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Pretreatment of sugarcane bagasse autoclave via assisted-alkali hydrogen peroxide and enzymatic hydrolysis on *mucor* circinelloides cultivation

Gregory Hope Soegiantoro
Sepuluh Nopember Institute of Technology, Department of Engineering Physics, Surabaya, Indonesia, gsoegiantoro@gmail.com
Akhmad Faruq Alhikami
University of Islam Malang, Department of Mechanical Engineering, Malang, Indonesia, alhikami@unisma.ac.id
Azzah Dyah Pramata
Sepuluh Nopember Institute of Technology, Department of Material and Metallurgical Engineering, Surabaya, Indonesia, azzah@its.ac.id
Daffa Hibatullah
Sepuluh Nopember Institute of Technology, Department of Engineering Physics, Surabaya, Indonesia, daffahibatullah02@gmail.com
Fani Ahmad Refansah
Sepuluh Nopember Institute of Technology, Department of Engineering Physics, Surabaya, Indonesia, fani.refansah@gmail.com
Fernando Wijaya
Sepuluh Nopember Institute of Technology, Department of Engineering Physics, Surabaya, Indonesia, fernandowijaya365@gmail.com
Satria Rafif Rafidianto
Sepuluh Nopember Institute of Technology, Department of Engineering Physics, Surabaya, Indonesia, satriarff21@gmail.com
Mutiara Rizki Maulida
Sepuluh Nopember Institute of Technology, Department of Engineering Physics, Surabaya, Indonesia, mutiararizki212@gmail.com
Gunawan Nugroho
Sepuluh Nopember Institute of Technology, Department of Engineering Physics, Surabaya, Indonesia, gunawan@ep.its.ac.id
Nur Laila Hamidah *
Sepuluh Nopember Institute of Technology, Departement of Engineering Physics, Surabaya, Indonesia, nurlaila@its.ac.id

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* *Corresponding Author*

Abstract: This study aims to evaluate the characteristics and feasibility of the autoclave assisted-alkali hydrogen peroxide delignification and enzymatic hydrolysis pretreated sugarcane bagasse for *Mucor circinelloides* cultivation to produce biofuels. The experimental setup consists of unpretreated sugarcane bagasse (SCB), delignified SCB, hydrolyzed SCB, and delignified-hydrolyzed SCB. The characterization was done using FTIR, XRD, and HHV calorimeter. The pretreatment of sugarcane bagasse using autoclave-assisted alkali hydrogen peroxide delignification was able to remove the lignin and hemicellulose. At the same time, the following enzymatic hydrolysis was able to increase the digestibility of sugarcane bagasse's cellulose, making it suitable for Mucor circinelloides cultivation. The cultivation of Mucor circinelloides was done in the 100 ml sugarcane bagasse hydrolysate medium for 72 hours and a 250 rpm stirring rate produced approximately 9.1 grams of lipid.

Keywords: *Pretreatment, lignocellulose, mucor circinelloides, sugarcane bagasse*

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1. INTRODUCTION

Currently, fossil fuels have become the main energy source since they fulfill 80% of the energy demand [1,2]. However, the depletion of fossil fuel and increase of greenhouse gas emissions has urged the exploration and utilization of renewable energy sources. Petroleum, coal, and gas reserve are expected to last only until year 2046, 2206 and 2076, respectively [3]. Moreover, fossil fuel combustion contributes 90% of total global CO_2 emissions [4]. Biofuels have become an attractive fossil fuel substitution as it has neutral carbon producing, sustainable supply, producible in rural areas, and promoting diverse energy sources. Biofuel can be produced from various biomass divided to four generations biofuel. The first-generation biofuel uses edible crops: Palm oil, corn, soybean, etc. Second generation biofuel uses inedible crops: Rice husks, corncob, switchgrass, etc. Third generation biofuel uses algae and other microorganism, while fourth generation biofuel use genetically modified microorganism [5]. Currently, the main raw material to fulfill biofuel demand in Indonesia is edible crops. This can trigger fluctuations in food and biofuel prices, disrupt food security, and lead to an increase in land conversion to crops that can damage Indonesia's biodiversity and ecosystem balance [6].

On the other hand, sugarcane is one of the leading agricultural commodities in Indonesia. The area of sugarcane plantations has keep increasing since 2019. In 2021, sugar production increased by 10.6% from the previous year [7]. However, in sugar production, 90% of sugarcane mass will become waste in the form of sugarcane bagasse (SCB). Although there are some alternatives on SCB utilization, the economic value is very low [8]. Utilization of SCB in renewable energy production has been done to produce bioethanol at both laboratory and industrial scale [9]. While biodiesel production using SCB has only been done outside Indonesia at laboratory scale [10,11,12,13,14]. The high productivity of sugar in Indonesia has created great potential for biodiesel production using SCB.

One of the most prominent oleaginous microorganisms researched for biofuel production using SCB is *Mucor circinelloides* as it is easy to cultivate on various carbon sources, having fast life cycle, and it's able to accumulate high lipid content [15]. This lipid can use to produce biofuel, especially biodiesel. Studies has shown that *Mucor circinelloides* was able to produce 12.5 gram dry biomass per liter, with lipid concentration of 25% and 96.2% maximum biodiesel conversion yield [11]. Another research has also showed that *Mucor circinelloides* can produce up to 51% lipid content using used cooking oil as carbon source [16]. However, lignin and hemicellulose content in SCB can inhibit the hydrolysis of fungi. Therefore, media preparation in the forms of delignification and enzymatic hydrolysis is required to increase the lipid production yield [17]. In this research, the effect of autoclave assisted-alkali hydrogen peroxide delignification and enzymatic hydrolysis on the lipid yield characterization of *Mucor circinelloides* growth is studied.

2. METHODS

The experimental setup in this study consists of 4 independent variables, namely unpretreated SCB, pretreated SCB with autoclave assisted-alkali hydrogen peroxide delignification (D-SCB), pretreated SCB with enzymatic hydrolysis (H-SCB), and pretreated SCB with consecutive autoclave assisted-alkali hydrogen peroxide delignification and enzymatic hydrolysis (DH-SCB).

2.1. Material & Chemicals

SCB was obtained from a sugarcane drink stall in Surabaya, Indonesia. Sodium hydroxide, hydrogen peroxide, and other chemical materials were purchased from PT. Nirwana Abadi in Surabaya, Indonesia.

2.2. Delignification SCB Sample

Delignification of SCB was done to dissolve the lignin and bleach the SCB. This step is essential to ensure access to the cellulose and hemicellulose layer in the next steps by removing the lignin that protects the cellulose and hemicellulose. This step was conducted using 3% sodium hydroxide and 6.25% hydrogen peroxide with a solid to liquid ratio of 1:10 in an autoclave for 30 minutes and temperature 121℃ [18]. Delignified SCB was then washed, filtered, and oven-dried at 40° C for 8 hours [19], [20].

2.3. Hydrolysis SCB Sample

Hydrolysis was conducted by the addition of 5 times citric acid buffer solution and 20 FPU cellulase per gram of SCB with 200 rpm stirring time for 24 hours at 50°C in a batch reactor [21]. This process will produce a solution of simple sugar and the precipitate. Both are used for *Mucor circinelloides* cultivation.

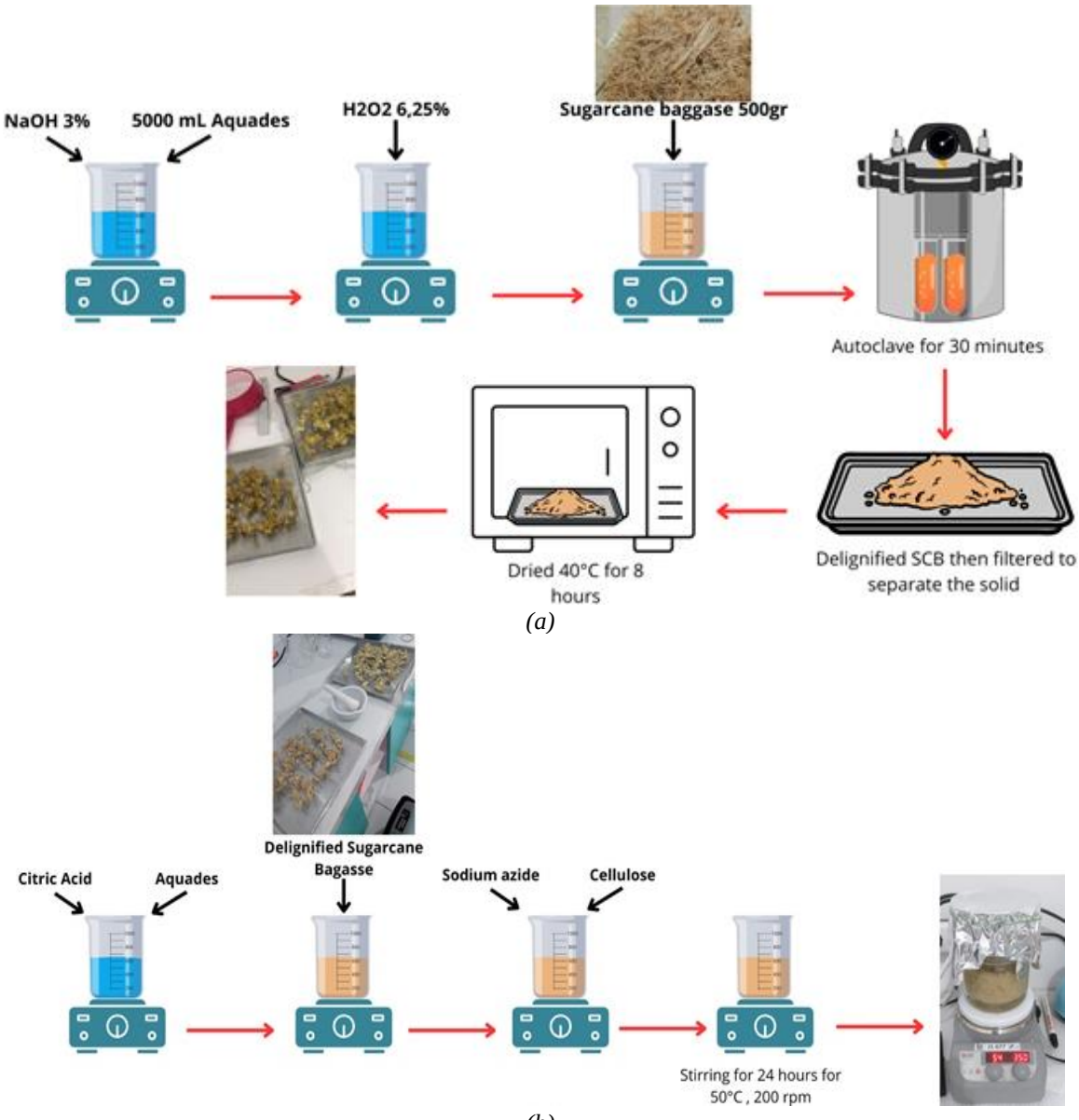

Figure 1. SCB pretreatment schematic: (a) Delignification and (b) hydrolysis.

2.4. Fourier Transform Infrared Spectroscopy (FT-IR)

The SCB samples were characterized using FTIR analysis by Thermo Scientific Nicolet iS10. The peaks identified were then compared to the literature to determine the chemical bonds in the samples [22].

2.5. X-ray Diffraction (XRD)

XRD analysis was performed by using an XRD type PanAnalytical type Xpert PRO to analyze the crystallinity of the samples. The XRD measurements were performed under the conditions an angular range of 5 − 80°, using CuKα wavelengths of 1.54056 Å, Ni filter, 40 kV, and 30 mA. The samples were analyzed in the range of 2θ =5 − 50° scanning speed 0.5° C/min and 0.05°/step at room temperature. The crystallinity of the samples was calculated using Gaussian peak fitting without a linear background (Gaussian peaks), where the area of crystalline peaks was used to calculate the peak using the equation

$$C = \frac{A_{cr}}{A_{sample}} = \frac{\int_{2\theta_1}^{2\theta_2} I_{cr} d2\theta}{\int_{2\theta_1}^{2\theta_2} I_{sample} d2\theta} \quad (1)$$

where A_{cr} is the area of the crystalline peak and A_{sample} is the area under the sample intensity curve [23].

2.6. Bomb Calorimeter

A bomb calorimeter is an apparatus used to measure heat produced by a combustion process. It is an enclosed space covered in conductor metal named 'Bomb' that is surrounded by water. It is equipped with an electrical heating device to start the combustion, stirrer, and thermometer. A bomb calorimeter can be used to measure the heat produced by the combustion of solid or liquid fuel, biomass, food, etc. Using gas reactant in the bomb, the sample tested will be combusted and produce heat. The heat produced in this process will be applied to the water surrounding the bomb vessel. A stirrer is used to maintain uniform water temperature and the thermometer is used to measure the temperature change by the combustion process [24]. This testing was performed using a digital bomb calorimeter Athena Technology with sample masses ranging from 0.73 to 0.9 grams.

2.7. Gas Chromatography-Mass Spectrometry (GC-MS)

Agilent 6980N Network gas chromatograph mass spectrometer was used to identify the main composition of SCB and D-SCB in the methanol-soluble portion. HP-5MS (30 m long, 0.25 mm inner diameter, 0.25 μm film thickness) was used as a capillary column. The temperature program was 1.0 min at 40° C, 5.0° C/min to 300° C, and 1.0 min at 300° C. The Flow rate in the column was constant at 1.5 ml/min. Injector and detector temperatures were both 300° C [25]. 500 mg of each SCB and D-SCB were extracted with 3 ml methanol. Both samples were vortexed for 2 minutes and sonicated for 15 minutes. SCB and D-SCB solution was filtered with 0,2 μm membrane filter and directly injected into GCMS.

2.8. Pre-cultivation for *Mucor circinelloides*

Pre-cultivation of *Mucor circinelloides* was done in slanted PDA media in test tubes for 72 hours. Subsequently, the accumulated spore was calculated using the total plate count method. The total plate count was conducted by adding 10 ml aquades into the slanted media test tube to make a spore solution. Then, 1 ml of the spore solution was mixed with aquades to make a 10% TPC solution. This step was iterated 6 times up to 10^{-6} TPC solutions. 10% of each solution was spread on a PDA plate and labeled. After 24 hours, individual spores in each plate were observed and calculated.

2.9. Cultivation for *Mucor circinelloides*

The cultivation of *Mucor circinelloides* was conducted in 100 ml delignified SCB hydrolysate mixed with 5% molasses and 15% Tween 80, both relative to the volume of the hydrolysate, in an enclosed beaker glass, having a stirring rate of 250 rpm and 0.5 liter/min oxygen flow. The cultivation was kept at a pH range of 4-5 and temperature under 35° C for 3 days. After 3 days, 0.08% of the cultivation solution was extracted for total plate count using the same method as used in the pre-cultivation calculation.

3. RESULTS AND DISCUSSION

3.1. XRD Result

The XRD analysis was used to determine the crystallinity of each sample. Fig. 2 shows the XRD diffractogram of SCB, D-SCB, H-SCB, and DH-SCB. The peak identified from SCB, D-SCB, and H-SCB samples was observed around 16.2º and 21.9º, corresponding to the XRD analysis of SCB [26], [27]. The crystallinity of SCB was initially increased from 59.6% in the unpretreated SCB to 87% in the D-SCB. This increase was caused by the removal of amorphous lignin and hemicellulose, and not due to the change in the cellulose's crystallinity [27].

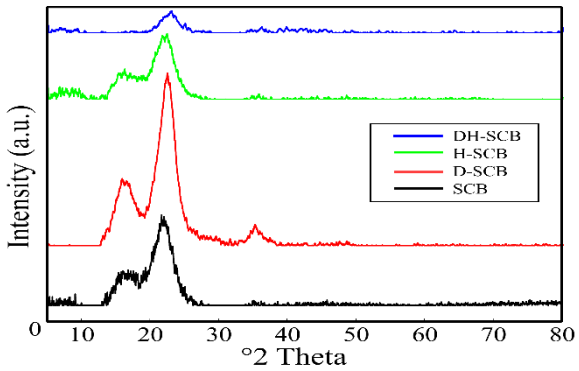

Figure 2. XRD results of SCB.

The H-SCB sample has a crystallinity of 62%, while the DH-SCB has the lowest crystallinity of 24.2%. Crystallinity is an important property of cellulose that indicates the accessibility of cellulose for enzymatic digestion by microorganisms. Lower crystallinity indicates a higher amorphous value, which increases the digestibility of cellulose in SCB. The XRD results also confirm that the autoclave assisted-alkali hydrogen peroxide delignification was able to break the amorphous lignin and hemicellulose in SCB, exposing the crystalline cellulose for hydrolysis. The increase of crystallinity in the H-SCB sample indicates that without delignification, the enzymatic hydrolysis failed to convert cellulose into individual sugars due to the presence of lignin and hemicellulose as barriers. While the low crystallinity in the DH-SCB sample confirms that the enzymatic hydrolysis reaction, following the autoclave assisted-alkali hydrogen peroxide delignification increases the digestibility of SCB cellulose for *Mucor circinelloides* cultivation [28], [29]

3.2. FT-IR Result

FTIR analysis was conducted to analyze the molecule structure of unpretreated and pretreated samples. It is shown in Figs. 3 and 4. The FTIR spectra of unpretreated, delignified, and hydrolyzed, compared with delignified and hydrolyzed SCB, summarized in Table 1.

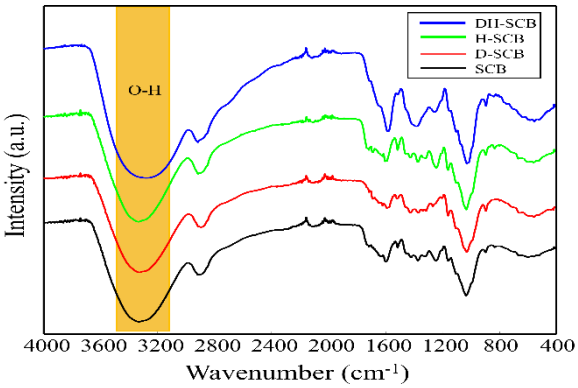

Figure 3. FT-IR of SCB.

The O-H alcohol stretching at the wavenumber 3335 cm^{-1} was widened in the DH-SCB, while increased in the H-SCB. The hemicellulose functional group was observed in 1716 and 1688 cm^{-1} peaks, identified as C=O. These peaks were observed to be increased in the D-SCB and disappear in the H-SCB and DH-SCB, indicating the increasing exposure of hemicellulose in D-SCB due to the removal of lignin, and conversion of hemicellulose into xylose in the hydrolysis process. The lignin functional group was observed at 1620, 1590, 1516, 1338, and 1317 cm^{-1} peaks, identified as C=C bond, C=C aromatic vibration, C-O bond, sryngyl ring, and G unit. The 1620 cm^{-1} peak was narrowed in D-SCB and H-SCB, while disappearing in DH-SCB. The peak around 1590 cm^{-1} was stretched in D-SCB and H-SCB, while shifted and stretched to 1583 cm^{-1} in DH-SCB. The 1516 cm^{-1} peak was reduced in D-SCB, stretched in H-SCB, and shifted in DH-SCB. The 1317 cm^{-1} peak stretched in D-SCB and disappeared in DH-SCB, while the 1338 cm^{-1} peak only appeared in D-SCB. These results indicate the removal of lignin specific in the delignification process, while showing that hydrolysis process was unable to remove lignin. The peaks related to cellulose content are observed at 2920, 1424, 1373, and 1241-833 cm^{-1}, identified as C-H stretching, O-H bending, C-O stretching, C-O-C asymmetric deformation, glucose ring asymmetric stretching, C-O stretching, C-O-C stretching, and the vibrations of the guaiacyl units. The 2920 cm^{-1} peak was significantly stretched in DH-SCB.

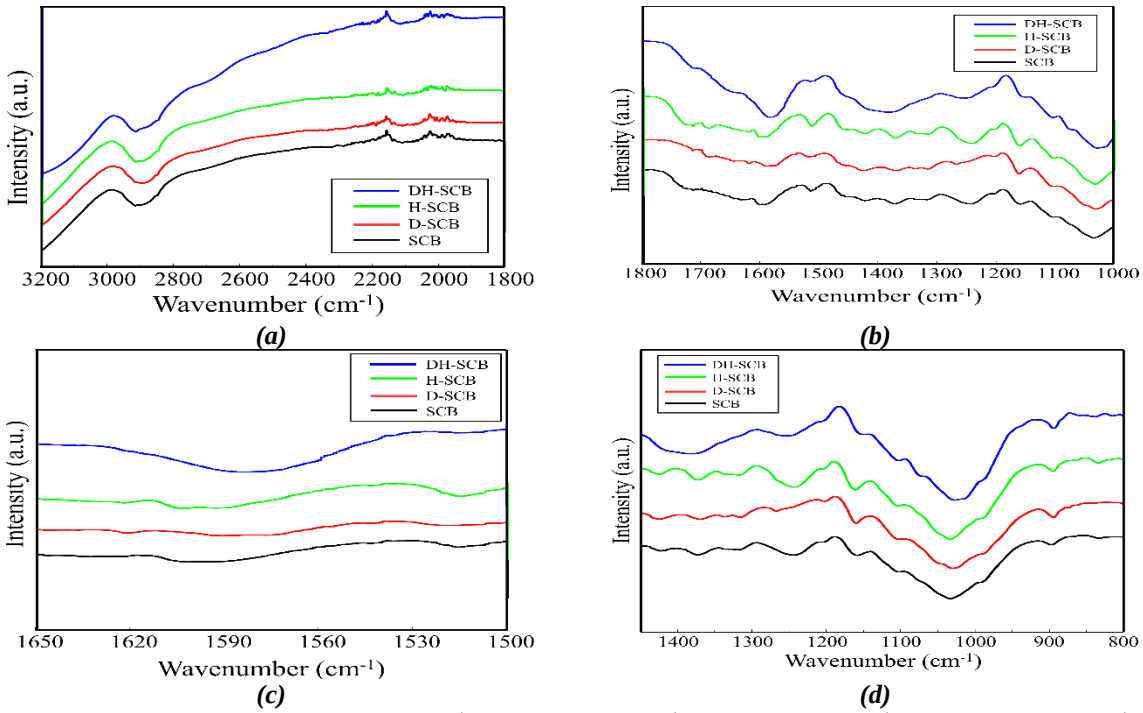

Figure 4. FT-IR of SCB in (a) 3200-1800 (cm^{-1}), (b) 1800-1000 (cm^{-1}), (c) 1650-1500 (cm^{-1}) and (d) 1450-800 (cm^{-1})

The 1424 cm^{-1} peak stretched in H-SCB and significantly stretched and shifted to the 1373 cm^{-1} peak in DH-SCB, resulting in a significantly higher stretch than other samples. 1241, 1159, and 833 cm^{-1} peak

was decreased in D-SCB and DH-SCB and increased in H-SCB, however, 1104, 1031, and 893 cm^{-1} peak was significantly increased in all pretreated samples, indicating the increase of accessible cellulose.

Table 1. List of FT-IR spectra

Functional Group	Wavenumber (cm^{-1})	References
O-H Alcohol Stretching	3335	[30], [31], [32], [33], [34], [35]
C-H stretching alkene	2920	[30], [35], [36]
C=O	1716	[32], [34], [36], [37], [38], [39], [40], [41], [42], [43]
C=O	1688	[35]
C=C	1620	[30], [31], [38], [44]
Aromatic ring vibration C=C	1590	[30], [32], [38], [39]
C-O	1516	[31], [32], [34], [38], [39], [40], [41]
OH bending	1424	[31], [32], [38], [39]
OH bending	1373	[31]
Sryngyl ring	1338	[31], [38]
G unit	1317	[31]
CO stretching	1241	[34]
COC asymmetric deformation	1159	[34]
Glucose ring asymmetric stretching	1104	[34]
CO stretching	1031	[34]
COC stretching	893	[34]
Vibrations of the guaiacyl units	833	[31]

These results indicate that the autoclave assisted-alkali hydrogen peroxide delignification process has successfully eliminated the lignin and hemicellulose of SCB without damaging the cellulose, while the enzymatic hydrolysis has successfully converted cellulose into individual sugar content.

3.3. Calorific Value Analysis

The calorific values of the pretreated SCB samples are shown in Table 2 and the graphical representation is presented in Fig. 5, below.

Table 2. Calorific value for SCB

Sample	Pretreatment	Calorific value (cal/gram)
A	Unpretreated	4759.97
B	Delignification	4247.44
C	Hydrolysis	4066.54
D	Delignification and Hydrolysis	3638.27

The calorific value of pretreated SCB varied from 3638.27 to 4247.44 cal/g. While the unpretreated SCB has a calorific value of 4759.97 cal/g. It is mentioned that delignification leads to reducing calorific value from the breaking of C-C bonds. The energy contained in C-C bonds are higher than in C-O or C-H bonds [45]. From the HHV test result, it can be inferred that the delignification and hydrolysis sample have the lowest calorific value from the breaking of complex molecules into simpler molecules.

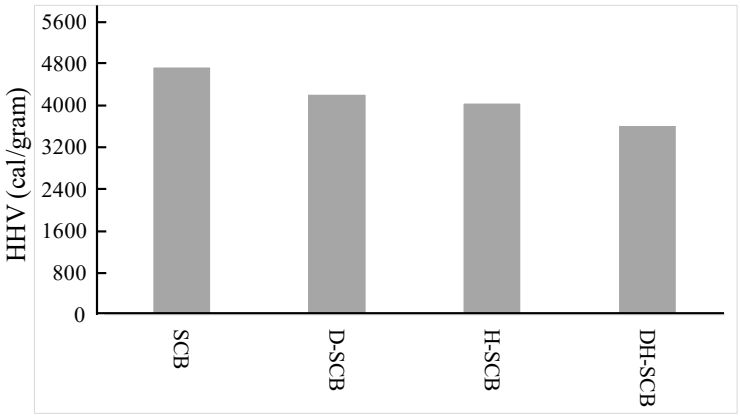

Figure 5. Caloric value graph for SCB.

This confirms the XRD and FTIR results that the pretreatment of SCB is successful. This simpler molecule will increase the digestibility of *Mucor circinelloides*. With the increase of digestibility, the fungal biomass will increase, resulting in higher oil yield [46].

3.4. Pre-cultivation and Cultivation Results

For pre-cultivation results in this study, the countable spores were on plate 5. It was calculated that in plate 5, there were 31 spores. Spores calculated were multiplied by 10 and 10 to the power of plate number. Therefore, it can be inferred that in 1 ml of spore solution, there were 31.10^6. Therefore, in the 8 test tubes, there were a total spore approximately 248.10^7 spores. For cultivation result in this total plate count, the spores calculated are 213 spores in the 10^{-6} plate. Therefore, it can be approximated that in the 100 ml growth medium, there are 213.10^9 spores.

3.5. Lipid Production from *Mucor circinelloides*

In the sugarcane bagasse hydrolysate media, the *Mucor circinelloides* undergo three physiological phases, that is balanced growth phase, the oleaginous phase, and reserve lipid turnover phase [47]. In the balanced growth phase, glucose is hydrolyzed by glycolysis pathways, creating pyruvate that is sent into mitochondria to be converted into acetyl-CoA by pyruvate hydrogenase. The acetyl-CoA then enters the tricarboxylic acid (TCA) cycle as the product of the oxaloacetate (OAA) reaction to generate citrate. This process provides energy for cellular activity, ATP from complete oxidation [48]. The carbon source was converted into cell mass, rich in protein and polysaccharide, while the lipid production was low [47]. When the nitrogen runs out, the fungi undergo the second phase, which is the oleaginous phase to accumulate storage material [49]. When nitrogen runs out, the protein in the TCA cycle experiences down regulation [50]. The citrate produced from OAA and accumulated in mitochondria was transported to cytosol. In there, the citrate can be transformed back into OAA in mitochondria to produce malate, while some citrate was converted into acetyl-CoA to produce triacylglycerol [47], [49], [50]. The sugarcane bagasse hydrolysate acts as a carbon source in the high carbon/nitrogen ratio media. While the molasse acts as a nutrient source, having an average composition of 62.3% sugars, 1.42% acotinic acid, 6.10% lactic acid, 0.1% malic acid, 0.34% pyrocarbonic acid, 0.44% acetic acid, 1.39% calcium, 0.43% magnesium, 0.08% sodium, 1.82 potassium, 2.03% phosphates, 464 mg/kg nitrates, and 60 mg/kg chlorides [51]. It has been reported that *Mucor circinelloides* have a mycelium length of around 1 μm^3 and a radius of 80 nm and a lipid yield of 17% kg/m^3 [52], [53]. Therefore, it can be approximated that the lipid production of *Mucor circinelloides* per cell is $0.004.10^{-14}$ kg. Therefore, the approximate lipid produced in the 100 ml culture media was 9.1 grams.

4. CONCLUSION

The pretreatment of sugarcane bagasse using autoclave-assisted alkali hydrogen peroxide delignification was able to remove the lignin and hemicellulose. At the same time, the following enzymatic hydrolysis was able to increase the digestibility of sugarcane bagasse's cellulose, making it suitable for *Mucor circinelloides* cultivation. The cultivation of Mucor *circinelloides* in the 100 ml sugarcane bagasse hydrolysate medium has produced approximately 9.1 grams of lipid. This further proved that the pretreatment of sugarcane bagasse using autoclave-assisted alkali hydrogen peroxide enzymatic hydrolysis is necessary for the cultivation of *Mucor circinelloides*.

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