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Enzymatic hydrolysis of cellulose banana stem (alkaline microwave-assisted pre-treatment)

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Abstract
The banana stem waste holds immense promise as a readily available and abundant source of lignocellulosic biomass, making it a compelling alternative for biofuel and biochemical applications. Therefore, this study investigates the impact of both time and substrate loading on the enzymatic hydrolysis of banana stem cellulose that has undergone alkaline microwave-assisted pre-treatment. The pre-treatment method involves subjecting the biomass to 5% KOH at 80 °C, 1 atm for 30 min, followed by microwave exposure at 300 W for 5 min, which enhances cellulose accessibility. Enzymatic hydrolysis experiments were carried out utilizing cellulase enzymes derived from Aspergillus niger, with variations in hydrolysis times (ranging from 5 to 45 h) and enzyme-to-substrate ratios (ranging from 1:1 to 1:10). The results of this investigation revealed a substantial improvement in hydrolysis efficiency, owing to the synergistic effects of alkaline microwave-assisted pre-treatment, signifying enhanced cellulose accessibility due to the removal of lignin. Notably, the highest concentration of reducing sugars (1.3 mg mL^{-1}) was achieved at a substrate-to-enzyme ratio of 1:1 and a hydrolysis duration of 45 h. These findings provide valuable insights into converting lignocellulosic biomass, emphasizing the potential of integrated pre-treatment strategies for sustainable biorefinery applications. This research advances our understanding of lignocellulosic biomass utilization, offering a promising avenue for biofuel and biochemical production from banana stem waste.

Keywords:
Alkaline microwave-assisted pre-treatment:
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Enzymatic hydrolysis;
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INTRODUCTION

There was a 1.9% rise in the intake of renewable fuel sources across various industries between 2019 and 2020 [1]. According to Cheng and Brewer [2], biofuels, including bioethanol, are anticipated to account for 50% of the overall biofuel production by the year 2050. Bioethanol, a renewable alternative to fossil fuels, is gaining attention as a key component in addressing environmental concerns and reducing reliance on non-renewable resources [3]. Bioethanol production from lignocellulosic-based renewable resources is being explored as a competitive and eco-friendly solution [4]. Second-generation bioethanol, derived from lignocellulose such as forest waste and crop straw, shows promise as an ideal biofuel. One abundant source of lignocellulosic biomass is banana stem waste, which, according to 2021 statistics from South Sumatra, amounts to approximately 9.7 million tons due to suboptimal utilization [5].

Cellulose is a natural product represented by the formula $(C_6H_{10}O_5)_n$. The high cellulose content of banana stems holds great potential for bioethanol production. The cellulose percentage of banana stems varies in the different studies. In one study by Iliyin et al. [6], the

highest cellulose content of 86.43% was obtained using a microwave at 800 W for 14 min. Another study by Y. Nurhaliza et al. reported that banana stem fiber has a high cellulose content of 46.3% [8]. A study by Taslima Ferdous et al. found that banana stem has a good amount of α-cellulose ranging from 40-45% [9].

Lignocellulosic structure on biomass necessitates pre-treatment to enhance cellulose accessibility and improve cellulose-to-sugar conversion during hydrolysis by removing lignin, modifying the lignin structure, reducing cellulose crystallinity, and increasing the porosity and surface area of the biomass [10]. Alkaline pre-treatment of lignocellulosic biomass has been studied as an effective method for biomass conversion. Traditional physical and chemical pre-treatment methods are unsustainable and have negative environmental impacts. However, alkaline pre-treatment using KOH can overcome these problems [11]. KOH treatment has been shown to effectively reduce lignin content in lignocellulosic biomass and increase the overall sugar yields [12]. Microwave radiation further complements the pre-treatment by applying heat selectively to the structure, effectively breaking down lignin structures [13][14].

Cellulose enzymes from *Aspergillus niger* are suitable for enzymatic hydrolysis of biomass lignocellulosic due to their high activity and stability [15][16]. *Aspergillus niger* is known to produce cellulases, including endoglucanases, exoglucanases, and β-glucosidases, which are essential for the breakdown of cellulose into glucose [17]. Enzymatic hydrolysis, particularly using *Aspergillus niger*, is chosen for its environmental friendliness and efficiency [18][19].

Enzymatic hydrolysis of lignocellulosic biomass is a complex process influenced by various factors. The recalcitrance of different parameters, including enzyme and substrate properties, affects enzyme adsorption and hydrolysis. Previous studies on cellulose extraction from banana stems used various methods [20][21], but limited research has combined alkaline microwave-assisted pre-treatment with enzymatic hydrolysis using *Aspergillus niger*. This study addresses this gap and aims to contribute to sustainable and renewable fuel sources by optimizing banana stem waste processing. The proposed model to describe the bioethanol production from banana stems is described in Figure 1.

MATERIAL AND METHOD

Material

Banana stem was taken from Pulomas Subdistrict, Pulo Gadung District, East Jakarta, Special Capital Region of Jakarta. The chemical and reagents for this study include KOH, $(NH_4)_2SO_4$, KH_2PO_4, Sucrose, HCl, Urea, $MgSO_4 \cdot 7H_2O$, Biuret Reagent (NH_2CONH_2), H_2SO_4 72%, Citric Acid, Trisodium citrate, CMC Substrate, DNS Reagent (3.5-Dinitrosalicylic acid), NaOH, Rochelete Salt, and Glucose were purchased from Merck. *Aspergillus niger* was acquired from Indilab, Indonesia.

Banana Stem Preparation

Figure 2 illustrates banana stem pieces that stems were cleaned, cut into 2x2 cm pieces, and dried in an oven for 4 days at a 50°C. After drying, the banana stems were reduced in size and finely ground using a blender, followed by sieving through a 40-mesh sieve.

Alkaline Microwave-assisted Pre-Treatment

The prepared banana stem was put into Erlenmeyer, then a 5% solution of KOH (1:10) was inserted. It was warmed at 80°C, 1 atm (30 min, 150 RPM), followed by microwave heating at 300 Hz in 10 min. The mixture, with a pH of 13, was filtered with a vacuum filter and neutralized with deionized water to reach a pH of 7 (neutral).

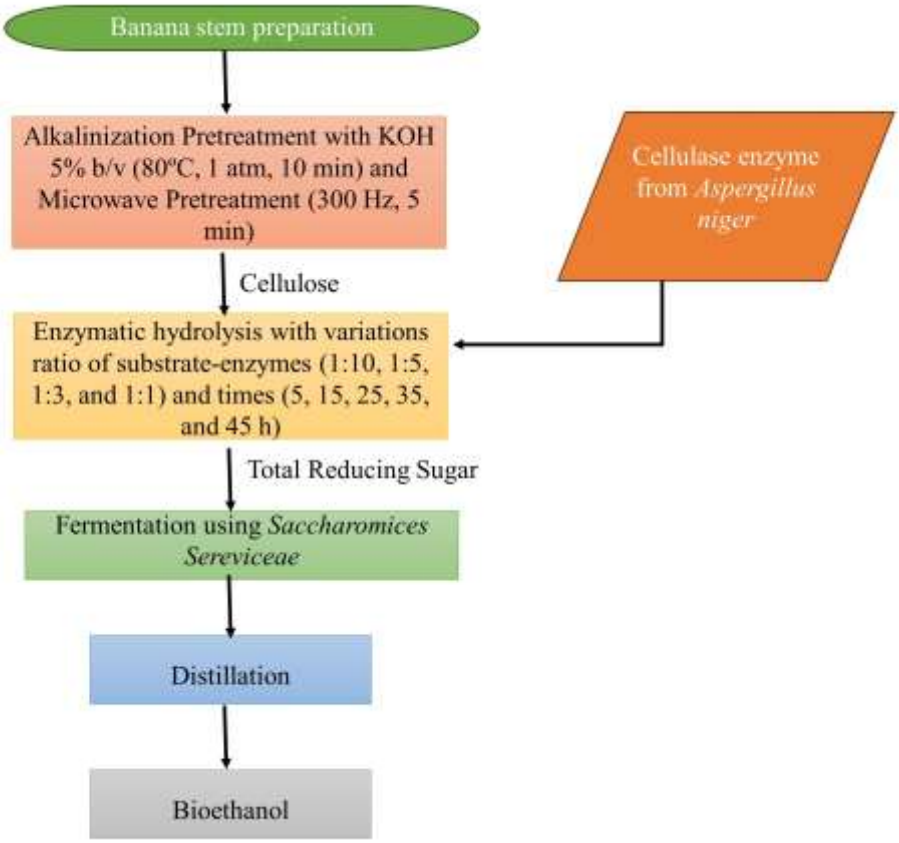

Figure 1. Flow chart of the bioethanol production from banana stem

Figure 2. Banana Stem Preparation

Then, the sludge was dehydrated in an oven at 50°C till it reached a persistent weight. Subsequently, the solid phase is used for the enzymatic hydrolysis process and analyzed for cellulose, hemicellulose, and lignin levels using the Chesson method.

Culture Inoculation

A culture of *Aspergillus niger* was established in a zig-zag pattern on Potato Dextrose Agar (PDA) media under strict aseptic conditions, as shown in Figure 3. The culture was then incubated for 120 h at a controlled temperature of around 30°C.

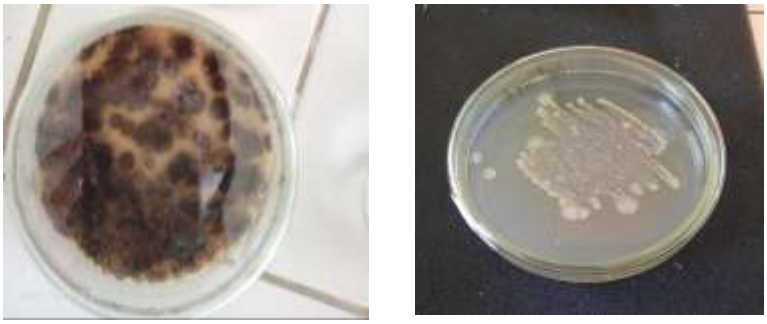

Figure 3. The culture of *Aspergillus niger* in a zig-zag pattern on Potato Dextrose Agar (PDA) media.

Inoculum Preparation

A 100 mL fluid medium was prepared, comprising 12.5% sucrose, 0.25% $(NH_4)_2SO_4$, and 0.2% KH_2PO_4, with the liquid pH adjusted to 3 using HCl. The equipment and liquid medium were neutered using an autoclave at 121°C for 15 min. Using an inoculating loop, a fungal inoculum (fluid culture) was created by aseptically transferring fungal isolates from PDA media into the liquid medium. It was then incubated for 24 h at approximately 30°C in an incubator.

Cellulase Enzyme from *Aspergillus niger* Preparation

First, the enzyme growth medium was prepared by 20 pre-treated banana stems with added nutrients in the form of 0.03 g of urea, 0.005 g of $MgSO_4 \cdot 7H_2O$, and 0.0023 g of KH_2PO_4, followed with added 80 mL of demineralized water (pH of 5). Both the equipment and enzyme growth medium were neutered with an autoclave. Secondly, the enzyme growth media was injected with 10 mL of the formulated inoculant and was nurtured at room temperature for 96 h. The fermentation liquid was filtered using 100 mL of demineralized water and mixed in a rotary shaker at 150 rpm for 1 h. After that, it was filtered with a centrifuge at 4000 RPM for 30 min at 4 °C. The crude enzyme obtained as a liquid phase is used for the enzymatic hydrolysis process. Cellulase enzyme activity, both endoglucanase and exoglucanase, is analyzed using the DNS method. While the protein content of the enzyme is determined using the Biuret method.

Enzymatic Hydrolysis

All equipment was sterilized using an autoclave at 121°C for 60 min. The crude enzyme from *Aspergillus niger* and banana stem were placed in each Erlenmeyer with different ratios of the enzyme to banana stem, specifically 1:10, 1:5, 1:3, and 1:1 (1:10 signifying 6 mL of enzyme for every 60 g of pre-treated dry biomass). The ratio of banana stem to deionized used is 1:10 (w/v) at pH 5, adjusted using sodium citrate buffer. The samples were heated at 50°C, stirred at 200 RPM, and varied the saccharification time of 5, 15, 25, 35, and 45 h, respectively. After hydrolysis, the filtrate was cooled and the glucose level was determined by using the DNS method and a UV-Vis Spectrophotometer.

RESULTS AND DISCUSSION

Effect of Pre-treatment Process on Banana Stems

The banana stems underwent a delignification process as a pre-treatment step to reduce the lignin content in the banana stems, thus making cellulose more accessible to enzymes during the hydrolysis process. The Chesson technique examined the composition of banana

stems before and after pre-treatment to determine the lignin, cellulose, hemicellulose, High Water Soluble (HWS), and ash content. The composition data for banana stems before and during delignification is shown in Table 1.

Cellulose remains relatively stable during the 5% KOH (alkaline) pre-treatment, albeit with a slight increase of 5.97%. Alkali acts as a chemical bond-breaking agent in hemicellulose and lignin, potentially altering their structures and rendering them more water-soluble. Consequently, a partial breakdown of lignin and hemicellulose is observed, indicated by a 4.73% reduction in lignin and a 1.95% reduction in hemicellulose. High Water Soluble (HWS) content increases by 1.63%, possibly due to the release of hemicellulose and lignin into the aqueous phase during alkali treatment.

Microwave pre-treatment contributes to further reductions in hemicellulose and lignin. Microwave heating can cause structural damage to cell walls and accelerate chemical reactions associated with the degradation of hemicellulose and lignin [22]. The increase in cellulose during microwave treatment is more pronounced than during alkali treatment, with a notable increase of 11.87%. Microwave treatment also has a greater impact on lignin than alkali, resulting in an 8.7% reduction. Lignin experiences a significant decrease during microwave treatment, as microwave heating accelerates chemical reactions that disrupt the lignin structure. High Water Soluble (HWS) content remains relatively stable during microwave treatment, with a minimal 0.47% reduction, suggesting that microwave treatment may not significantly affect the solubility of lignin in water. Meanwhile, ash content remains low and stable throughout both stages of treatment, as these treatments do not significantly impact the ash content in the sample.

Overall, 5% KOH and microwave treatments affect the chemical composition of banana stems. Cellulose composition increases significantly after both stages of treatment, indicating that the 5% KOH and microwave processes reduced other components such as hemicellulose and lignin, thus enhancing the proportion of cellulose in the sample. Lignin experiences a drastic reduction after both stages of treatment, demonstrating the effectiveness of 5% KOH and microwave treatments in removing lignin from banana stems. Hemicellulose also decreases after both stages of treatment, indicating significant hemicellulose degradation.

The composition of High Water Soluble (HWS) remains relatively stable after both stages of treatment, with minimal changes, suggesting that these treatments do not significantly affect lignin solubility in water. Meanwhile, ash composition remains low and stable throughout both stages of treatment, indicating that these treatments do not substantially impact the ash content in the sample. Thus, the pre-treatment results indicate that 5% KOH and microwave treatments can be used to reduce lignin and hemicellulose in banana stems, increase the proportion of cellulose, and maintain HWS composition in banana stems.

Table 1. Configuration of Banana Stems before and After Pre-Treatment

Composition	Percentage		
	Before Treatment (%)	After KOH Treatment (%)	After Microwave Treatment (%)
Cellulose	59.72	65.68	77.55
Hemicellulose	13.10	11.16	8.56
Lignin	14.89	10.16	1.46
HWS	11.22	12.85	12.38
Ash	1.07	0.15	0.05

Cellulase Enzyme Characterization

The cellulase enzyme was produced through a 96 h fermentation process by *Aspergillus niger* isolate. The cellulase enzyme system comprises three types, namely endo-β-glucanase, exo-β-glucanase, and β-glucosidase, working together to hydrolyze cellulose into reducing sugars, particularly glucose, as the end product. Enzyme activity was measured in U/mL, indicating how much enzyme is required to hydrolyze 1 micromole of cellulose into reducing sugars per minute.

Cellulase activity analysis was conducted using two main methods: the CMCcase method and total enzyme activity. The CMCcase method was employed to identify the activity of endo-β-glucanase in randomly cleaving cellulose chains. The products of this cleavage were subsequently acted upon by exo-β-glucanase to produce cellobiose, which was then hydrolyzed into glucose. Meanwhile, analysis with the Whatman No. 1 filter paper substrate was utilized to measure the activity of exo-β-glucanase in glucose formation.

Cellulase activity analysis using the Dinitrosalicylic Acid (DNS) technique was carried out to determine the concentration of reducing sugars produced during the reaction. Reducing sugar groups reacted with DNS to yield an orange color, which was measurable using a spectrophotometer. In addition to enzyme activity analysis, this research also measured the level of soluble protein in the cellulase enzyme. This was crucial since the enzyme used was a crude extract. The Bradford method was used to determine the total protein concentration in the enzyme solution.

The analysis results demonstrated the cellulase enzyme's capability to hydrolyze cellulose into reducing sugars, with the results measured colorimetrically using a spectrophotometer, as presented in Table 2. The obtained protein concentration was 85.9 µg mL^{-1} from an absorbance of 0.485, with a glucose content of 0.086 mg mL^{-1} from the analysis result.

Different methods used to determine the activity of cellulase can yield different results. The activity of crude cellulase enzymes produced from any agricultural waste is influenced by various factors, including the cellulose content in the material, type of substrate, media, substrate concentration, pH, and temperature [15]. The small cellulase enzyme activity test results may be caused by the low concentrations of chemicals and small-volume samples with minimal sample preparation compared to a previous study [23].

Influence of Saccharification Time and Substrate to Enzyme Ratio on Total Reducing Sugar Content

Enzymatic hydrolysis was conducted to convert cellulose into glucose, utilizing enzymes as catalysts. The glucose produced during the enzymatic hydrolysis process was analyzed using a spectrophotometer by the dinitrosalicylic Acid (DNS) method as illustrated in Figure 4. The data shows that the cellulase enzyme activity steadily increases with prolonged hydrolysis time across all variations of enzyme-to-substrate ratios. This suggests that enzymes require time to break down the banana stem substrate and enhance cellulose conversion into more straightforward products.

Table 2. Activity of Cellulase Enzyme

Cellulase Enzyme	Absorbance	Glucose Concentration (mg mL^{-1})	Activity (U mL^{-1})
endo-β-glucanase	59.72	65.68	77.55
exo-β-glucanase	1.07	0.15	0.05

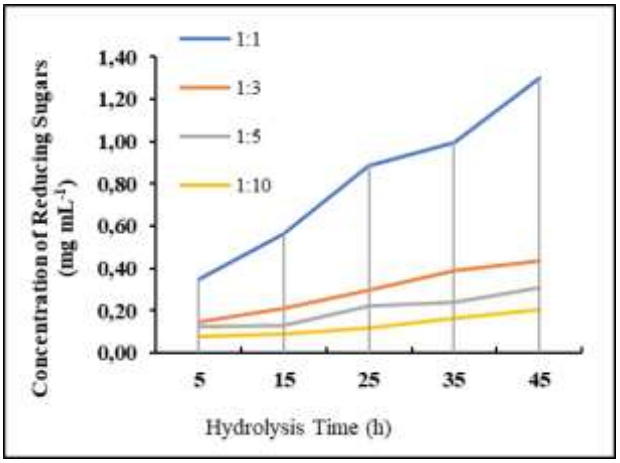

Figure 4. Total Reducing Sugar at Various Hydrolysis Time and Enzyme-to-Substrate Ratio.

The enzyme-to-substrate ratio is crucial in determining cellulase enzyme activity because it influences enzyme-substrate binding, enzymatic reaction conditions, and substrate suitability for enzyme production [24]. Accordingly, considering the impact of different enzyme-to-substrate ratios at certain time points, variations in enzyme activity were observed. This is shown at a hydrolysis time of 25 h with a 1:3 ratio; the resulting reduced sugar concentration was only 0.3 mg mL^{-1}, whereas at a shorter time of 5 h with a 1:1 ratio, it already reached 0.35 mg mL^{-1}. This indicates that the substrate-to-enzyme ratio significantly influences cellulase enzyme activity, with lower substrate ratios yielding higher reduced sugar concentrations in a shorter time. This is further shown by the highest reducing sugar content observed at a 1:1 ratio for 45 h, which reached 1.3 mg mL^{-1}. While others, ratios of 1:3, 1:5, and 1:10, ranging from 0,21-0,43 mg mL^{-1}.

Table 3. Comparison of a Total Reducing Sugar concentration for numerous pre-treatment biomass conditions with the current research

Feedstock	Pre-treatment Condition	Enzymatic Hydrolysis conditions	Total Reducing Sugar Concentration	Reference
Corncob biomass	alkaline hydrogen peroxide	enzyme combination of 43.8% cellulase, 41.8% hemicellulose, and 14.4% pectinase, fed-batch, 48 h	110.47 mg mL^{-1}	[25]
Poplar wood chips	dilute acid pre-extraction and chemical-assisted mechanical refining	*Cellic CTec2*, pH 4.8, shaking incubator at 50°C, 180 rpm, 72 h.	21.41 mg mL^{-1}	[26]
corn stover	Hydrothermal at 170 °C	*Cellic CTec2* (cellulase) from Sigma-Aldrich, pH 4.8, in an incubator shaker, at 50°C 150 rpm for 72 h	Yield 80.36%	[27]
Rice Hull	Aqueous Ammonia Soaking (AAS) 20% at 100 °C, 5 h, and dilute acid 2%, at 85 °C for 75 min	A crude enzyme from *Aspergillus niger*, in Erlenmeyer, at 200 rpm, 50 °C for 24 h, pH 5	241.8 mg mL^{-1}	[28]
Rice husk	Combined hydrogen peroxide-aqueous ammonia	A crude enzyme from *Aspergillus niger*, in Erlenmeyer, at 200 rpm, 50 °C for 24 h	15.11 mg mL^{-1}	[23]
Banana Stem	Alkaline Microwave-Assisted Pre-treatment	A crude enzyme from *Aspergillus niger*, at 200 rpm, 50 °C for 24 h	1.3 mg mL^{-1}	This study

At the maximum hydrolysis time of 45 h, cellulase enzyme activity tends to reach saturation, where further substrate reduction no longer significantly enhances enzyme activity. This suggests that the banana stem substrate may have been fully hydrolyzed or reached a saturation point where enzymes cannot work more efficiently. These results highlight that selecting an appropriate enzyme-to-substrate ratio can significantly impact process efficiency.

In this study, a lower reducing sugar concentration is observed from the enzymatic hydrolysis result compared to another research as shown in Table 3. It may be due to the performance of enzymatic hydrolysis. The activity and quality of the cellulase enzymes used in this research have lower activity or purity than previous studies, which may result in less efficiency for breaking down cellulose and resulting in lower sugar concentrations. It may also be due to a significant amount of reducing sugars being lost during the pre-treatment or insolubility of macromolecular that may hinder the efficient recovery of reducing sugars [27, 29, 30].

CONCLUSION

The results of this study demonstrate that KOH and microwave alkali treatments can be employed to reduce lignin and hemicellulose in banana stems, increase the proportion of cellulose, and maintain HWS composition. It reveals a significant improvement in hydrolysis efficiency due to the KOH-microwave pre-treatment, suggesting enhanced cellulose accessibility. The substrate-to-enzyme ratio significantly influences cellulase enzyme activity, with the highest reducing sugar content being 1.3 mg mL^{-1}, at a ratio of 1:1 for 45 h. Further research on the kinetics of this process is necessary to gain deeper insights and understanding into the underlying mechanisms.

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