutrients with pre-culture and it was adding with pretreated banana stem (dry) substrat: 60 g/L substrat, 3 g/L of urea, 10 g/L $(\text{NH}_4)_2\text{SO}_4$, 3 g/L KH_2PO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/L CaCl_2 . The main culture (100 mL) medium of *Z. mobilis* was containing: 60 g/L substrate of pretreated banana stem (dry), 10 g/L yeast extract, 1 g/L $(\text{NH}_4)_2\text{SO}_4$, 1 g/L KH_2PO_4 , and 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Prior to incubation, the main culture mediums were autoclaved at 121°C and a pressure of 0.18 MPa for 20 minutes. The main cultures of *A. niger* 6018, *T. reesei*, *Z. mobilis* were incubated for 72 hours at 30°C with shaker speed of 120 rpm.

2.3. Simultaneous Saccharification and Fermentation (SSF)

The Simultaneous Saccharification and Fermentation (SSF) of banana stem pretreated was carried out in 600 ml fermentation medium with a pretreated banana stem concentration of 10 % (w/v). A 0.05 M

sodium citrate buffer solution was used to maintain the pH (4; 5; 6). The pretreatments were carried out using autoclave at 121°C and pressure 0.18 MPa for 20 minutes. The sterilized SSF samples were inoculated with mixed cultures of *A. niger*, *T. reesei*, *Z. mobilis* using a total loading of enzymes volume was 300 ml. The effect of microbes ratios of *A. niger*: *T. reesei*: *Z. mobilis* were (1: 1: 1), (1: 2: 1), (1: 2: 2); (1: 1: 2) in SSF were carried out using a substrate of acid pretreated banana steam. SSF was conducted for 6 days at a temperature of 30°C with a shaker speed of 150 rpm.

2.4. Analysis of Lignin

The content of lignin in biomass was determined by using the method of "Klason lignin" (TAPPI T222 om-88) [11]. 1 gram of biomass was hydrolyzed using a 72% H₂SO₄ solution of 15 cm³ for 2 hours at room temperature. Then the sample was diluted until the concentration of sulfuric acid up to 3 wt% by adding water as much as 560 cm³ water. Samples boiled for 4 hours. Then the sample was separated between solid and liquid by filtration. Lignin was washed using hot water until it reaches a neutral pH. The content of lignin was obtained from the amount of dried residue.

2.5. Analysis of Holocellulose

Milled wood samples (4 g) was added with acid solution (Sodium acetate solution 160 cm³) at 75 °C for 5 hours. Then sodium chlorite solution (4 cm³) was added every hour for 4 hours. The solution was then cooled and the residue was filtered. The residue was washed with the first liquid (1 dm³) and with acetone (15 cm³). The residue was finally dried at room temperature. For the determination of holocellulose aliquots were dried at 105 °C and it was weighed.

2.6. Analysis of Cellulose

5 g holocellulose was added 100 cm³ of NaOH solution (17.5 wt%) at room temperature for 30 minutes. The residue was filtered and washed clean twice with water (with 200 cm³) and then filtered again. Then, a acetic acid solution (10% by weight of 15 cm³) was added to hydrolyze cellulose and degraded hemicellulose. Hemicellulose is more easily hydrolyzed than cellulose. The residue was filtered and washed with hot water (500 cm³), and dried at 105 °C. Cellulose was counted ultimately determined by gravimetry.

2.7. Analysis of Bioethanol

The ethanol was obtained by distillation of fermentation broth at 78 °C until the impurities removed. Ethanol concentration was analyzed by Gas Chromatography (GC Shimadzu GC-8A, Japan), equipped with a Flame Ionisation Detector and a polar factor four capillary column (GP 106 SP 1200). Detector temperature was set at a temperature of 250°C. The carrier gas N₂ was kept at 40 ml/min. Velocity gases flow rate of H₂ was set at 40 ml/min and O₂ at 300 ml/min. Each sample was analyzed in duplicate.

3. Results and discussion

Banana stem was analyzed its chemical composition as shown in Table 1. The result shows that the lignin, cellulose and hemicellulose contents of dry banana steam are 19.39%, 47.74%, 36.01%, respectively.

Table 1. Composition of Banana Stem.

Component	Percentage
Lignin	19.4%
Cellulose	44.6%
Hemicellulose	36.0%

This result seem to be suitable with the previous studies reported in the literature. It has been reported that banana stem contains considerable amount of cellulose 47%, hemicellulose 13%, lignin 13 %, ash 8.2% and extractives 3.05% [5]. Due to its high lignocellulosic material contents therefore banana stems has been considered as raw material for bioethanol. Cellulose and hemicellulose content in

banana stem can be converted to be fermentable sugar by enzymatic hydrolysis. However lignin content was a barrier for the successful of hydrolysis. Pretreatment was used to remove the lignin content.

3.1. The effect of delignification

In this study, the pretreatment process was carried out using acid and alkali pretreatment in an autoclave at 121°C for 30 minutes with 2% H₂SO₄ and 2% NaOH. Based on the composition of pretreated banana stem in figure 1, the lignin and hemicellulose of pretreated banana stem was significantly reduced as compared to untreated banana stem. The lignin content of pretreated banana stem using acid and alkali solution were 15.92% and 16.34%, respectively. Figure 1 shows the slightly different of lignin for the banana stem pretreated using autoclaving of 2%(w/v) acid and alkali solution. The result showed that all pretreatments were successful in degrading the lignin of banana stem. The results show that cellulose contents of the treated banana stem was increased as compared to untreated stem.

The acid pretreatment has a higher efficiency in degrading lignin than alkaline pretreatment which produced the cellulose of 52.11% and 50.6%, respectively and the hemicellulose of 28.45% and 28.83%, respectively. This results are in accordance with results found in the literature [7], [12],[13]. Pretreatment of cactus pear biomass using hydrogen peroxide, NaOH and H₂SO₄-alkaline delignification at 121 °C for 30 min was reported. The pretreatment resulted an increased of cellulose content, however an increase in lignin and hemicellulose content of treated cactus pear biomass was found using all chemical treatments. The greater delignification values were obtained with alkaline peroxide pretreatment[7]. Another work studied on the Kans (*S. Spontaneum*) grass pretreated with 2% sulfuric acid for 90 min at 120 °C followed by saccharification of the pretreated kans grass. The treated biomass with dilute acid (2% H₂SO₄, 90 min, and 120 °C) removed 27.63% of the total lignin along with 77.05% of the total hemicellulose and 19.7% of total cellulose content [12]. In this work the simultaneous saccharification and fermentation (SSF) was employed where the hydrolysis and fermentation process taken place in one process simultaneously. In this work the SSF process was using mixed cultures of *Aspergillus niger*, *Trichoderma Reesei*, and *Zymomonas Mobilis* with the microbes ratios 1:1:1. The SSF of treated and untreated banana stem lignocellulose to ethanol production was studied at different pH (pH 4 and 5) and temperature of 30 °C and the result was shown in figure 2.

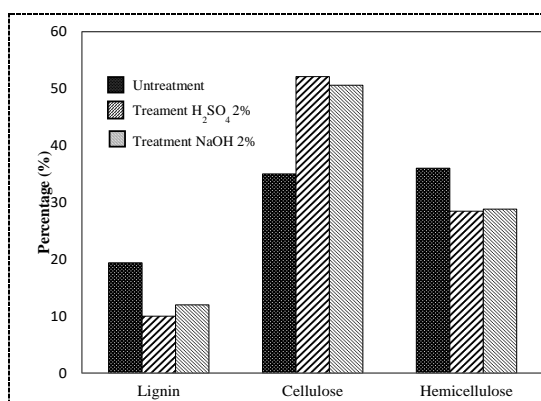


Figure 1. The results of the analysis of lignin, cellulose and hemicellulose before and after pretreatment

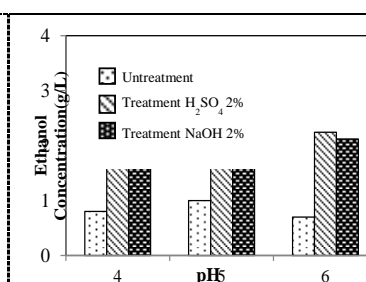


Figure 2. Effect of acid and alkaline pretreatment and pH on ethanol production

The highest ethanol was observed at pH 5 (3.493 g/L) for the treated and untreated banana stem raw materials. While at pH 4 and 6, a decreased in ethanol was observed. Previous studied reported the effect of initial pH value of food waste hydrolysate fermentation on ethanol production by *Z.mobilis* at pH range 3.5-6. The highest ethanol was found at pH 4 a decreased in ethanol was observed when pH

increased up to 6. This was due to food waste raw material contained lactic acid bacteria that produced high concentration of contaminants such as acetic acid and lactic acid which inhibited ethanol formation [14]. Figure 2 shows the acid and alkaline pretreatment banana stem resulted the higher ethanol concentration than untreated banana stem. Previous work reported SSF of delignification of bagasse steam pretreated sweet sorghum (SSB) as a substrate indicated that delignification of SSB could increase ethanol concentration obviously [15]. Steam-pretreatments were employed for delignification of sweet sorghum bagasse (SSB) and Douglas-fir (DF). In SSF, the delignified SSB and DF showed an increase final ethanol concentration to 2.76 g/L (SSB) and 3.43 g/L (DF) with an increase of 26% and 157% compared with controls [15]. A co-fermentation process of hydrothermally pretreated wheat straw as substrate was studied in which *F. oxysporum* culture (submerged or solid-state) was added at the SSF stage along with *S. cerevisiae*. The addition of solid *F. oxysporum* culture increased ethanol production by 19%, leading to a final ethanol concentration of 58 g L⁻¹ [16].

3.2. The effect of mixed culture ratios in Simultaneous Saccharification and Fermentation of acid pretreated banana stem

Simultaneous saccharification and fermentation of acid pretreated banana stem was studied at temperature 30°C using pH 4 and 5. The influence of mixed culture ratios of *Aspergillus niger*, *Trichoderma Reseei*, and *Zymomnas Mobilis* (1:1:1; 1:2:1, 1:2:2, 1:1:2) were studied. The lowest ethanol was observed at ratios microbes A. Niger : T. Reesei : Z. Mobilis 1: 1: 1. Figure 3 shows the highest ethanol concentration at pH 5 (8.51 g/L) was observed at ratio of microbial A. Niger : T. Reesei : Z. Mobilis (1: 1: 2). The higher ethanol concentrations were obtained on pH 5 than that on pH 4. The ethanol concentration for the ratios of A. Niger : T. Reesei : Z. Mobilis of (1:2:1), (1:2:2), and (1:1:2) at pH 4 were 2.78 g/L, 6.25 g/L, and 6.67 g/L while at pH 5 were 3.93 g/L, 7.41 g/L, 8.51 g/L, respectively. Based on this result mixed cultures of A. Niger : T. Reesei : Z. Mobilis produced higher concentration of ethanol from pretreated banana stem. This research is consistent with the results of research conducted by [16] that the higher the levels of concentration Z. mobilis ethanol produced will be higher levels of ethanol. Mixed cultures of A. Niger and T. Reesei was examined for the production of enzyme complex of β -glucosidase and cellulase enzymes for the conversion of lignocellulosic material to ethanol. The higher β -glucosidase activity was reported for the cocultures as compare to monoculture A. Niger and monoculture T. Reesei [9]. The simultaneous saccharification and fermentation to ethanol of microcrystalline cellulose using co-cultures of *Klebsiella oxytoca* and strains of *Saccharomyces pastorianus*, *Kluyveromyces marxianus* and *Z. mobilis* showed that more ethanol and less time fermentation was observed for co-cultures than pure cultures of the above organisms. This was due to the higher thermotolerance of *Klebsiella oxytoca*, which permitted the use of a temperature (37 °C) more favourable to cellulase activity in the yeast SSFs [17].

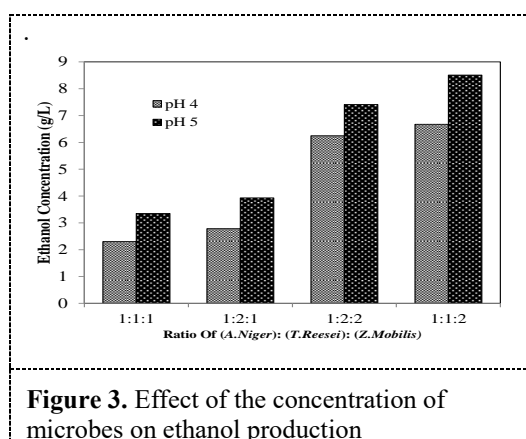


Figure 3. Effect of the concentration of microbes on ethanol production

4. Conclusion

Simultaneous saccharification and fermentation (SSF) of acid pretreated banana stem was carried out using different ratios of mixed cultures of *A. niger*, *T. reesei* and *Z. Mobilis* enzymes. At the culture ratios *A. Niger*: *T. Reesei* : *Z. mobilis* of 1:1:2 yielded the highest ethanol concentration. Prior to SSF, banana stem pretreatments were carried out using acid (2 % w/v H₂SO₄) and alkaline (2% w/v NaOH) solution and heated in autoclaved 121°C for 30 minutes. The highest ethanol was obtained at pH 5 as compared to pH 4 and pH 6. The highest ethanol (8.51 g/L) was obtained at SSF condition of pH 5 with a ratio mixed cultures of 1:1:2.

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