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Research Article

Investigating the potential of avocado seeds for bioethanol production: A study on boiled water delignification pretreatment

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Abstract. The increasing need for alternative fuels to replace fossil fuels has made bioethanol a promising option. Although numerous sources of sugar generation and agricultural wastes can be converted into ethanol, Avocado Seeds (AS) are particularly attractive as raw materials due to their abundance, high carbohydrate content, and lack of interactions with the food chain. Therefore, this study investigated the potential of AS for bioethanol production using several steps, including boiled water delignification pretreatment, catalytic hydrolysis, and fermentation with Saccharomyces cerevisiae. The delignification pretreatment of AS involved soaking in 4% (w/v) sodium hydroxide liquor for 24 hours. Then the mixture was heated to 80°C and stirred slowly for 2.5 hours and after that washing with boiled water at 100 °C for 1.5 hours and screening the mixture. Subsequently, catalytic hydrolysis and fermentation were carried out using two different concentrations of Saccharomyces cerevisiae as yeast, namely 10% (w/v) and 15% (w/v). Qualitative sample analysis was conducted using scanning electron microscopy (SEM) to observe the effect of delignification pretreatment, while FTIR analysis using Thermo Scientific Nicolet iS50 was used to test for glucose functional groups. Quantitative analysis was performed using gas chromatography 7890b mass spectrophotometry 5977A, Agilent DBVRX to determine hydrolysate fermentation. The results revealed that the highest ethanol yield was achieved through fermentation with 15% (w/v) yeast and 40% (v/v) catalyst, resulting in an ethanol yield of 83.755% of the theoretical maximum.

Keywords: agricultural waste; enzyme; fermentation; hydrolysis

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1. Introduction

The global economy is in need of a transition towards more sustainable and environmentally friendly energy sources, as the reliance on fossil fuels remains high (Deby *et al*., 2014; Fadhil *et al*., 2017). Therefore, biofuels are being considered as potential replacements for traditional fuels such as petrol, diesel, and aerostatic fuels (Ahlgren *et al*., 2017; Rahman Herliati *et al*., 2018). Simulation studies of the world energy systems predict that biofuels could contribute significantly, ranging from 10% to 40% of the market, in the long term, indicating a substantial increase in their utilization (Acevedo-García *et al*., 2018). In Indonesia, the production of biofuels at a competitive price is being explored to support the economic and energy security of the country (Raza *et al*., 2021). Several studies have considered biomass as the most promising renewable carbon source for biofuels (Yu *et al*., 2020; Zhao *et al*., 2009). The National Research and Innovation Agency of Indonesia predicts that new renewable energy, including biomass, will increase power and heat generation by 2035 (Frankowski *et al*., 2022; Rahman *et al*., 2019; Sluiter *et al*., 2011).

Bioethanol, derived from fruit waste biomass such as avocado seeds, is a viable biofuel option (Dong *et al*., 2019; Frankowski *et al*., 2022; Salehi *et al*., 2018). This biomass has several advantages, such as low cost, low dependence on the food chain, and colossal availability (Risyad *et al*., 2016; Muhammad *et al*., 2020; Mueansichai *et al*., 2022). According to the Central Bureau of Statistics Indonesia (BPS), 307.3 tons of avocados were produced in Indonesia in 2014 (Marlina *et al*., 2018; Sukaryo & Sri Subekti, 2017). The production rate continues to increase yearly, at a growth level of 24.48%, raising the number of avocado seeds (Sukaryo & Sri Subekti, 2017). Currently, avocado seeds are indiscriminately discharged into the environment, thereby leading to pollution (Risyad *et al*., 2016; Sluiter *et al*., 2011). However, there is growing interested in utilizing avocado seeds as a crucial biomass resource for bioethanol production, given their significant quantity and high cellulose content (Baruah *et al*., 2018; Paredes-Sánchez *et al*., 2021). According to the Food and Agriculture Organization (FAO), avocado (Persea Americana) is a tropical or subtropical fruit native to South America and widely grown in Asia (60%), including Indonesia (Hurtado-Fernández *et al*., 2018; Janice *et al*., 2018). The primary waste is avocado seeds, with a ratio of about 0.33 kg of seeds/kg of avocado (Acevedo-García *et al*., 2018; Ruiz *et al*., 2013). Due to its availability and high cellulose content (Baruah *et al*., 2018), [19], AS is regarded as an expected raw material for bioethanol.

The first step in ethanol manufacturing is the generation of glucose or simple sugars from biomass and avocado seeds (Acevedo-García *et al*., 2018; Ruiz *et al*., 2013). Additionally, in 2004, Werby and Petersen stated that biomass can yield 12

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building blocks of sugars through biochemical routes, further highlighting the potential of biomass as a renewable resource for ethanol production.

The compositions of AS are cellulose, hemicellulose, and lignin, forming a complex composition (Janice *et al*., 2018). However, due to their high molecular weight, these components are challenging for microorganisms to efficiently digest into tinny molecular-weight sugars (Krajang *et al*., 2021). Lignin can prevent microorganisms from producing ethanol in subsequent processes. Therefore, pretreatment of AS is necessary to separate lignin and make carbohydrates more accessible for hydrolysis and fermentation (Ghazanfar *et al*., 2022; Sultan *et al*., 2022). This typically involves stages such as boiled water delignification and catalytic hydrolysis using liquor HCl, followed by fermentation, making it a promising area of study for bioethanol production.

Numerous studies have explored diverse biomass pretreatment methods for obtaining carbohydrate materials from agricultural residues (AS). Some of the methods examined are Steam Explosion (SE) (Acevedo-García *et al*., 2018; Ruiz *et al*., 2013), enzymatic hydrolysis (Zakaria *et al*., 2014), liquid hot water hydrolysis (Pérez *et al*., 2008), and enzymatic hydrolysis combined with ultrasound (Subhedar & Gogate, 2013). Previous researchers using Steam Explosion during biomass pretreatments to obtain specific sugar raw materials. However, some disadvantages of SE 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 (Chen *et al*., 2022). So, this study using boiled water (100°C) during the pretreatment which is more interesting from an ecological and economic perspective.

This study chose hydrochloric acid as a catalyst for the hydrolysis stage due to its cost-effectiveness compared to other acids, such as phosphoric and sulfuric acid, which can increase production costs (Acevedo-García *et al*., 2018; Ruiz *et al*., 2006). Furthermore, hydrochloric acid has been found to exhibit significant effectiveness in digesting the hemicellulose fraction in dilute concentrations (Velmurugan & Muthukumar, 2011). The use of liquor hydrochloric acid as a catalyst in the hydrolysis of AS for obtaining some sugar has never been carried out in previous studies.

Treating all the pretreated biomass in subsequent steps of catalytic hydrolysis and fermentation is environmentally and economically beneficial. The concentration of lignocellulosic chemicals in the pretreated biomass tends to vary in accordance with factors such as biomass source, pretreatment parameters (duration and temperature), and the presence or absence of base cooking liquor (Acevedo-García *et al*., 2018). In some studies, the slurry was filtered and washed, and then the solid cellulose-rich material was separated from the liquid phase. The liquid phase typically contains small amounts of acetic acid, hemicellulose, lignin, degraded carbohydrates, and other substances (Subhedar & Gogate, 2013; Zakaria *et al*., 2014).

Preliminary studies stated that after biomass pretreatment, catalytic hydrolysis and fermentation should be carried out simultaneously in a single reactor (Liu & Dien, 2022). 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, corresponding to the inefficient activity of cellulolytic enzymes. According to Chen *et al*. (2022), performing saccharification as a separate step prior to in-situ hydrolysis-fermentation is an option, as it can lower the viscosity of the slurry at high substrate concentrations. The glucose in the biomasses can be improved by using a suitable microorganism (Kim, 2018).

According to Ghazanfar *et al*. (2022), saccharomyces cerevisiae ferments glucose through hydrolysis. Previous studies have shown that this yeast can produce high yields of ethanol from various biomass materials, achieving up to 0.24 g ethanol per gram of biomass (Acevedo-García *et al*., 2018; Ruiz *et al*., 2013; Song *et al*., 2021). The primary objective of this study is to explore the production of next-generation bioethanol using AS pretreated with BD, followed by the subsequent steps of hydrolysis and fermentation using Saccharomyces cerevisiae.

2. Materials and Methods

2.1 Raw Materials Preparation

A total of 500 grams of avocado seeds sourced from cafes and restaurants in Bogor, West Java, Indonesia, were thoroughly washed to remove impurities. The seeds were sliced into thin pieces and dried in an oven at 105°C for 24 hours (Fülöp & Ecker, 2020). Additionally, the oven-dried seeds were ground in a laboratory hammer mill using a Herzog grinding device, and then sieved to a pass size of 50 mesh before being stored at room temperature. The moisture content and raw material composition of the prepared avocado seeds were determined using the standard technique of the National Renewable Energy Laboratory (NREL) (Sluiter *et al*., 2011). The prepared raw material is shown in Figure 1.

2.2 Experimental Process

The experimental process started with weighing 100 grams of ASP, which was then mixed with 150 mL of 4% NaOH solution and 1350 mL of distilled water and soaking for 24 hours. The mixture was heated to 80°C and stirred slowly for 2.5 hours. Afterward, it was filtered and rinsed with boiled water at 100°C to remove the lignin content. The filtered solids were then dried in an oven at 105°C for 24 hours to obtain dried ASP, which was characterized using SEM with an Olympus Type Quanta 650. Another part of the experiment involved the hydrolysis of avocado seeds that were previously treated to remove lignin (Chhouk *et al*., 2017). The hydrolysis reaction was carried out in a 500 mL reaction vessel at 60°C, using 10 g of ASP, 100 mL of 30% and 40% HCl solutions. Once the hydrolysis reaction was completed in about an hour, the resulting output, which contained high sugar concentration, was

a. Fresh Avocado seeds b. Flake Avocado seeds

c. Powder Avocado seeds (50 Mesh)

Fig 1 Avocado Seed Powder (ASP) Preparation

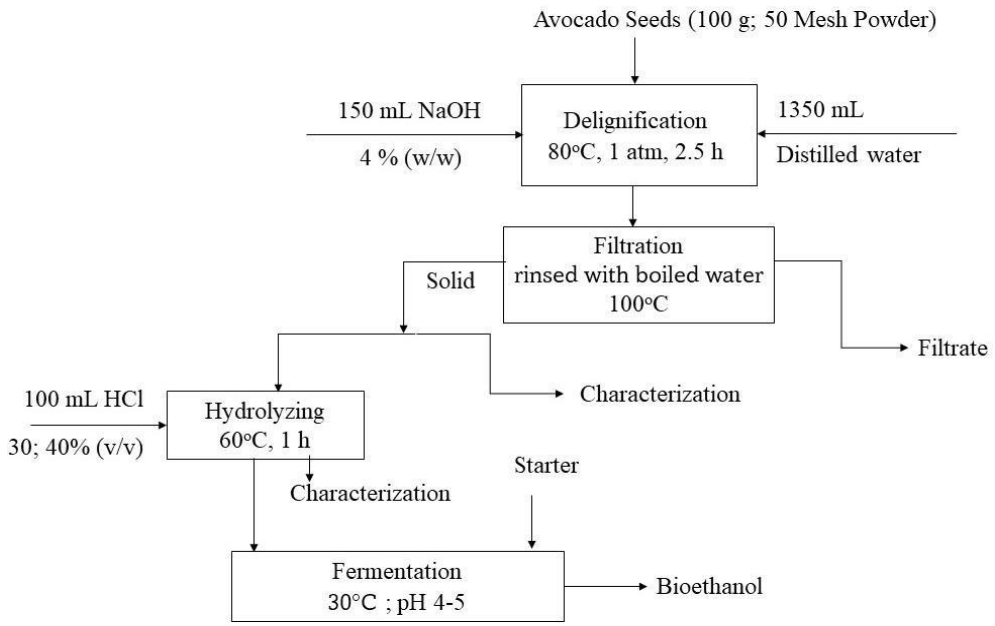

Fig 2 Study Flowchart for bioethanol production from AS

transferred to a fermenter. Saccharomyces cerevisiae (SS) yeast was used for fermentation, with two different concentrations of SS, 10% and 15%, along with additional nutrients such as ammonium sulfate and urea as a nitrogen source ($CO(NH_2)_2$) to support microorganism growth. The overall experimental steps are shown in Figure 2.

2.3 Testing Methods

The NREL laboratory analytical process, was employed in this study to quantify the amount of lignin and carbohydrates in biomass (Sluiter *et al*., 2011). FTIR was utilized to analyze organic molecules within the IR range of 4000 cm^{-1}- 400 cm^{-1}, specifically focusing on the structure and functional groups of glucose analytes. The FTIR instrument used in this study was Thermo Scientific Nicolet iS50, equipped with a multi-coated, conical-shaped germanium tip crystal with a 350-micron spherical finish, a single reflection, throughput > 50%, and a 27° average angle. It also featured an ATR (Attenuated Total Reflectance) detector with a liquid nitrogen-cooled MCT-A detector (Fülöp & Ecker, 2020).

2.4 Microorganism and Growth Conditions

This study was carried out using SS, a bacterium known for its ability to ferment glucose (Mishra *et al*., 2016). One gram of each sample was diluted in buffered saline and plated on tributyrin agar (TBA) plates in 100 µL aliquots. The TBA plates were composed of 0.5% peptones, 0.3% yeast extract, 1% agar, and 0.1 ml tributyrin, with the pH adjusted to 5.5. Prior to use, tributyrin was sterilized through membrane filtration and the filtrate was added to the basic growth medium. After 24 hours of incubation at 30°C, each colony was selected and streaked to obtain pure cultures (Godoy *et al*.,2018).

2.5 Characteristics of the Fermentation

The experiments were conducted in duplicate using 500-mL fermenters made of glassware equipment from Pyrex, Germany, with a working volume of 250 mL. The initial substrate concentration of yeast was set at 10% (w/v) and 15% (w/v) for different trials. In addition, the pH of the substrate was adjusted from 4 to 5. Temperature, rpm and pH were the only parameters that were set and controlled for each fermentation. Periodic monitoring and analysis were carried out by examining 1 mL samples from the fermenters. Prior to analysis using GC-MS, the samples were prepared according to the established protocol.

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 (Acevedo-García *et al*., 2018):

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

where [G], $[G]_0$, [C] and [0.03] denote the quantity of glucose produced by the reaction, the amount of glucose in the feed raw material, the amount of carbohydrate presents in 100 g of the original material, and the amount of catalyst at 4% (w/v), respectively.

Fermentation yields were determined using Equation (2) (Acevedo-García *et al*., 2018):

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

where [G] and [C] are the substrate's starting concentrations of glucose and cellulose (mg/mL), and [E] is the concentration of ethanol in the fermentation broth (mg/mL) (Acevedo-García *et al*., 2018).

3. Results

3.1 Samples Composition and BD-Pretreatment

Table 1 shows the carbohydrate and lignin content of the AS used, which exhibit slight variations from other studies due to

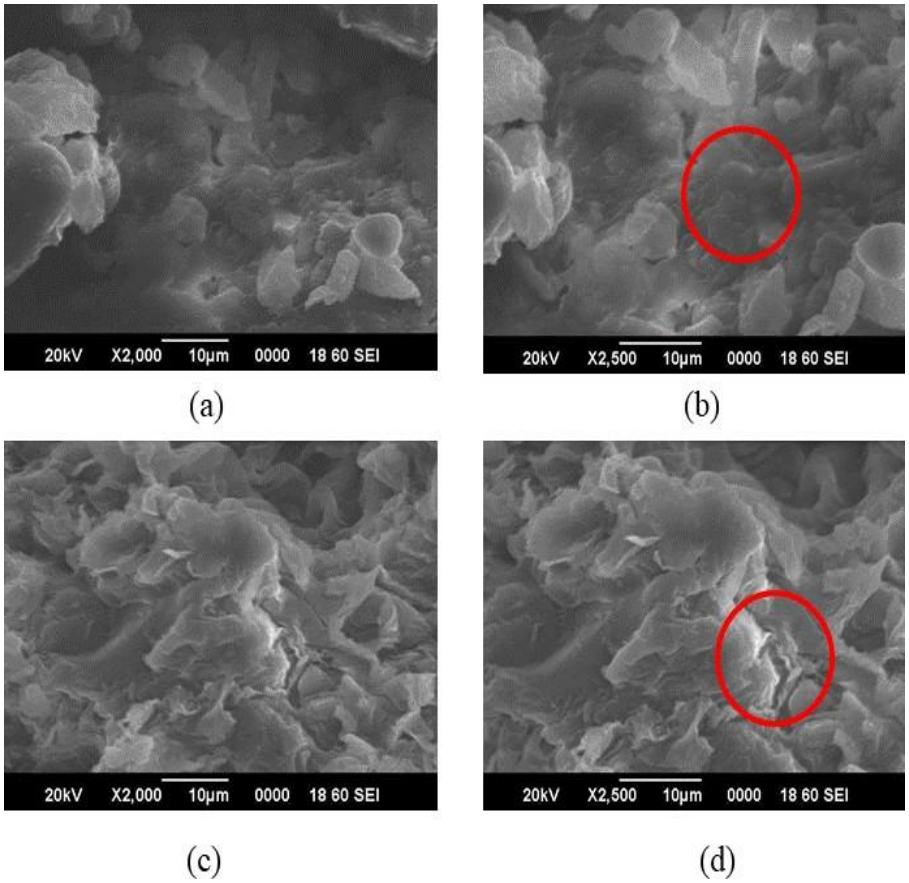

Fig 3 SEM Analysis of AS Without BD (3a, 3b) and with BD Pretreatment (3c, 3d)

Table 1
Composition of AS (n=3 ± SD)

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

factors such as diverse growing locations, farming practices, and analytical procedures (Ifesan *et al*., 2015).

According to FTIR analysis, the dry AS showed a total carbohydrate percentage of over 50%. However, some organic chemicals and trace elements, such as starches, resins, and gums, present in smaller concentrations, were not examined (Acevedo-García *et al*., 2018). This study is comparable to those described by preliminary studies (Ji *et al*., 2022; Liu & Dien, 2022; Pérez *et al*., 2008), where the percentages for carbs and lignin ranged from 50 to 75% and 17 to 20%, respectively.

This carbohydrate composition indicates that AS is a potential material for bioethanol production (Ji *et al*., 2022). However, the lignin fractions in AS may hinder converting sugars to bioethanol, which can be alleviated through boiled water lignification pretreatment.

Figure 3 shows the impact of BD pretreatment on the lignin content, as observed through SEM micrograph images. Based on the micrograph image, at magnifications of 1000x (Figure 3a) and 5000x (Figure 3b), AS samples without pretreatment showed less cracks or pores in the lignin matrices. However, samples subjected to BD pretreatment Figures 3c and 3d at magnificent of 100x and 5000x, exhibited more cracks or pores within the lignin matrices. This result indicates a reduction in the lignin fraction, primarily due to the base solution of sodium hydroxide and thermally degradable lignin (Ji *et al*., 2022).

3.2 Hydrolysis

After the delignification stage, the filtrate was subjected to hydrolysis by adding hydrochloric acid (HCl) in a ratio of 2:1 to carbohydrates. The mixture was stirred using a magnetic stirrer and heated at 60°C for 2.5 hours. Two different concentrations of HCl, 30% and 40% (v/v), were used as catalysts to accelerate the hydrolysis reaction rate. The hydrolysis reaction was carried out in excess water to ensure a pseudo-first order reaction, where changes in carbohydrate concentration determine the reaction rate. At the end of the hydrolysis reaction, the resulting mixture was cooled to room temperature, filtered, and the filtrate, rich in glucose, was used as the feed for the fermentation process. Prior to fermentation, the filtrate was pasteurized at 70°C for 15 minutes and adjusted to pH 5 as required. Different concentrations of solid substrate (SS), 10% (w/v) and 15% (w/v), were added to each sample, and then the samples were incubated at 30°C for 4 to 6 days in a tightly closed environment to allow anaerobic fermentation. The results of the reaction were analyzed using a refractometer, and Table 2 shows that the glucose content in the hydrolyzed samples using 30% and

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

Catalyst Concentration	Glucose (% Brix)
HCl 30 %	27.5 ± 0.05
HCl 40 %	29.5 ± 0.05

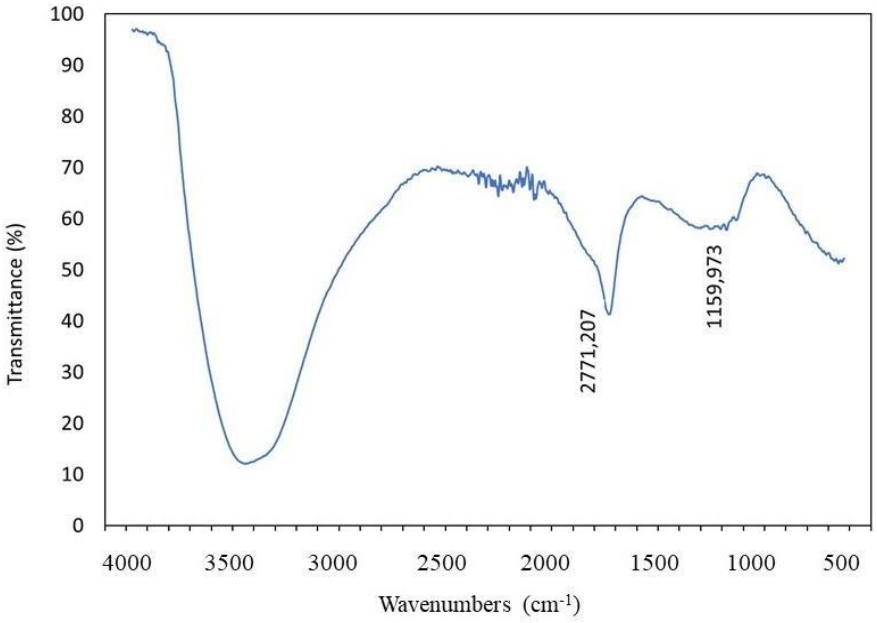

Fig 4 FTIR Spectrum of glucose

40% HCl were 27.5% and 29.5% Brix, respectively. This result indicates that the hydrolysis process to convert carbohydrates into glucose has been successful.

In addition, the functional groups of glucose were analyzed using FTIR analysis with a Thermo Scientific Nicolet iS50 type. The wave number range for glucose functional groups was determined to be between 2500-4000 cm^{-1}, which includes three main functional groups, namely C-H, N-H and O-H with wave numbers of 2800-3000 cm^{-1}, 3300-3600 cm^{-1}, and 3300-3600 cm^{-1}, respectively. Even though N-H and O-H groups have similar wave numbers, both can be differentiated by their spectral shapes. A widened and halved spectral shape characterized the N-H group, while the O-H group also has a broadened but not halved spectral form. In addition, the wave numbers 2200 cm^{-1}, 2100 cm^{-1} and 1600-1800 cm^{-1} are in C≡N, C≡C and three functional groups, namely C=O, C= C, and C=N (R *et al*., 2017; Yu *et al*., 2020). Figure 4 shows the spectrum of glucose, confirming its presence in the hydrolysate with a molecular formula of $C_6H_{12}O_6$, indicated by a single broad-spectrum alcohol functional group (O-H) at a wave number of 3340.79 cm^{-1}. Therefore, the test results shown in Figure 4 confirm that the hydrolysate contains the dominant glucose.

3.3 Fermentation of Hydrolysate

The fermentation of glucose in the hydrolysate is the subsequent process after catalytic hydrolysis. In this study, the effect of substrate concentration on the fermentation reaction was investigated using Saccharomyces cerevisiae at concentrations of 10% and 15% (w/v). *S.* cerevisiae is an acidophilic microbe that thrives at a maximum pH of 5 (Rahman *et al*., 2018). Therefore, fermentation was carried out under acidic conditions with a pH range of 4-5. Analysis of the fermentation results indicated that a slightly higher ethanol content was obtained at a substrate concentration of 15% (w/v) compared to 10% (w/v). The fermented ethanol was subsequently separated by distillation and analyzed to determine the ethanol content obtained. Table 3 revealed that the highest bioethanol content of 83.755% was achieved at a concentration of 10% substrate with a fermentation time of 6 days. In contrast, the bioethanol content at a substrate concentration of 10% was lower than that of the 15% substrate. This observation is attributed to the low initial yeast population, which was unable to optimally break down the available glucose into alcohol due to an insufficient number of cells proportional to the medium used, resulting in an extended lag phase (Tan *et al*., 2019). Additionally, temperature played a critical role in ethanol production, as it influenced the yeast growth rate. When 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 (Mishra *et al*., 2016).

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

Quantitative testing of fermented ethanol content using GC-MS was carried out by injecting the sample using a syringe into the injector. The sample was then evaporated and carried by the carrier gas to the separation column, where an interaction occurs between the mobile and stationary phases. Each component in a mixture interacts at a different rate depending on the volatility of that compound. The most volatile component interacts the fastest with the stationary phase, exiting the column first, followed by the other, heavier components. Each component is bombarded with electrons through the ionizing chamber resulting in ionization. The ion fragments are then received by the detector and displayed as a mass spectrum, as shown in Figures 5 and 6, which depict the ethanol chromatogram from the fermentation. The chromatograms indicate that the ethanol yield was slightly higher at 15% SS concentration compared to 10% SS, as seen from the area of the chromatogram. However, these results are also better than those reported by other researchers who investigated different biomass and fermentation bacteria, such as olive tree pruning

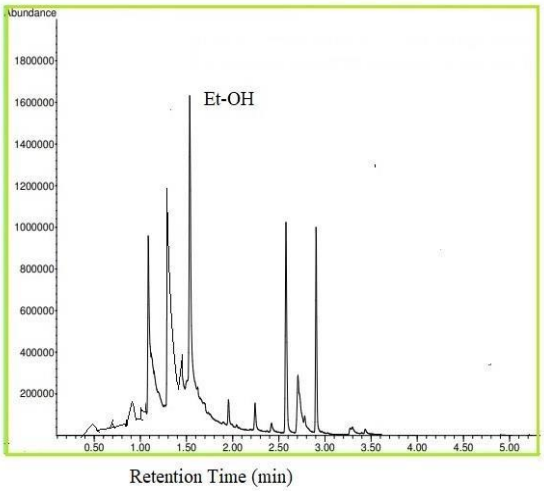

Figure 5 Ethanol chromatogram using SS 10%

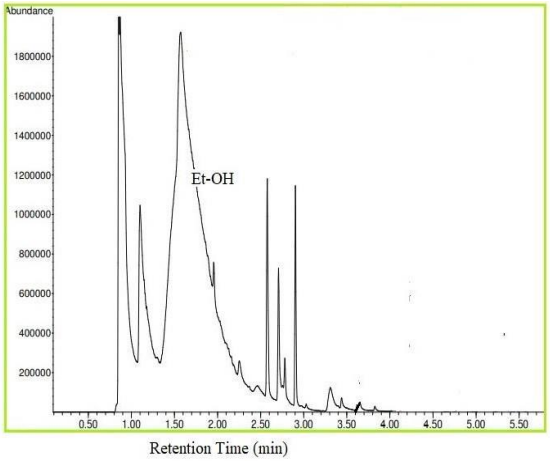

Figure 6 Ethanol chromatogram using SS 15%

by e-coli, which achieved 72-82% of the theoretical maximum (Ruiz *et al*., 2006).

4. Conclusions

In conclusion, this study examined the feasibility of producing bioethanol from avocado seeds through a series of tests. The result showed that avocado seeds are a promising raw material for next-generation ethanol production due to their high sugar content. The study employed boiled water delignification pretreatment of the avocado seeds prior to hydrolysis and fermentation, which allowed for the extraction of lignin from the raw material in subsequent steps. The maximum bioethanol yield from the hydrolysate was found to be 83.755% when compared to the theoretical yield.

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Conflicts of Interest: The authors declare no conflicts of interest to report regarding the present study.

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