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Investigating the Potential of Avocado Seeds for Bioethanol Production: A Study on Steam Delignification, Catalytic Hydrolysis, and Fermentation

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Abstract: The need for alternative fuels as a replacement for fossil fuels is becoming increasingly necessary, and bioethanol is a promising option. Meanwhile, numerous sources of sugar generation and agricultural wastes can be converted into ethanol. In this study, Avocado Seeds (AS) are attractive raw materials because they are abundant, have high carbohydrate content, and have no interactions with the food chain. Therefore, this study aimed to investigate the potential of AS for bioethanol production using several steps, such as steam delignification, catalytic hydrolysis, and fermentation with *Saccharomyces cerevisiae*. The method used to convert AS into ethanol involved steam delignification, pretreatment at (100°C, 1.5 h) of AS initially, 24h soaking in the sodium hydroxide liquor 4% (w/v), and washing and screening of the mixture. This is followed by catalytic hydrolysis and fermentation using two different concentrations of *Saccharomyces Cerevisiae* as yeast. In terms of ethanol yield, the best results were obtained after the fermentation process with 15% (v/w) yeast with 40% catalyst, and these processes yield 83.755% of the theoretical maximum.

Keywords: Agricultural Waste; Fermentation; Hydrolysis

1. Introduction

The reliance on fossil fuels is high, but a transition in the global economy to more sustainable and environmentally friendly energy sources is necessary [1]. Subsequently, biofuels are being considered as a replacement for petrol, diesel, and aerostatic fuels [2], [3]. According to simulations of the world's energy systems, it is predicted that biofuels would contribute between 10% and 40% to the market over the long term, indicating a massive increase in their use [4]. In Indonesia, the production of biofuels at a competitive price is being explored to support the country's economic and energy security [5]. Several researchers reported that biomass is considered the most promising renewable carbon source for biofuels [6], [9]. The National Research and Innovation Agency of Indonesia predicts that new renewable energy, including biomass, will increase power and heat generation by 2035 [10].

Bioethanol is a suitable biofuel option where the feedstocks are from fruit waste biomass, such as avocado seeds [4], [8], [11]. This biomass has several advantages such as low cost, low dependence on the food chain, and colossal availability [12], [13]. In 2014, According to the Central Bureau of Statistics Indonesia (BPS), 307.3 tons of avocados were produced in Indonesia per year [14], [15]. The production of avocados in Indonesia continues to increase yearly, with a production growth level of 24.48%, and as a result, the number of avocado seeds is also increasing [14]. Currently, avocado seeds are thrown away and pollute the environment [7][16]. There is a great deal of interest in exploiting fruit seeds, which are a crucial biomass resource utilized to produce bioethanol, which are obtained in vast quantities [17]. In addition, according to the Food and Agriculture Organization (FAO), the avocado (*Persea Americana*) is a tropical or subtropical fruit native to South America. It was a large fruit plant grown at 60% found in Asia, including Indonesia [18]. The primary waste of avocados is seeds, with a ratio of about 0.33 kg of seeds/kg of avocado [19]. Due to its availability and high cellulose content [17], [20], AS is regarded as an expected raw material for bioethanol as a result of the significant amount of AS involved.

The first step in the manufacturing of ethanol can be the generation of glucose or simple sugars from biomass in general, and avocado seeds in particular [1]. In addition, in 2004, Werby and Petersen also identified that biomass could contribute to 12 building blocks of sugars through biochemical routes. Numerous conversion methods succeeded economically, and each method offers a substitute for fossil raw materials [21].

The compositions of AS are cellulose, hemicellulose, and lignin, forming a complex composition [19]. Meanwhile, it was strenuous for microorganisms to digest into tiny molecular weight sugars [22]. Lignin can prevent microorganisms from producing ethanol in subsequent processes. Moreover, to ensure hydrolysis and

fermentation proceed as expected AS requires a pretreatment stage to separate lignin and make the carbohydrates available [23], [24]. Therefore, the presentation of bioethanol is carried out using the following stages: pretreatment- steam delignification, catalytic hydrolysis using liquor HCl, and fermentation, which can be an exciting study.

Several researchers have investigated a considerable variety of biomass pretreatments to obtain specific sugar raw materials, catalyzed Steam Explosion (SE) [25], enzymatic hydrolysis [26], liquid hot water hydrolysis [27], and enzymatic hydrolysis combined with ultrasound [28]. This study proposes Steam-Delignification (SL) as an economical pretreatment of carbohydrate materials of AS. This assigns its robust biomass distraction to farmed residues, and one of the strongest, low chemical consumption and low power consumption prompted SL to investigate pre-treatments. However, some disadvantages of SL include modifying the lignin compounds into chemicals by hemicellulose-derived sugars that can inhibit the following steps; and the prospect possibility of extractives breaking down during the pretreatment [29].

Since hydrochloric acid is more affordable than other acids, such as phosphoric and sulfuric acid, which increase production costs, it was utilized in this study as a catalyst for the hydrolysis stage [9]. In addition, in dilute concentrations, hydrochloric acid shows a large amplitude for the digestion of the hemicellulose fraction [30]. The use of liquor hydrochloric acid as a catalyst in the hydrolysis of AS for obtaining some sugar has never been carried out by previous researchers.

It is more interesting from an ecological and economic perspective to treat all the pretreated biomass in the subsequent steps of catalytic hydrolysis and fermentation. Depending on the biomass source, the pretreatment parameters (mostly duration and temperature), and the presence or absence of base cooking liquor, the concentration of lignocellulosic chemicals changes after pre-treatment [9]. In some studies, the slurry obtained is then filtered and washed. Then, the solid, rich in cellulose, separates from the liquid phase. The solution includes trace amounts of acetic acid, hemicellulose, lignin, degraded carbohydrates, and other substances [26][28].

According to other researchers, after the biomass pretreatment, the following steps are catalytic hydrolysis and fermentation, possibly simultaneous, carried out in one reactor [31]. However, the fundamental drawback of the aforementioned step in comparison to a single hydrolysis-fermentation arrangement is that it is typically carried out at low operation temperatures, which corresponds to the inefficient activity of cellulolytic enzymes. Additionally, doing a step in advance of the in-situ scenario, such saccharification, is an option. The slurry's viscosity is lowered at high substrate stock as a result [29], [32]. Moreover, the glucose in the biomasses contains can be improved by using a suitable microorganism [32].

Saccharomyces cerevisiae ferments glucose to hydrolyze it, as demonstrated by this study [33]. In previous experiments, this bacterium produced high yields of ethanol from different biomass materials, achieving 0.24 g ethanol/g biomass [34].

The main objective of this study is to investigate the production of next-generation bioethanol from AS pretreated with SL, then continue the process of hydrolysis and fermentation by Saccharomyces cerevisiae.

2. Materials and Methods

2.1. Raw Material

Avocado seeds are obtained from farmers in the Bogor area (West Java, Indonesia). A total of 500 grams seeds were washed thoroughly to remove impurities. The prepared avocado seeds are cut into thin slices and then dried in the oven at 105°C for 24 hours. Additionally, avocado seeds that had been oven-dried were ground in a lab hammer mill (a Herzog grinding device), sieved with a pass size of 50 mesh, and kept at room temperature. Using NREL's normal technique, the moisture and the raw material composition were determined [7]. The prepared raw material can be seen in Figure 1.



Figure 1. Avocado Seed Powder (ASP) Preparation.

2.2. Study Process

ASP weighed 100 grams, then combined with 150 mL of 4% NaOH and 1350 mL of distilled water. The mixture was then heated at 80°C, stirred slowly for 2.5 hrs, then filtered and rinsed with hot water (100°C) to digest the lignin content. Furthermore, the filtered solids were dried using an oven at 105°C for 24 hours to obtain dried ASP. The ASP content is characterized by SEM (Olympus Type Quanta 650). Another part of the experiment is a hydrolysis process of avocado seeds lignin-free [36]. The reaction is carried out in a 500 mL reaction vessel at 60°C, containing 10 g ASP, 100 mL of 10%, and 15% HCl solution. After the hydrolysis reaction is completed in about an hour, reactor output, which contains high sugar, is fed into the fermenter using *Saccharomyces Cerevisiae* (SS) as yeast. The concentration of SS was applied at two different types, 30%, and 40%, with some nutrition contributing to microorganism growth, such as ammonium sulfate and urea as a nitrogen contributor ($\text{CO}(\text{NH}_2)_2$). The complete study steps as shown in Figure 2.

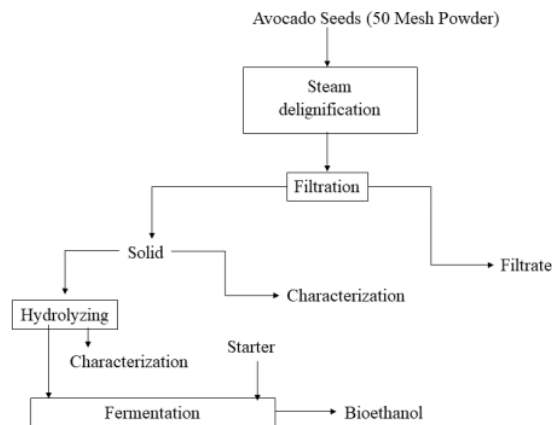


Figure 2. Study Flowchart for bioethanol production from AS.

2.3. Testing Methods

In this work, the AS utilized to measure the amount of lignin and carbs in biomass is determined by the NREL laboratory analytical process [7]. This study uses FTIR to analyze organic molecules with an IR range of 4000 cm^{-1} - 400 cm^{-1} , specifically related to glucose analytes' structure and functional groups. FTIR (Thermo Scientific Nicolet iS50) was used to determine the hydrolysis product's sugar content (glucose) from the resulting hydrolysis reaction. Prior to FTIR determinations, quantitative analysis using a Brix refractometer was also performed. The type of FTIR has a multi-coated, conical-shaped germanium tip crystal with a 350-micron spherical finish, a single reflection, throughput > 50%, and a 27° average angle, as well as an ATR detector with a liquid nitrogen-cooled MCT-A detector.

2.4. Microorganism and Growth Conditions

We employed SS, a bacterium that can ferment glucose. In a medium [35] made up of glucose, $(\text{NH}_4)_2\text{HPO}_4$,

and urea, the substrate was produced. They were agitated on a shaker for 24 hours at 200 rpm while the growth temperature was between 27 and 30 C.

2.5. Characteristics of the Fermentation

All experiments were duplicated in 500-mL fermenters glassware equipment (Pyrex, Germany) with a space volume of 250 mL. The starting substrate concentration of yeast was 30 % (w/v) and 40 % (w/v). In addition, the pH of the substrate was adjusted from 4 to 5. Temperature, rpm, and pH are the only three parameters that are set for each fermentation. 1 mL of fermenter samples were examined for periodic monitoring and analysis, and the sample was prepared before being added to the GC-MS apparatus.

2.6. Yields Calculation

The yield from hydrolysis was calculated using Equation (1) and is expressed as g glucose per 100 g carbohydrate present in the original material [36]:

$$Yield = \frac{(G-G_0) \times 0.03 \times 100}{C} \times 100 \quad (1)$$

where [G] denotes the quantity of glucose produced by the reaction, [G]0 is the amount of glucose in the feed raw material, [C] denotes the amount of carbohydrate present in 100 g of the original material, and [0.03] denotes the amount of catalyst at 4% (w/v).

Fermentation yields were determined by using Equation (2) [37]:

$$Yield = \frac{[E]}{[G]+[C]} \times 100 \quad (2)$$

where [G] and [C] are the substrate's starting concentrations of glucose and cellulose, and [E] is the concentration of ethanol in the fermentation broth.

3. Results

3.1. Samples Composition and SL-Pretreatment

Table 1 shows the carbohydrate and lignin content of the AS used. These results differ slightly from others due to different growing locations, farming practices, and analytical procedures [38].

The total carbohydrate percentage of the dry AS, based on FTIR analysis, was more than 50 %. Some organic chemicals and some trace elements, such as starches, resins, and gums, which may be present in smaller concentrations, were not examined [19]. This study is comparable to those described by other researchers in a prior study [27], [31], and [39], where the percentages for carbs ranged from 50 to 75% and for lignin from 17 to 20%.

Table 1. Composition of AS.

Compounds	% Dry weight
carbohydrate	54.85 ± 0.74
Lignin	19.32 ± 1.20

Mean values and standard deviations of duplicate determinations

This carbohydrate composition indicates that AS is a potential material for bioethanol production [40]. However, the lignin fractions in AS may hinder converting sugars to bioethanol, and steam lignification pre-treatment should ease these obstacles.

After SL pre-treatment, the lignin content decreased, as shown in Figure 3. The results show the images obtained by SEM on AS without and using pre-treatment. Based on the micrograph image in Figures 3a (magnificent 1000x), 3b (magnificent 5000x), 3c (magnificent 1000x), and 3d (magnificent 5000x), it can be seen that samples with SL pre-treatment (Figure 3c and 3d) presented more cracks or pores inside the lignin's matrices compare to Figure 3a and 3b. This result indicates a reduction in the lignin fraction, primarily due to the base solution of sodium hydroxide and thermally degradable lignin [39].

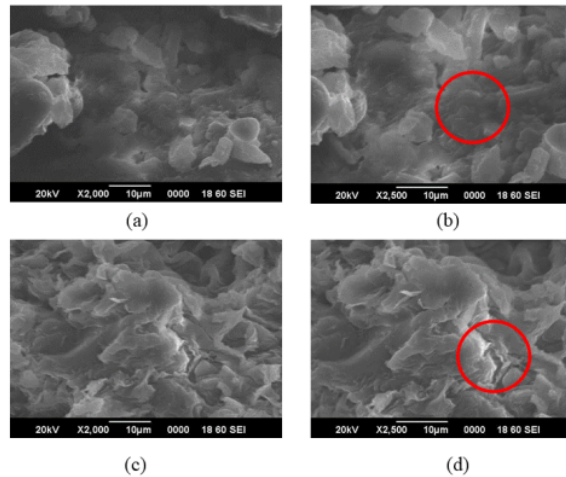


Figure 3. SEM Analysis of AS Without SL (3a, 3b) and with SL Pretreatment (3c, 3d)

Table 2 shows that higher glucose levels were obtained at a 15% concentration variation of HCl catalyst compared to 10% concentration, which was 29.5% Brix. This yield shows that HCl as a catalyst can convert carbohydrates into glucose, which is a source of nutrition for *Saccharomyces cerevisiae* in the fermentation stage [41].

Table 2. Analysis of hydrolysate from Pretreatment of AS (n=3 ± SD)

Catalyst Concentration	Glucose
HCl 10 %	27.5 ± 0.05
HCl 15 %	29.5 ± 0.05

In addition, glucose functional groups were tested using FTIR analysis of the Thermo Scientific Nicolet iS50 type. The glucose functional group is in the wave number range 2500-4000 cm^{-1} where there are three main functional groups, such as C-H with a wave number of 2800-3000 cm^{-1} ; N-H with wave numbers 3300-3600 cm^{-1} , and O-H with wave numbers 3300-3600 cm^{-1} [6]. Figure 4 shows that the test produces a glucose spectrum. Subsequently, glucose is the raw material for the fermentation process to produce ethanol, with the molecular formula $\text{C}_6\text{H}_{12}\text{O}_6$, which is shown at a wave number of 3340.79 cm^{-1} with a single broad-spectrum alcohol functional group (O-H). Therefore, the test results shown in Figure 4 confirm that the hydrolysate contains the dominant glucose.

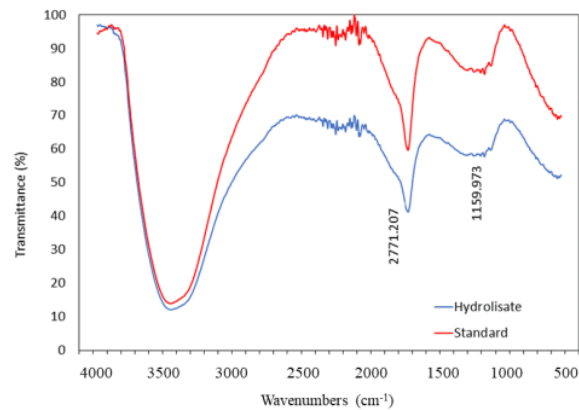


Figure 4. FTIR Spectrum of glucose

3.2. Fermentation of Hydrolysate

After the catalytic hydrolysis, the next process is the fermentation of the glucose in the hydrolysate. At this

step, two different concentration of SS plays on the fermentation. The fermented ethanol is then separated by distillation and analyzed to determine the ethanol content obtained. Table 3 shows that the highest bioethanol content was obtained at a concentration of 15% SS with a fermentation time of 6 days of 83.755%. At a substrate concentration of 10%, the bioethanol content was lower than that of the 15% substrate. This situation is due to the low initial yeast population, hence, it cannot maximally break down the available glucose into alcohol. In other words, the number of cells present is not proportional to the medium used, hence, the cells will experience a more extended lag phase [42]. In addition, the temperature is also one of the most critical parameters in ethanol production because the fermentation temperature can increase the yeast growth rate under certain conditions. However, if the temperature is too high, above 37°C, it can cause the deactivation of the yeast itself. Since the fermentation reaction is exothermic, the heat released must be controlled using a cooling system [43].

Table 3. Ethanol Yield Analysis (n=3±SD)

Reaction Time (Day)	SS Concentration (%)	
	10	15
4	83.145±2.0275	83.320±0.2748
6	84.345±0.8154	83.755±0.0686

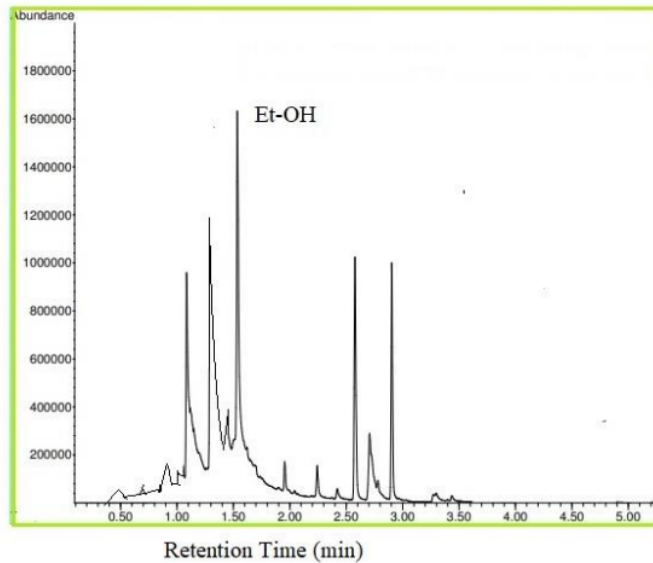


Figure 5. Ethanol chromatogram from the fermentation using SS 10%

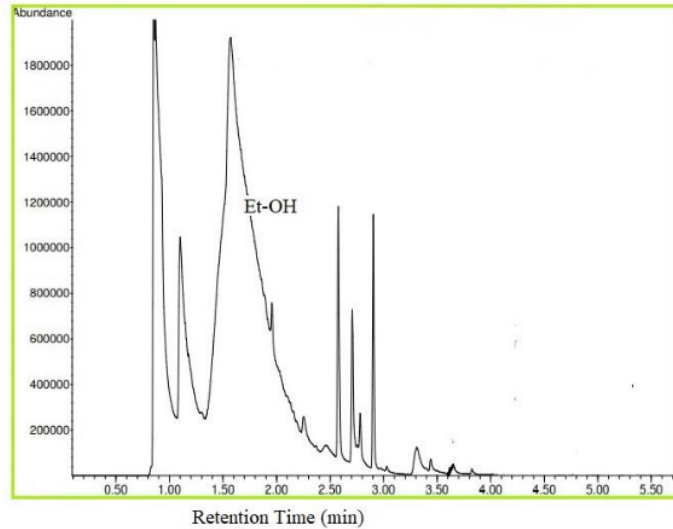


Figure 6. Ethanol chromatogram from the fermentation using SS 15%

Figure 6 indicates that the ethanol yield was higher at 15% SS concentration than at 10% SS, as shown in Figure 5. However, these results strongly agreed with those reported by other researchers. They investigated different biomass, such as sweet corn and olive tree pruning, which have (72–82% of the theoretical maximum) [25].

4. Conclusions

In this study, the test series for the production of bioethanol from avocado seeds were examined. The results show that avocado seeds have excellent potential as a raw material for the production of next-generation ethanol due to their pure sugar content. The steam delignification pretreatment of the SA before hydrolysis and fermentation then leads to the extraction of the lignin contained in the raw material as in the following steps. Then, compared to the theoretical, the maximum bioethanol yield from hydrolysate is 83.755%.

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