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Research Paper

Effectiveness of HNO_3 and NaOH Pretreatment on Lignin Degradation in Areca Leaf Sheath Fibre (*Areca catechu* L.) for Bioethanol Production

Wahyuti[1], **Abdul Karim**[1] ✉, **Rugaiyah Andi Arfah**[1], **Muhammad Zakir**[1], **Syarifuddin Liong**[1], **Maming**[1], **Maswati Baharuddin**[2], **Mirna**[1]

[1]Department of Chemistry, Hasanuddin University, Makassar 90245, Indonesia
[2]Department of Chemistry, Alauddin State Islamic University of Makassar, Makassar 92118, Indonesia

✉ karimkimia@yahoo.com

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Abstract

Areca leaf sheaths are underutilized waste but have a high cellulose content of 72.27%, so they can be utilized for bioethanol production. This research aims to utilize areca leaf waste for bioethanol production through acid (HNO_3 5%) and alkaline (NaOH 10%) pretreatment processes, enzyme hydrolysis, and fermentation. Pretreatment using 5% HNO_3 and 10% NaOH solutions is carried out because it can break down the lignin bond and release it from cellulose and hemicellulose fibers. The enzymatic hydrolysis process uses cellulase enzymes at 37 °C for 48 hours to produce glucose. Glucose content analysis uses the DNS method and UV-Vis spectrophotometry instruments because it is accurate and can detect glucose in low concentrations. The fermentation process is carried out using *Saccharomyces cerevisiae* as a fermentation microorganism because it has high efficiency in bioethanol production for a duration of 3, 5, and 7 days. Based on the results of the analysis, pretreatment with HNO_3 5% solution reduced the level of lignin in areca leaf sheaths by 2.31%. Meanwhile, pretreatment using a 10% NaOH solution lowered lignin levels to 1.81%. Reduced sugar levels after hydrolysis after pretreatment with HNO_3 5% and NaOH 10% were 25.08 mg/mL and 16.37 mg/mL, respectively. The highest concentration of bioethanol in the 5% HNO_3 pretreatment was achieved on the 7th day at 16.75%, while that of 10% NaOH on the 5th day was 14.75%. This difference is influenced by the availability of fermentable sugars, where HNO_3 substrates take longer to decompose by *S. cerevisiae* than NaOH substrates. Based on the analysis, the bioethanol contains ethanol, thus the areca leaf sheath fibre feedstock has the potential to assist in the advancement of a sustainable biorefinery process that can reduce dependence on fossil fuels and increase added value.

Keywords: Areca leaf sheath; Pretreatment; Hydrolysis; Fermentation; Bioethanol

1. Introduction

The areca nut (*Areca catechu* L.) is a plant that thrives in tropical and subtropical areas, including Indonesia with a plantation area of about 145.890 hectares. One of the parts of the plant that has not been optimally utilized is the areca leaf sheaths, which are often plantation waste [1], [2]. However, areca leaf sheaths have great potential as value-added waste, which can be used for various products such as bioethanol, industrial raw materials, and other products. The use of areca leaf sheaths can reduce waste and increase economic value [3].

Based on laboratory analysis, areca leaf sheaths contain high levels of lignocellulose, namely 72.27% cellulose, 13.28% hemicellulose, and 12.84% lignin [4]. When compared to other lignocellulose materials, such as straw which only contains cellulose at 34.26% [5] and bagasse at 37.5% [6]. Based on the study, the high cellulose

content of areca leaf sheaths has the potential to be an alternative source of renewable energy. In addition, areca leaf sheaths have advantages, such as being available in abundant quantities if production is done on an industrial scale. Bioethanol is a renewable energy source that plays an important role in reducing dependence on fossil fuels. In addition, it can reduce carbon emissions by producing cleaner combustion. Produced from organic waste such as areca leaf sheaths. Bioethanol, with its environmentally friendly and renewable characteristics, is a strategic choice in global efforts to reduce carbon footprint and accelerate the transition to sustainable energy [7], [8].

Bioethanol is a chemical compound generated via the fermentation process of glucose facilitated by microorganisms, be it fungi, bacteria, or yeast. Bioethanol has the molecular formula C_2H_5OH, commonly written as EtOH. Bioethanol can be derived from biological resources that have high carbohydrate content, such as simple sugars, homopolymers (starch and cellulose), and heteropolymers (hemicellulose and seaweed) [9]. Bioethanol is one of the renewable energies being developed in Indonesia due to its abundant and easily available raw materials. The use of bioethanol can reduce the use of fossil fuels [10]. In addition, bioethanol produces less CO_2 than fossil fuels. However, bioethanol is sold at a very high price, so to reduce these costs, we use raw materials with low economic value, such as areca leaf sheath waste [11].

The utilization of areca leaf sheaths as a source of bioethanol feedstock goes through several important stages: pretreatment, hydrolysis, fermentation, and distillation. The pretreatment process is carried out because cellulose and lignin, the main components of lignocellulose, have complex, stable, and strong structures. Therefore, mechanical, chemical, or biological forces to break these structures are pretreatment [12]. The pretreatment process can use acidic (HNO_3) and basic (NaOH) solvents. The purpose of pretreatment is to remove residual lignin that can inhibit the work of enzymes in the hydrolysis stage that produces glucose, which will later go through the fermentation process [13].

The hydrolysis stage is known as the sugar production stage or saccharification stage. Hydrolysis aims to convert cellulose into glucose through an enzymatic or chemical process [14]. Enzymatic hydrolysis uses enzymes such as cellulase enzymes to break down cellulose and hemicellulose from lignocellulose pretreatment [15]. Enzymatic hydrolysis can produce a higher ethanol yield than chemical hydrolysis. The glucose produced from this stage of hydrolysis then becomes the main substrate in the fermentation process to produce bioethanol [16].

Saccharomyces cerevisiae (*S. cerevisiae*) is a microorganism that is commonly used in the bioethanol fermentation process because it is able to convert simple carbohydrates such as glucose into bioethanol efficiently. These microorganisms grow quickly and have a high tolerance to extreme environmental conditions such as pH and limited oxygen levels [17]. In this study, fermentation was carried out using *S. cerevisiae* at temperature of 30 °C, pH 4.5 - 5.0, and anaerobic conditions for a duration of 3, 5, and 7 days to optimize bioethanol production [18].

Several studies using the *S. cerevisiae* yeast strain in fermentation show that the ethanol concentration obtained is 9.5 g/L, resulting from pretreatment using 5% nitric acid. In addition, a study [19] revealed that gas chromatography analysis showed a bioethanol content of 6.08% after distillation. Meanwhile, research [20] showed that pretreatment using 2% NaOH resulted in lignin removal of 74.7% with glucose levels produced around 93.2%. The fermentation results obtained using *S. cerevisiae* are around 86.4%. Therefore, this research aims to evaluate the impact of various HNO_3 and NaOH pretreatments methods on lignin degradation for bioethanol production from areca leaf sheath fiber biomass.

2. Materials and Methods

2.1. Apparatus and Materials

The materials used in this research are areca leaf sheath waste, distilled water, NaOH p.a (Merck), HNO_3 p.a (Merck), glucose p.a (Merck), commercial cellulase enzyme, yeast (*S. cerevisiae*). For the fermentation process, a fermentation medium consisting of KH_2PO_4, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$, and $K_2Cr_2O_7$ is used with a pH of 4.5-5.0. Fermentation is carried out 30 °C in anaerobic conditions for 3, 5, and 7 days. Other materials such as H_2SO_4 p.a (Merck), ethanol p.a (Merck), ADF and NDF solutions, aluminium foil, acetate

buffer pH 5, DNS reagent, and Whatman filter paper no.42.

The tools used in this study were gas chromatography-mass spectroscopy (GC-MS), Fourier Transform Infrared (FT-IR), UV-Vis spectrophotometer, refractometer, oven, autoclave, magnetic stirrer, hotplate, analytical balance, incubator, distillation apparatus, grinder, sieve shaker, desiccator, pH meter, and common glassware in the laboratory.

2.2. *Preparation of Areca Leaf Sheaths Fibre Powder*

Areca leaf sheaths were collected in Sidenreng Rappang Regency, South Sulawesi, Indonesia. These samples are biomass waste from areca nut plantations. The samples were washed to remove dirt attached to the areca leaf sheaths. Once clean, they were cut into small pieces and dried in the sun. Then, they were mashed using a grinder at the Forest Products Processing and Utilisation Laboratory at Hasanuddin University. Samples with a size of 100 mesh were dried at 70 °C for 3 hours.

2.3. *Pretreatment with HNO_3 5% and NaOH 10%*

Pretreatment is carried out by taking 50 g of areca leaf sheath fiber powder with 500 mL of HNO_3 5% and NaOH 10% respectively, so that the ratio of ingredients to solution is 1:10 (b/v). The pretreatment process for HNO_3 treatment is carried out at a temperature of 50 °C, while for NaOH at 120 °C, each for 90 minutes is stirred using a magnetic stirrer. This selection of temperature and time aims to optimize the decomposition of lignin without damaging the cellulose structure, where low temperatures are quite effective for acid treatment, while high temperatures are required to increase the effectiveness of alkaline pretreatment. The precipitate was filtered and rinsed with distilled water until a neutral pH of 7 was achieved. Once neutralized, it was dried in an oven at 70 °C, ground using a blender, and then sieved to a size of 100 mesh.

2.4. *Hemicellulose, Cellulose, and Lignin Content Analysis*

The van Soest method was employed to assess lignocellulose content. This technique measures the levels of cellulose, hemicellulose, and lignin by analyzing neutral detergent acid (NDF) and acid detergent fiber (ADF) [21].

2.5. *Characterization with FT-IR*

FT-IR spectrum analysis of areca leaf fiber powder and pretreatment results were made into pellets by adding dry KBr. Then, it was measured with an FT-IR instrument in wave numbers 500-4000 cm^{-1}.

2.6. *Moisture Content*

The porcelain dish was cleaned and heated in an oven at 105 °C for 1 hour to remove moisture. After drying, it was allowed to cool in a desiccator for 15 minutes before being weighed (a). We weighed 1 g each of areca leaf sheath fiber flour, and pretreatment resulted in a porcelain cup with known weights (b). The sample was then placed in an oven at 105 °C for 2 hours, cooled in a desiccator for 30 minutes, and process was repeated until a constant weight was achieved (c). The water content was determined using the following formula Eq. (1).

$$Moisture\ content\ (\%) = \frac{b-c}{b-a} \times 100\% \qquad (1)$$

where, a = weight of empty cup (g); b = cup weight + sample weight (g); c = weight of cup + dry sample (g)

2.7. *Enzymatic Hydrolysis Process*

The pretreatment results of HNO_3 5% and NaOH 10% of 10 g each, were put into Erlenmeyer, and then added 150 ml distilled water. The mixture was sterilized using an autoclave at 121 °C for 15 minutes and cooled in a laminar airflow. After that, 0.1 M acetate buffer pH 5 and 15 mL of a commercial cellulase enzyme (activity of 100 U/mL, derived from *Trichoderma reesei*) were added. The mixture is then covered with cotton and aluminium foil and incubated for 48 hours at 37 °C and 105 rpm using an incubator shaker. The condition was chosen to optimize the activity of cellulase enzymes that work effectively at pH 4.5 - 5.5 and temperature of 35 – 50 °C. The sample was centrifuged at 10,000 rpm for 30 minutes, and the resulting supernatant (hydrolysate) was used to test glucose using the dinitro-salicylic acid (DNS) method.

2.8. *Analysis of Glucose Content by Dinitro Salicylic Acid (DNS) Method*

A 1.5 mL hydrolysate sample was transferred to a test tube and mixed with 1.5 mL of DNA reagent. The mixture was then homogenized,

heated in boiling water for 15 minutes, and cooled to room temperature. If the sample is too concentrated, it can be diluted first before measuring the absorbance with a UV-Vis spectrophotometer at a wavelength of 513 nm. Blank measurements were performed with distilled water. Calibration curves were generated using glucose standard solutions with concentrations of 0.025; 0.05; 0.1; 0.2; and 0.4 mg/mL. The calculation of glucose levels can be known in Eq. (2), the standard linear regression equation of glucose.

$$y = ax + b \quad (2)$$

where, y = absorbance; a = slope; b = intercept; x = glucose concentration (mg/mL)

2.9. *Bioethanol Manufacturing of Fermentation*

Erlenmeyer containing hydrolysate was added with nutrients such as KH_2PO_4, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$ each 0.1 g and homogenized. Then, it was sanitized in an autoclave at 121 °C for 15 minutes and then allowed to cool, *S. cerevisiae* yeast of 3 g was added. Next, it was put into a shaker incubator for 2 hours at 150 rpm. It continued the fermentation process with a variation of 3, 5, and 7 days at room temperature. It was covered with aluminium foil equipped with a rubber hose and put into water. The fermentation mixture was centrifuged at 10,000 rpm and 4 °C for 30 minutes. The resulting supernatant (bioethanol) was then continued in the distillation stage.

2.10. *Distillation Process*

A series of distillation equipment was installed, and the fermented liquid was put into the flask. The distillation process was conducted at temperatures between 78 °C and 80 °C until the distillate no longer dripped. Then, the distillate obtained is put into a closed bottle.

2.11. *Bioethanol Qualitative Analysis*

Distilled bioethanol was analyzed qualitatively using the potassium dichromate ($K_2Cr_2O_7$) oxidation method. The qualitative test is done by pipetting 2 mL of potassium dichromate 2% into a sample tube. Then, 5 drops of concentrated sulfuric acid were added and homogenized. Then, 1 mL of the sample is added to be tested. The positive results of bioethanol showed a colour change from orange to blue.

2.12. *Quantitative Analysis of Bioethanol Using a Refractometer*

Measurement of bioethanol content was carried out using a refractometer. The prism surface of the refractometer is cleaned using water and tissue. After cleaning, the sample is dripped on the surface of the prism, then closed, and the light beam is allowed to enter and pass through the sample. The prism is arranged so that the light in the refractometer layer becomes two separate colours or with a clear boundary. Then the boundary mark is shifted using the adjustment knob found on the refractometer until it intersects the intersection point of two diagonal lines that intersect each other. Then, the refractive index scale found on the refractometer will be observed and read, and the results will be recorded. Then, open the cover and clean the surface of the prism from the sample until it is clean. Calculation of bioethanol content using Eq. (3).

$$y = ax + b \quad (3)$$

where, y = refractive index; a = slope; b = intercept; x = bioethanol concentration % (v/v)

2.13. *Bioethanol Qualitative Analysis Using GC-MS*

Qualitative bioethanol analysis was also conducted using GC-MS. The analysis involves injecting 1µL of distillate into the column through the injection site; then, the chromatogram and mass spectrum will be seen. The resulting compound can be determined from the retention time [22].

3. Results and Discussion

3.1. *Determination of Lignocellulose Content by van Soest Method*

The composition of lignocellulosic biomass was analyzed using the method developed by van Soest. The measured fiber fractions consisted of acid detergent fiber (ADF), neutral detergent fiber (NDF), cellulose, hemicellulose, and lignin. The selection of this method is based on the fact that van Soest method has the advantage of separating plant cell wall components in a systematic, structured, and accurate manner [23]. In addition, this method is suitable for coarse lignocellulose materials such as straw, plant stems, pulp, and fronds. It is particularly suitable for analyzing agricultural waste, including areca leaf sheaths.

The results of biomass composition analysis before and after pretreatment are presented in **Table 1**.

The data in **Table 1** shows that the content of areca leaf sheath fiber samples obtained cellulose content of 35.48%, hemicellulose of 10.40%, and lignin of 3.68%. Pretreatment was carried out to increase cellulose content and reduce lignin and hemicellulose levels contained in the areca leaf sheath fiber. **Table 1** shows the effectiveness of lignin degradation from the pretreatment process. Cellulose content after pretreatment increased to 53.62% using 5% HNO_3 solvent. Other studies have shown that an HNO_3 solvent with a concentration of 5% is considered optimal for increasing cellulose levels [24]. Meanwhile, the use of 10% NaOH solvent increased by 75.75%. This is to other studies, which show that high concentrations of NaOH can produce high cellulose levels as well.

As in other studies, the concentration of 4% NaOH [25] and 2% NaOH [26], resulted in low cellulose content. This is because the use of NaOH with a high concentration will increase hydroxyl ions in the liquid, which disrupts the bonds within the fundamental structure of lignin so that it will break down more and more lignin from the cellulose chain [27]. The results obtained by comparing the research results show that using HNO_3 solvent in the pretreatment process removes hemicellulose, while NaOH solvent removes lignin [28]. This indicates that the reaction after pretreatment with HNO_3 and NaOH solvents is indicated by an increase in cellulose content and a reduction in hemicellulose and lignin content.

Pretreatment with HNO_3 5% and NaOH 10% significantly lowers lignin levels and increases cellulose levels in areca leaf sheaths. Lignin is known to inhibit the work of cellulase enzymes by forming a physical barrier on the cell wall structure. Therefore, the reduction of lignin can improve the enzyme's accessibility to cellulose during the enzymatic hydrolysis process [29]. The cellulose content increases relatively speaking, indicating that the pretreatment successfully changes the structure of the biomass to be more open and reactive. This increase in cellulose content has a direct impact on the hydrolysis and fermentation process, as it provides more glucose as a fermentation substrate so that it can increase bioethanol yield [30].

3.2. *Characterization Using FTIR*

FTIR characterization is used to identify functional groups. Areca leaf sheath fiber is lignocellulose, composed of hemicellulose, lignin, and cellulose. Generally, the three components consist of alcohol, alkane, ester, and aromatic [26]. The FTIR spectra of areca leaf sheath fiber the condition before and after pretreatment is depicted in **Figure 1.**

Figure 1 and **Table 2** show that alkaline pretreatment, namely NaOH, causes loss of functional groups. In addition, there are differences in peak intensity in each functional group identified [31]. The absorption peak of the areca leaf fiber functional group before pretreatment was 3404.36 cm^{-1}, and after NaOH and HNO_3, pretreatment had a sharper wave frequency, namely, 3448.72 cm^{-1} and 3415.93 cm^{-1}. This suggests the existence of O-H bonds that experience stretching due to the influence of alkali and acid pretreatment [32]. Alkaline pretreatment

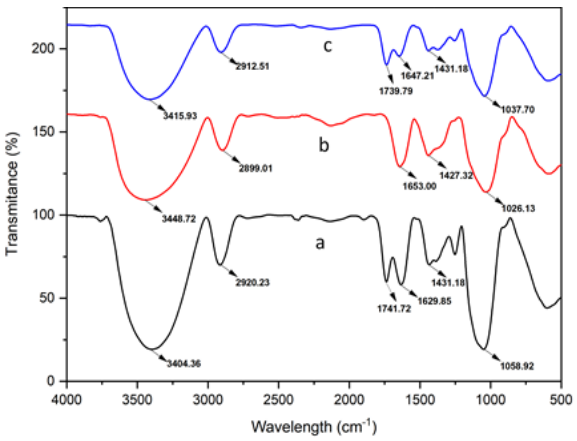

Figure 1. FTIR spectra of areca leaf sheath fiber before (a), after pretreatment NaOH (b) and pretreatment HNO_3 (c)

Table 1. Composition of areca leaf sheath fiber before and after pretreatment

Composition	Initial areca leaf sheath fibre (%)	Areca leaf sheath fiber after pretreatment (%)	
		HNO_3	NaOH
Cellulose	35.48	53.62	75.75
Hemicellulose	10.40	3.90	4.90
Lignin	3.68	2.31	1.81

Table 2. Functional groups of areca leaf sheath fiber before and after pretreatment [33], [34]

Wavenumbers (cm^{-1})			Functional Groups	Range (cm^{-1})
Before pretreatment	NaOH pretreatment	HNO_3 pretreatment		
3404.36	3448.72	3415.93	O-H stretching	3550-3200
2920.23	2899.01	2912.51	C-H stretching	3000-2840
1741.72	-	1739.79	C=O	1745-1720
1629.85	1653.00	1647.21	C=C	1900-1500
1431.18	1427.32	1431.18	C-H bending	1439-1399
1058.92	1026.13	1037.70	C-O	1260-1000
898.83	896.90	896.90	CH_2 (glycoside)	895-899

can disrupt hydrogen bonds through the reaction of hydroxyl groups with sodium hydroxide, which can increase the peak of O-H groups when compared to fibers before pretreatment [35]. This suggests a rise in cellulose after pretreatment. The peaks around 3448.72 cm^{-1} to 2899.01 cm^{-1} correspond to α-cellulose [36].

The results of FTIR analysis on areca leaf sheath fiber before pretreatment and after HNO_3 pretreatment produced peaks at wavenumbers 1741.72 cm^{-1} and 1739.79 cm^{-1}, this signified the presence of C=O groups [37]. Meanwhile, NaOH pretreatment causes the loss of the C=O functional group. This indicates the loss of some lignin compounds from the areca leaf sheath fiber after NaOH pretreatment. This is consistent with research [38] that the significantly reduced peak at wavenumbers around 1748 cm^{-1} is hemicellulose and lignin, which indicates effective removal during the pretreatment or delignification process.

Peaks at wavenumbers around 1653.00 cm^{-1} to 1629.85 cm^{-1} indicate the existence of C=C functional groups in the aromatic structure, a structural group in lignin [39]. In addition, the peak at wavenumber around 1431.18 cm^{-1} indicates the existence of C-H and bending functional groups of OH or CH in hemicellulose [33]. The peaks observed at specific wavenumbers 1026.13 cm^{-1} to 1058.92 cm^{-1} are cellulose constituent components derived from vibrations of the pyranose ring group, indicating the existence of C-O bonds. The peak appears sharp and visible [26], [37]. The peaks at wave numbers 898.83 cm^{-1} and 896.90 cm^{-1} indicated the existence of glycoside bonds. This shows that the cellulose produced is not susceptible to degradation by chemical solutions, in this case, NaOH and HNO_3 solvents [33], [40].

3.3. Determination of The Moisture Content

Moisture content is analysis to determine the percentage of water in a sample [41]. Analysis of the moisture content of a sample is important in the bioethanol production process. A good moisture content in a sample should not exceed 10%. This is because the high moisture content is easier to use as a medium for microbial growth [42], [43]. The moisture content of the research carried out can be seen in **Figure 2.**

Figure 2 shows the overall percentage of moisture content in this study. It can be seen that the moisture content obtained in the areca leaf sheath fiber powder sample before pretreatment is higher than that of the sample after the pretreatment process. This is because the structure of lignocellulose is complex, so it can hold moisture [44]. However, after pretreatment using either NaOH or HNO_3 solvent, the percentage of moisture content of the sample will decrease. This is because after the pretreatment process, the sample lost lignin and some hemicellulose, so the structure of the lignocellulose also became more porous and was unable to hold water anymore [45]. In addition, the long drying stage after pretreatment also has an effect so that the water

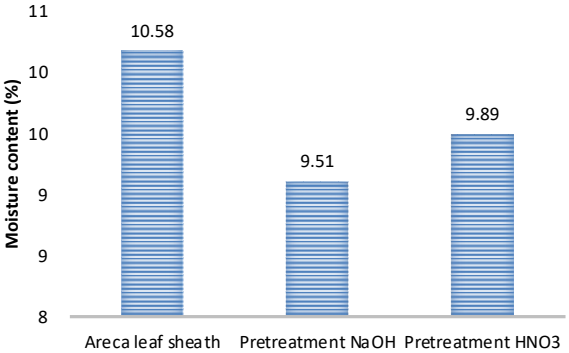

Figure 2. The moisture content of areca leaf sheath fiber before and after pretreatment

content can also be reduced [46]. Based on research conducted from several samples, such as straw, a moisture content of 13.80% was obtained [47]. In addition, the sample of kapok banana leaf obtained a moisture content of 16.85% [48].

3.4. *Glucose Content*

The hydrolysis process to produce reducing sugars is enzymatic, using cellulase enzymes [49]. Hydrolysis is the process of converting complex carbohydrates into simpler sugars. Cellulase enzymes have an important role as a catalyst in hydrolysis [50]. The glucose content in the HNO_3 and NaOH pretreatment samples is shown in **Table 3**. While shows that enzymatic hydrolysis of HNO_3 pretreatment reduces sugar value by 25.08 mg/mL, and NaOH pretreatment is 16.37 mg/mL. The difference in hydrolysis results between HNO_3 and NaOH pretreatment indicates that HNO_3 is 5% more effective in breaking down lignocellulose structures and increasing the availability of cellulose for enzymatic hydrolysis. Nitric acid (HNO_3) works by hydrolyzing hemicellulose and lignin again, making the cellulose structure more open and easily accessible to cellulase enzymes [51].

The enzymatic hydrolysis process is influenced by several factors, namely pH, temperature, and enzyme ratio. This is based on research conducted in the enzymatic hydrolysis process of adding 26.57 mL of cellulase enzyme, resulting in glucose levels of 38.96% [52]. This shows that the more cellulase enzymes are added, the more it will help accelerate the hydrolysis reaction process to produce a lot of glucose [53]. This study showed that areca leaf sheath fiber with HNO_3 pretreatment had higher enzymatic digestibility than NaOH pretreatment. This indicates that HNO_3 increases enzyme accessibility to cellulose so it can be converted into glucose [54].

The study on artichoke biomass pretreatment using a HNO_3 5% solution, yielded 38.5 g/L of glucose after hydrolysis which is equivalent to 89% of the theoretical yield, and increased the ethanol yield to 77-82% of the maximum potential [51]. This shows that HNO_3 is not only effective in removing lignin, but also improving the efficiency of enzymatic hydrolysis and fermentation. In contrast, pretreatment with NaOH is indeed effective in removing lignin, but its effect on the opening of cellulose structures is not as efficient as HNO_3, so the glucose levels resulting from enzymatic hydrolysis are lower [55]. This is due to the difference in the mechanism between acids and bases in breaking down the structure of lignocellulose.

Glucose obtained from hydrolysis is the result of cellulolytic enzymes. The cellulolytic enzyme system consists of three main groups. Firstly, the endoglucanase enzyme hydrolyses the amorphous part of cellulose to produce oligosaccharides of varying lengths and new chain termini are produced on cellulose. Secondly, the exoglucanase enzyme targets the end of the polysaccharide chain, generating a disaccharide known as cellobiose. Third, the enzyme β-glucosidase breaks down cellobiose into two (2) glucose molecules [56].

Testing of glucose levels was carried out using the DNS method because it has advantages, such as high accuracy, so it can detect glucose even at small levels [57]. The DNS reaction to reduce glucose can be seen in **Figure 3**.

Table 3. Comparison of glucose content of areca leaf sheath fiber after pretreatment

No.	Hydrolysate	Glucose content (mg/mL)
1.	Pretreatment HNO_3	25.08
2.	Pretreatment NaOH	16.37

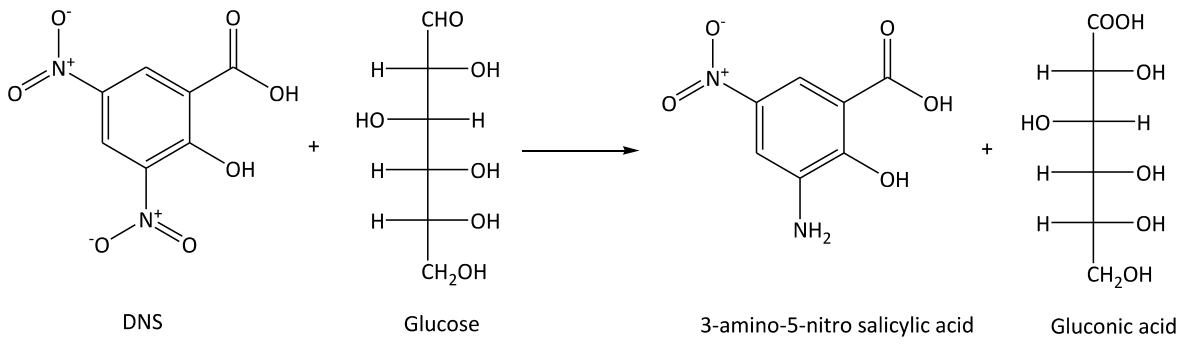

Figure 3. The reaction of glucose with DNS [58]

Figure 3 shows that reducing sugar is quantified using the standard DNS method that identifies the free carbonyl group (C=O) of reducing sugar. Glucose, as a reducing sugar, reduces 3,5-dinitro salicylic acid in an alkaline solution to 3-amino-5-nitro salicylic acid. In contrast, the aldehyde group on glucose as a reducer will accept electrons and turn into carboxyl. The colour change that occurs is from yellow to reddish-orange [59].

3.5. *Bioethanol Fermentation*

The fermentation stage in bioethanol production from areca leaf sheath fiber is carried out anaerobically, and *S. cerevisiae* yeast is added. The addition of *S. cerevisiae* aims to produce energy under anaerobic conditions by converting glucose into ethanol and CO_2 [60]. To determine the condition of bioethanol produced, the optimum fermentation time is needed. Fermentation time is the time required by *S. cerevisiae* to convert glucose from the enzymatic hydrolysis process into bioethanol [53]. The fermentation time variations in this study were 3, 5, and 7 days.

The results obtained in **Table 4** show that the highest bioethanol content was received at the fermentation time of HNO_3 pretreatment on days 7 or for 168 hours. This is because the contact time between *S. cerevisiae* and glucose is getting longer, so it can produce a lot of bioethanol [61]. Meanwhile, the optimum fermentation time from NaOH pretreatment is on days 5 or 120 hours. The bioethanol content from NaOH pretreatment decreased at 168 hours or days 7 of fermentation time. This is because after the optimum time, the substrate converted by yeast is exhausted, and the bioethanol obtained turns into organic acids. In addition, it is also because the nutrient concentration begins to decrease, so the growth of *S. cerevisiae* begins to decline or experience a stationary phase [62].

Therefore, the balance between substrate availability and fermentation time is an important factor in determining the final result of bioethanol. Thus, the consumption of the substrate determines how much of the raw material is available to be converted into ethanol, while the fermentation condition determines how efficiently microorganisms convert the substrate into ethanol [63]. The effectiveness of pretreatment greatly affects the fermentation stage. Higher concentration of reduced sugars after HNO_3 pretreatment leads to greater availability of fermentable substrates, which then results in bioethanol in higher concentrations.

3.6. *Qualitative Analysis with $K_2Cr_2O_7$*

The qualitative test with $K_2Cr_2O_7$ aims to confirm the bioethanol content using 2% potassium dichromate reacting with concentrated H_2SO_4. The positive result is that the bioethanol sample will change colour to a blue solution [64]. Based on the research results on the testing of distillate samples from bioethanol fermentation, the colour changes from orange to blue after adding 2% potassium dichromate and concentrated sulfuric acid. This indicates that the qualitative analysis of the fermentation distillate contains ethanol. The test results can be seen in **Figure 4**.

This is because potassium dichromate is a strong oxidizer, so when reduced, the orange-coloured solution containing dichromate (VI) ions will turn into a blue solution containing chromium (III) ions [65]. The reaction that occurs is as follows Eq. (4) to Eq. (6).

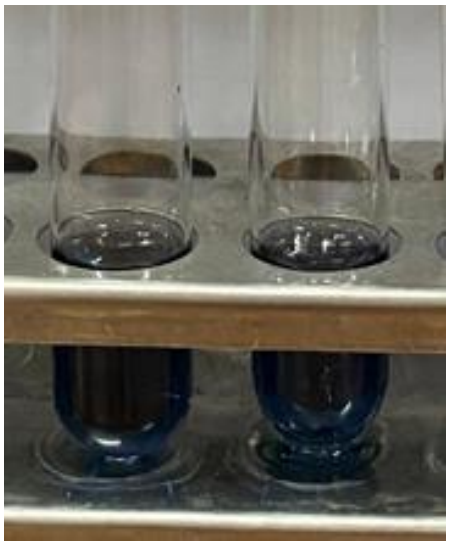

Figure 4. Qualitative test results with $K_2Cr_2O_7$

$$Oxidation: 3CH_3CH_2OH_{(aq)} + 3H_2O_{(l)} \rightarrow 3CH_3COOH_{(aq)} + 12H^+{}_{(aq)} + 12e \quad (4)$$

$$Reduction: 2CrO_7{}^{2-}{}_{(aq)} + 28H^+{}_{(aq)} + 12e \rightarrow 4Cr^{3+}{}_{(aq)} + 14H_2O_{(l)} \quad (5)$$

$$3CH_3CH_2OH_{(aq)} + 2Cr_2O_7{}^{2-}{}_{(aq)} + 16H^+{}_{(aq)} \rightarrow 3CH_3COOH_{(aq)} + 4Cr^{3+}{}_{(aq)} + 11H_2O_{(l)} \quad (6)$$

3.7. *Bioethanol Content using Refractometer*

Testing by measuring the refractive index is carried out to identify liquid compounds in addition to determining the purity of the tested liquid compounds [66]. In addition, the refractive index value can also choose the percentage of ethanol produced by the ethanol standard solution curve that has been made [60]. The refractive index value obtained from this study can be seen in **Table 4**, which shows that the refractive index in fermentation from HNO_3 pretreatment on day 7 has a high refractive index value of 1.3381, resulting in an ethanol concentration of 16.75%. Meanwhile, the fermentation from NaOH pretreatment on day 5 obtained a high refractive index value of 1.3373 with an ethanol concentration of 14.75%. The two methods used show that fermentation from HNO_3 pretreatment produces ethanol with a higher refractive index than NaOH. Compared to the refractive index value of standard ethanol based on Merc Index data, the resulting product is still far from the standard of good quality bioethanol.

3.8. *Analysis of GC-MS Bioethanol*

The glucose fermentation distillate obtained was identified using gas chromatography-mass spectroscopy (GC-MS) to ensure that the bioethanol product produced has ethanol content [67]. The GC chromatograms of the distillation results of HNO_3 and NaOH glucose fermentation on days 3, 5, and 7 and their mass spectra can be seen in **Figure 5** and **Figure 6**.

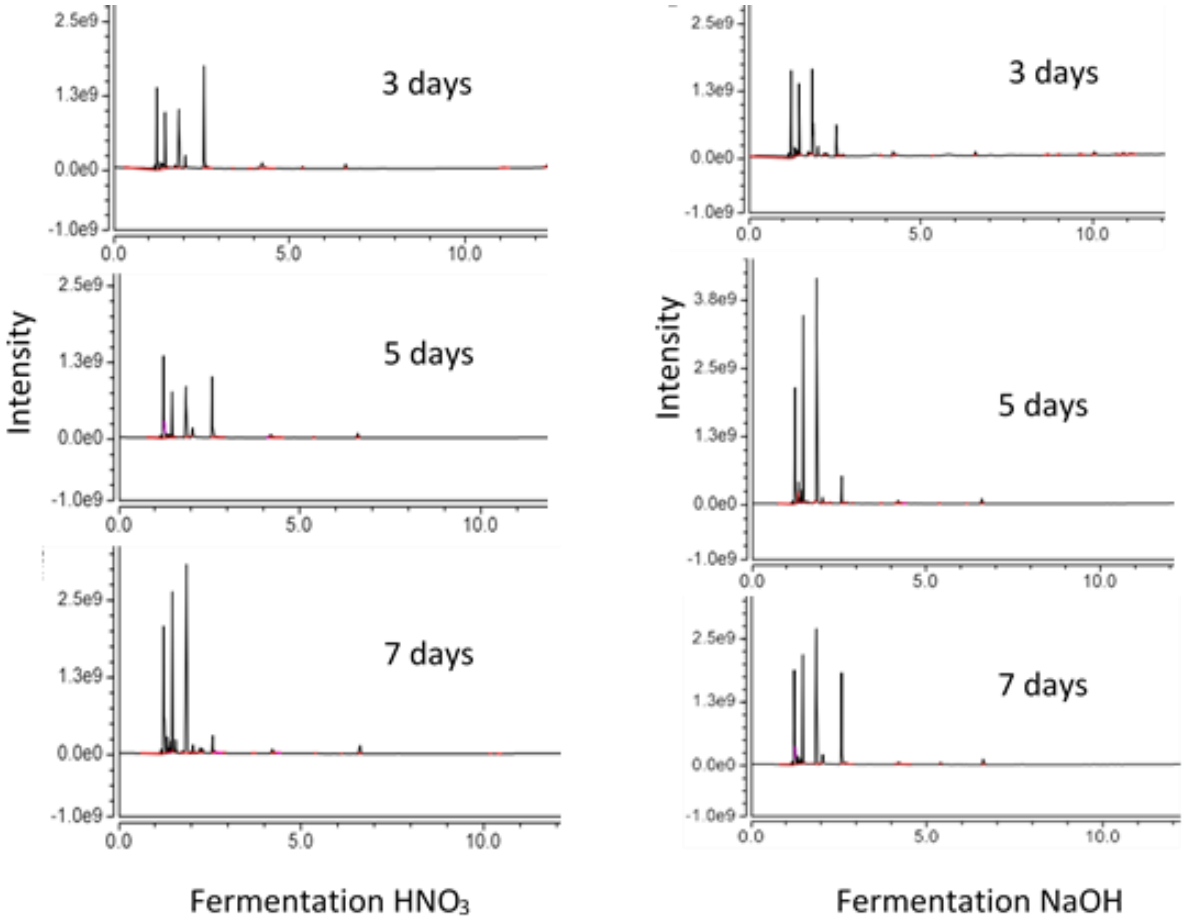

Figure 5. GC-MS chromatogram of fermentation results with HNO_3 and NaOH pretreatment on days 3, 5, and 7

Table 4. Refractive index value and bioethanol content of fermentation results

Pretreatment	Fermentation Days	Index of Refraction	Bioethanol Content (% v/v)
HNO_3	3	1.3362	12.00
HNO_3	5	1.3363	12.25
HNO_3	7	1.3381	16.75
NaOH	3	1.3355	10.25
NaOH	5	1.3373	14.75
NaOH	7	1.3363	12.25

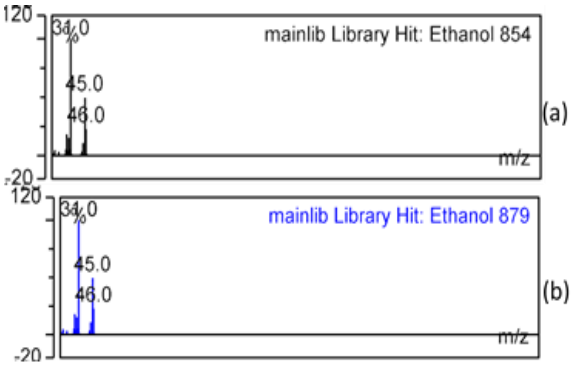

Figure 6. Mass spectra of fermentation products with (a) HNO_3 pretreatment day 7and (b) NaOH day 5

Based on the chromatogram results in **Figure 5** show that the highest glucose fermentation yield from enzymatic hydrolysis of cellulose from HNO_3 pretreatment was on day 7. This can be seen from the higher intensity of the chromatogram on day 7. The chromatogram of ethanol compounds from HNO_3 fermentation appeared at a retention time 1.275. In addition, based on the results of the mass spectrum in **Figure 6**, the reading on mass spectroscopy shows the molecular mass of the ethanol compound, which is Mr = 46 g/mol.

The GC chromatogram results of NaOH glucose fermentation distillation in **Figure 5** show that the highest glucose fermentation results were from the enzymatic hydrolysis of NaOH pretreated cellulose on day 5. The chromatogram of the fermentation results produced ethanol compounds. It appeared at a retention time of 1.282, and the mass spectrum shown in **Figure 6**, the ethanol compound appears as a molecular mass at Mr = 46 g/mol. This is based on the relative molecular mass of ethanol, which is 46 g/mol, as based on the National Centre for Biotechnology Information. Based on research on bioethanol production from rice straw, the chromatogram of ethanol compounds appeared at a retention time of 1,901, and the relative molecular mass of ethanol compounds is Mr = 46. Based on analysis using GC-MS, the distillate from enzymatic hydrolysis fermentation of cellulose pretreated with HNO_3 and NaOH showed that the bioethanol product contained ethanol.

4. Conclusion

This study showed that HNO_3 pretreatment was 5% more effective than 10% NaOH, producing glucose of 25.08 mg/mL and bioethanol of 16.75%, compared to NaOH which produced glucose of 16.37 mg/mL and bioethanol of 14.75%. The effectiveness of HNO_3 is due to its ability to aggressively depolymerize lignin and hemicellulose, resulting in more fermentable sugars. HNO_3 fermentation peaks on day 7 as glucose is released gradually, while NaOH peaks faster (days 5) due to faster but decreased glucose release due to substrate depletion. These results provide stone insights into the use of areca leaf sheath waste as a raw material for bioethanol and the importance of choosing pretreatment methods to optimize bioethanol production. To get the maximum benefit from bioethanol production from lignocellulosic biomass, it is necessary to conduct further research with biological pretreatment methods using white weathering fungi. Some studies have found that bioethanol production from different pretreatment processes show different results, so other pretreatment methods and microorganisms can be tried.

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Author's Declaration

Authors' contributions and responsibilities

The authors made substantial contributions to the conception and design of the study. The authors took responsibility for data analysis, interpretation and discussion of results. The authors read and approved the final manuscript.

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