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Comparative study of single and multiple pre-treatments of rice straw on cellulose content for bioethanol production

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OBJECTIVES Utilization of lignocellulosic biomass for ethanol production has increased. One source of lignocellulosic is rice straw which contains cellulose, hemicellulose, and lignin. Before being used as an ingredient for bioethanol production, it needs to be pre-treated with alkali or acid. One of the factors that affect pre-treatment is the concentration of alkali or acid. The purpose of this study was to determine the concentration of NaOH and H_2SO_4 in single and multiple pre-treatment which produced the highest cellulose content for bioethanol production. METHODS The concentration of NaOH and H_2SO_4 are 0.75 M; 1 M and 1.5 M. Pre-treatment was carried out at room temperature for 90 minutes. Cellulose, hemicellulose, and lignin content were analyzed using the Chesson Datta method. The Simultaneous Saccharification and Fermentation (SSF) was carried out at room temperature and analyzed for reducing sugar and bioethanol contents. RESULTS The results showed that the highest bioethanol content was obtained in the multiple pretreatments of 0.75 M H_2SO_4 – 1.5 M NaOH of 23%. was a combination of multi-pre-treatments H2SO4 0.75 M and NaOH 1.5 M, 68.14%, followed by a single pre-treatment NaOH 1.5 M, which was 60.79% and a single pre-treatment 0.75 M H2SO4 treatment of 44.89%. CONCLUSIONS The type of pre-treatment of rice straw that produces the highest bioethanol content is multi pre-treatment of 0.75 M H2SO4 and 1.5 M NaOH of 23%.

KEYWORDS bioethanol; cellulose; pre-treatment; rice straw; simultaneous saccharification and fermentation (SSF)

1. INTRODUCTION

Currently, Indonesia is still heavily reliant on fossil energy, which continues to dominate. However, this high dependence on fossil energy has significant environmental repercussions. This also has an impact on the depletion of energy reserves which is not in line with the increase in energy needs. This encourages us to look for new energy sources that can be an alternative through the energy transition (Febijanto 2012). One of the provisions of environmentally friendly alternative energy is biofuel. In the Regulation of the Minister of Energy and Mineral Resources 25 of 2013 concerning the Provision, Utilization, and Trade of Biofuels as Other Fuels, biofuels have been regulated to be required as fuel blending, specifically biodiesel and bioethanol (Anggita et al. 2021). Bioethanol, which can be produced biologically from various biomass, was accepted as an alternative to fossil fuels. Biomass-based ethanol production was proven not to increase CO_2 in the atmosphere, so it has no impact on global warming (Jin & Sutherland, 2016). Bioethanol combustion also results in relatively low emissions of volatile organic compounds such as CO and NO. This is also in line with Indonesia's goal to achieve net-zero emissions by 2060 as outlined in the Roadmap Towards Net-Zero Emissions in the Energy Sector in Indonesia.

Currently, the utilization of non-starch raw materials such as lignocellulose biomass for ethanol production has increased. Lignocellulose biomass is an abundant, inexpensive, and renewable source of sugar, which was considered a desirable raw material for the sustainable production of liquid fuels and chemical products through the biorefinery process (Phi Trinh et al. 2016). Lignocellulose material is a promising choice as a raw material for ethanol production because of its large availability in both tropical and temperate countries (Hidayat 2013). One of the sources of lignocellulose is from rice straw. Rice straw is one of the largest sources of lignocellulose biomass in the world and is a common and abundant agricultural waste in Asian countries. Straw contains cellulose (24.34%), hemicellulose (19.29%), lignin (5.11%), and coarse ash (10.22%). Rice straw has attracted attention because of its potential use in biofuel production because its high cellulose and hemicellulose content can be easily hy-

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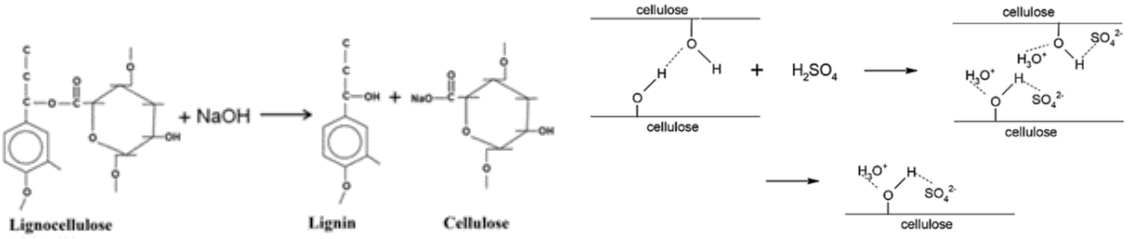


FIGURE 1. Delignification reaction with (a) acid (H2SO4); (b) alkali (NaOH) (Barman et al. 2020).

drolyzed into fermentable sugars (Phi Trinh et al. 2016).

Research on bioethanol has been carried out by many parties in the context of developing the utilization of bioethanol. Based on several studies that have been done before on the production of bioethanol, the results given are not satisfactory because the resulting yield can still be even higher by applying more diverse pre-treatment variations (Nata et al. 2014; Thakur et al. 2013; Weerasai et al. 2014). Therefore, there is an effort to pre-treat the rice straw waste substrate to increase the amount of yield produced. Many physical, chemical, and biological methods have been used for pretreatment of various agricultural by-products (Hidayat 2013; Weerasai et al. 2014). The physical pre-treatment process involves size reduction and heating using steam explosion, microwave, and ammonia fiber explosion. The advantage of this method is its environmental friendliness as it does not involve the use of chemicals, thus avoiding the production of hazardous residues. However, it requires a significant amount of energy, making it economically unfeasible. For biological pre-treatment processes, microorganisms such as soft rot fungi, white rot, and brown rot are typically utilized (Susmiati 2018). The advantages of this pretreatment include low energy consumption, absence of chemical usage resulting in minimal environmental impact, and absence of inhibitor formation. However, the hydrolysis rate in this method is still low, as most lignolytic microorganisms consume not only lignin but also hemicellulose and cellulose. The pretreatment process itself requires a long time, and the growth of microorganisms needs to be continuously monitored (Hidayat 2013). Physical and biological pre-treatments has limitations in technical and economic aspects (Mosier et al. 2005). Chemical pretreatment by acid and alkaline are simple and efficient processes for increasing biomass digestibility (Weerasai et al. 2014).

Single types of chemical pre-treatment are alkali pretreatment and acid pre-treatment (Weerasai et al. 2014). Compounds commonly used in alkali pre-treatment are NaOH because OH^- ions can separate the basic bond from the structure of lignin and Na^+ ions can bind to lignin, then form a phenolic salt that is easily soluble (Hidayat 2013). Because of this, NaOH solution can decompose lignin from cellulose (Novia et al. 2015). The commonly used compound in acid pretreatment is H_2SO_4. This is because the use of H_2SO_4 is better than other acid solutions based on the results of enzymatic hydrolysis (Hidayat 2013). The reaction involving the breakdown of lignin bonds with acid (H_2SO_4) and alkali (NaOH) is depicted in Figure 1.

The aim of this research is to determine the appropriate variations in alkali (NaOH) and acid (H_2SO_4) concentrations (0.75 M; 1M; 1.5 M respectively) in single and multiple pre-treatments of rice straw to achieve the highest cellulose content at room temperature for 90 minutes with a stirring speed of 200 rpm. Additionally, the study aims to investigate the effect of the type of pre-treatment (single and/or multi pre-treatments) of rice straw on the levels of bioethanol produced. This research is also expected to provide a reference regarding the pre-treatment process of lignocellulosic biomass, particularly rice straw, for bioethanol production.

2. RESEARCH METHODOLOGY

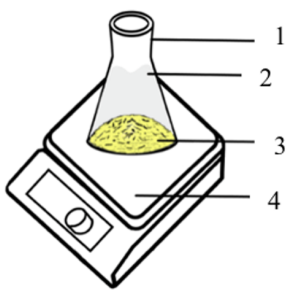
2.1 Materials

The material used is rice straw from Jl. Joyo Suko Agung Malang East Java, H_2SO_4 (technical grade 98%), NaOH (technical grade 98%), *Saccharomyces cerevisiae* (Food Microbiology Laboratory, Faculty of Agricultural Technology, Universitas Brawijaya), Cellulase enzymes (Novozymes®), Agar Nutrients Powder Oxoid CM0003, Broth Nutrients Oxoid CM0001 For Laboratory, Aquadest (technical grade 99%), Nelson A & B Reagents and Arsenomolibdat Reagents.

2.2 Procedures

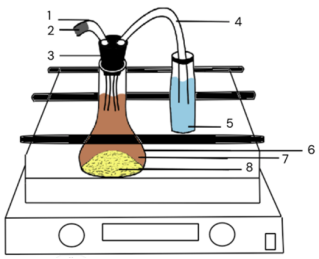
2.2.1 Raw material preparation

The preparation of raw materials was carried out as a uniform sample where biomass in the form of rice straw was



Description:
1. Erlenmeyer
2. *Pre-treatment solution*
3. Rice straw powder
4. *Hotplate & stirrer*

FIGURE 2. A series of pre-treatment tools.



Description:
1. Hose for sampling
2. Rubber stopper
3. Perforated plug rubber
4. Hose for CO_2 discharge
5. Aquadest
6. Erlenmeyer
7. *SSF* Solution
8. Rice straw substrate

FIGURE 3. Simultaneous Saccharification and Fermentation (SSF) toolset.

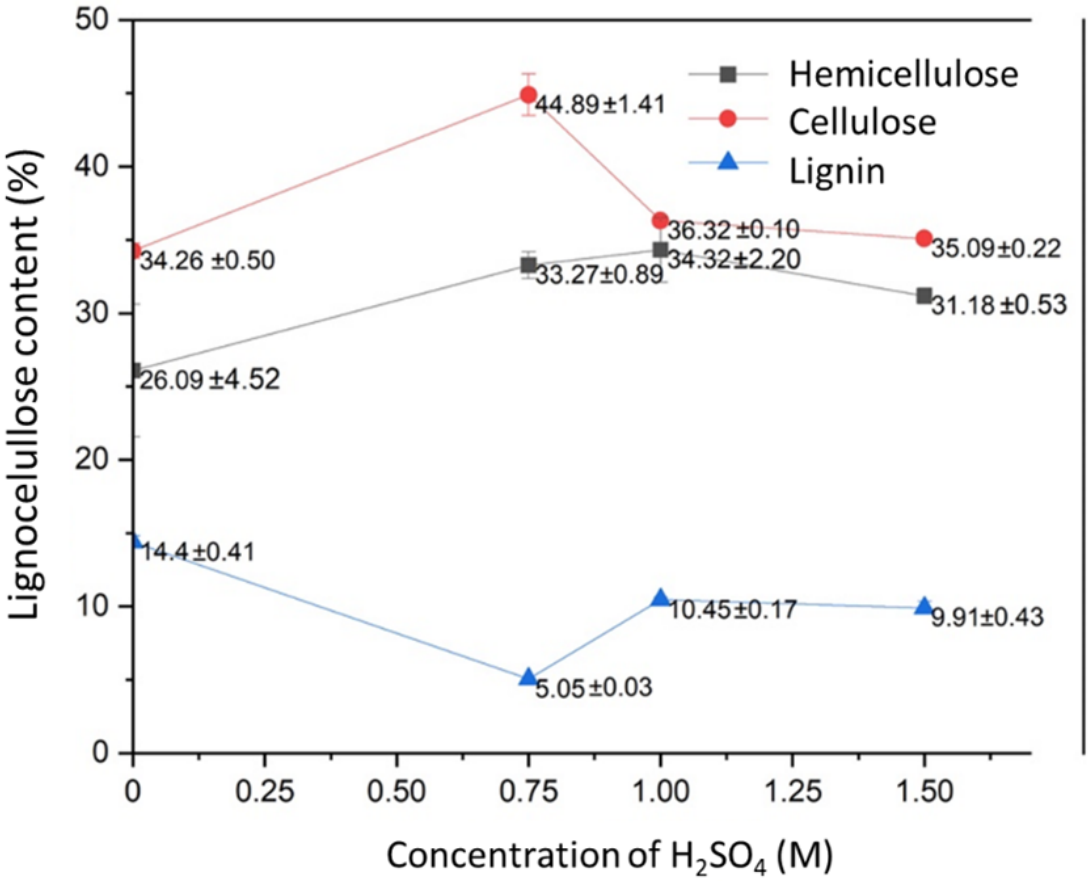


FIGURE 4. Lignocellulosic content of rice straw in H_2SO_4 pre-treatment.

mashed by blending. Then shifted using a 60-mesh screener and dried using a vacuum oven (Memmert VO29) at 70°C until the moisture content was below 10%. After that, the initial contents of cellulose, hemicellulose, and lignin were analyzed using the Chesson-Datta method.

2.2.2 Single pre-treatment

Rice straw powder was weighed as much as 30 g and put into Erlenmeyer. After that, 300 mL of H_2SO_4 0.75 M solution was added, then delignified at room temperature and stirring at 200 rpm (Digital Ceramic AREC Stirrer, Velp (F20500011) covered with aluminum foil. Following a 90-minute interval, the sample underwent filtration using a 100-mesh nylon sieve and was adjusted to a neutral pH using an Ohaus ST300 pH meter. Subsequently, it was dried in a vacuum oven (Memmert VO29) at 70°C. The series of pre-treatment tools was shown in Figure 2. Rice straw powder after pre-treatment was then analyzed for cellulose, hemicellulose, and lignin contents by the Chesson-Datta method. The procedure was repeated to a solution concentration of H_2SO_4 1 M; 1.5 M and NaOH 1 M; 1.5 M.

2.2.3 Multi pre-treatments

Rice straw powder was weighed as much as 30 g and put into Erlenmeyer. After that, 300 mL of 0.75 M H_2SO_4 solution was added, then delignified at room temperature and stirring at 200 rpm (Digital Ceramic AREC Stirrer, Velp (F20500011) covered with aluminum foil. After being left for 90 minutes, the sample was filtered and set until the pH neutral then the residual results from the filtration were continued with the second pre-treatment, which was added 300 mL of 1.5 M NaOH solution then delignified again at room temperature and stirring at 200 rpm (Digital Ceramic AREC Stirrer, Velp (F20500011) covered with aluminum foil. After being left for 90 minutes, the sample was filtered and set to a neutral pH and then dried in a vacuum oven at 70°C. Rice straw powder after multi pre-treatments then analyzed for cellulose, hemicellulose, and lignin contents by the Chesson-Datta method.

2.2.4 Rejuvenation of solid media (NA)

The source of the *Saccharomyces cerevisiae* isolate was inoculated in a zig-zag pattern on the oblique agar medium with aseptic transfer where the *Saccharomyces cerevisiae* isolate was taken using an ose needle and streaked on the NA medium starting at one end. Every time the ose was scratched on the medium for petri, the ose was preheated for the next quadrant. Then it was incubated with an incubator (Memmert/MEM-INE550) at 30°C for 24 hours and stored in the refrigerator (Lovibond/ET750 EX Type 500) at 5 °C.

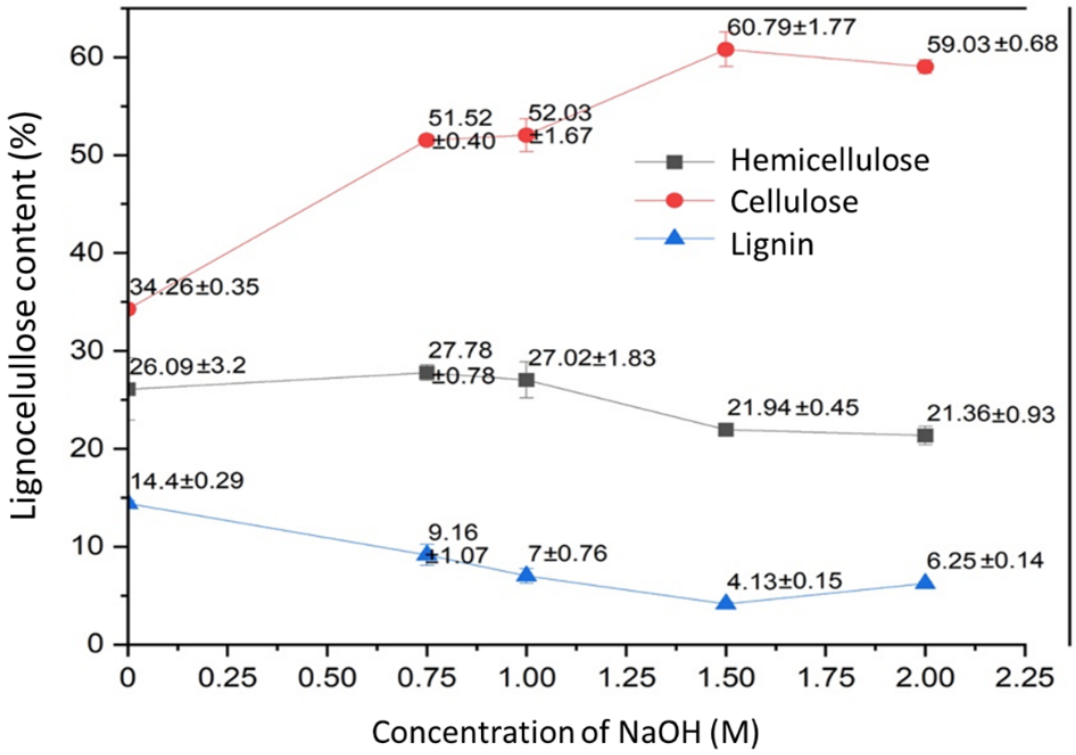


FIGURE 5. Lignocellulosic content of rice straw in NaOH pre-treatment.

2.2.5 Creation of NB (nutrient broth) media

NB media was prepared as follows: put into 100 mL aquadest then the solution was homogenized. After that, the NB media was sterilized in an autoclave with a temperature of 121°C for 15 minutes. After being sterile, NB solid media was ready to be used as a culture growth medium for *Saccharomyces cerevisiae*.

2.2.6 Making culture starter *Saccharomyces cerevisiae*

Saccharomyces cerevisiae 1 ose from oblique agar medium was incubated in a Nutrient Broth 100 mL medium with a temperature of 30 °C for 72 hours. After incubation, the liquid starter culture was ready to be used for the SSF process.

2.2.7 Simultaneous saccharification and fermentation (SSF)

The process of saccharification and fermentation is carried out simultaneously at one time so that it can occur efficiently, using saccharification microorganisms (cellulase enzymes) and fermentation microorganisms (*Saccharomyces cerevisiae*). Pre-treatment results of 15 g were put into the Erlenmeyer. Then, 200 mL of aquadest were added to the Erlenmeyer then a pH was measured and adjusted until the pH reached pH 5 with a citrate buffer. After that, an enzyme with 10% fermentation media and 10% *Saccharomyces cerevisiae* pre-culture was added to the Erlenmeyer which contains the pre-treatment results. Then the solution was stirred at 150 rpm until homogeneous. Erlenmeyer was covered with a rubber stopper equipped with two branches, one for sampling and the other for removing CO_2. The SSF process was carried out at room temperature and observations were made every 24 hours. Figure 3 shows Simultaneous Saccharification and Fermentation (SSF) toolset.

2.2.8 Cellulose, hemicellulose, and lignin contents test with Chesson-Datta Mmethod

The analysis of Lignocellulose (lignin, Hemicellulose, and cellulose) used the Chesson-Datta Method and through calculations in the following equations (Rusdianto et al. 2021):

$$\text{Hemicellulose content}(\%) = \frac{b-c}{a} \times 100\% \quad (1)$$

$$\text{Cellulose content}\ (\%) = \frac{c-d}{a} \times 100\% \quad (2)$$

$$\text{Lignin content}\ (\%) = \frac{d-e}{a} \times 100\% \quad (3)$$

Description:

1. a: Initial mass of lignocellulose biomass sample (g)
2. b: residual mass of the sample with hot water
3. c: residual mass of the sample after being heated with 0.5 M H_2SO_4 (V = 150 mL; T = 100 °C, t = 2 hours)
4. d: residual mass of the sample after being soaked with 72% H_2SO_4 (V = 10 mL; T = 30 °C, t = 4 hours)
5. e: Sample ash mass after furnace

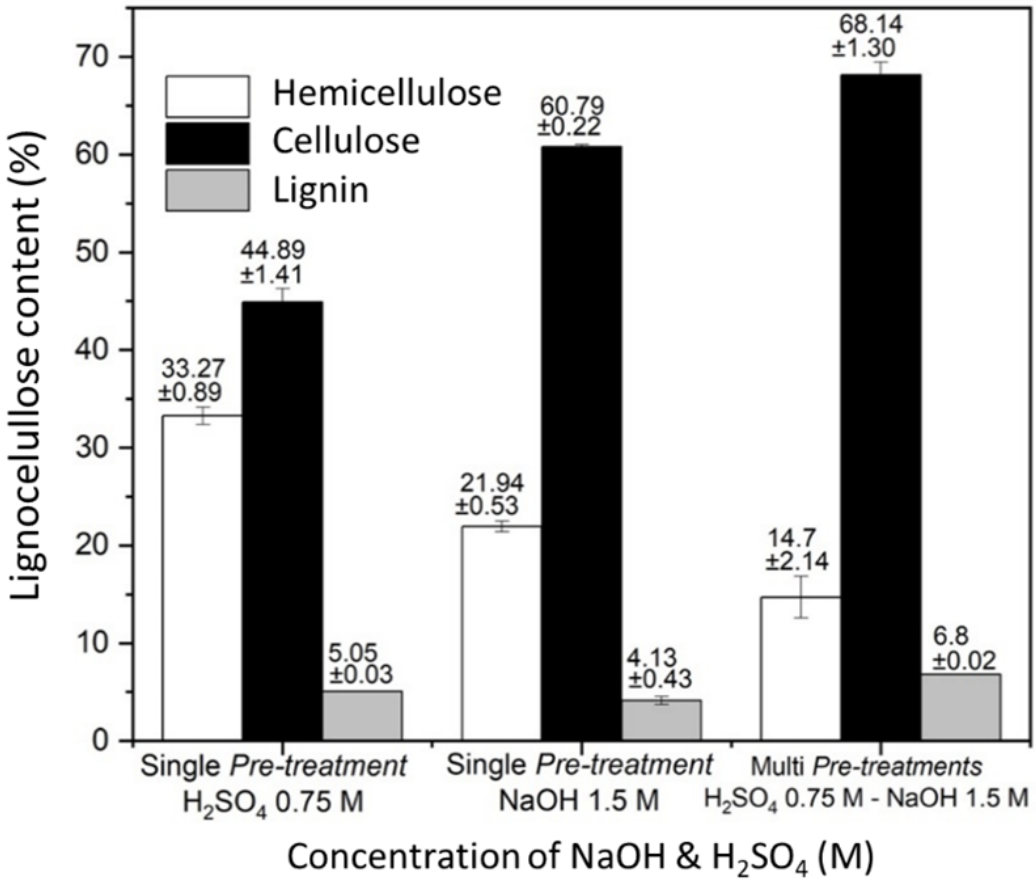


FIGURE 6. Lignocellulosic content of rice straw in single and multi-pre-treatments.

2.2.9 Sugar reduction test with Nelson-Somogyi method

The reduced sugar content in the sample can be known through the Nelson-Somogyi method. The nelson-somogyi method is used to measure reducing sugar by using copper reagents and arsenolmolibdat. The testing procedure begins with the creation of a standard solution, the determination of the maximum wavelength with the absorbance of the standard solution and the sample, the creation of the standard curve, and the determination of the glucose content in the sample by the resulting equation of the standard curve. The standard solution was prepared as much as 5 concentrations (0; 0.01; 0.02; 0.03; 0.04; and 0.05 g/L) which was prepared from the dilution of 1 mg/mL glucose standard solution dissolved in 10 mL of a volumetric flask. Sample preparation was carried out with 10^3 times dilution in a 10 mL volumetric flask of 1 mL of the sample. Dilution was done so that the sample can be easily analyzed and readable by a UV-Vis spectrophotometer (Optizen POP) (Kaczmarek 2014).

2.2.10 Bioethanol content testing

The fermentation bioethanol test procedure was carried out every day by sampling 10 mL of a fermented substrate with a syringe needle (18G). The sample was then transferred into a centrifugation tube and centrifuged with a centrifuge (Hettich EBA 20) at a speed of 4000 g for 5 minutes which aims to precipitate the solids on the fermentation substrate. The centrifugation fluid was filtered using a 0.22 µm syringe filter. The filtered sample liquid can be tested for bioethanol content with a digital refractometer (Atago PAL-34S) by dripping enough samples on the prism, then the results were immediately read on the refractometer screen.

3. RESULTS AND DISCUSSION

3.1 The effect of single and multiple pre-treatment of rice straw on lignocellulosic content

Pre-treatment is a crucial step in producing bioethanol from rice straw. In the pre-treatment process, the lignocellulosic structure of rice straw will be broken down to facilitate the process of separating cellulose and hemicellulose from the lignin complex bonds. Breaking down the lignocellulosic structure of rice straw will facilitate the process of enzymatic hydrolysis into cellulose to obtain simple sugars used in bioethanol fermentation. So that the presence of pre-treatment can increase the yield of bioethanol produced (Susmiati 2018). One of the pre-treatments carried out to process lignocellulosic rice straw is chemical pre-treatment. Chemical pre-treatment is conventional pre-treatment involving one stage of pre-treatment using an acid or alkaline solution which can also be called a single pre-treatment. Meanwhile, combination pre-treatment that combines two stages of pre-treatment is called multi-pre-treatment. Multi pre-treatments are considered to be able to break down the

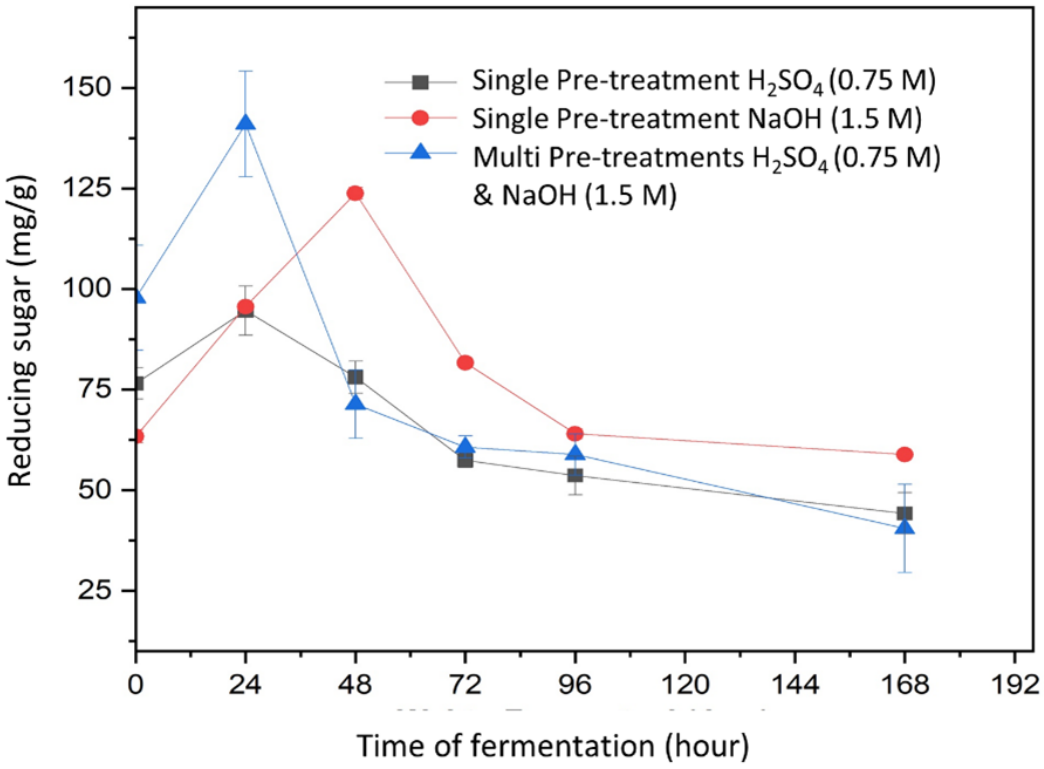


FIGURE 7. Reducing sugar levels during the SSF process.

lignocellulosic structure effectively (Weerasai et al. 2014). In this study, the initial of lignocellulosic content of rice straw was assessed using the Chesson-Data method, yielding cellulose levels of 34.26%, hemicellulose 26.09%, and lignin 14.40%. The lignocellulosic composition of rice straw varies depending on the variety, origin, rice straw cultivation, and analytical method used. The average straw content includes 30-45% cellulose, 20-25% hemicellulose, and 15-20% lignin (Sietske Boschma et al. 2013). In another study, the lignocellulosic content of rice straw was found to be 37.71% cellulose, 21.19% hemicellulose, and 16.62% lignin (Aprilyanti and Madagaskar 2020).

Single pre-treatment is a conventional pre-treatment involving one stage of pre-treatment using an acid or alkaline solution. The acid pre-treatment aims to dissolve some of the hemicellulose so that the enzymes in the hydrolysis process can then reach the cellulose structure (Hidayat 2013). Figure 4 shows that a single H_2SO_4 pre-treatment caused an increase in cellulose by 9% -18.8%. The same phenomenon also occurs in the relative changes of hemicellulose content influenced by changes in cellulose and lignin content. The results of the H_2SO_4 pre-treatment with a concentration of 0.75 M obtained the highest cellulose content 44.89%, accompanied by a decrease in lignin to 5.05%. The decrease in cellulose occurred at concentrations of 1 M and 1.5 M. This was because the pre-treatment concentration of H_2SO_4 which was too high caused cellulose to be degraded into its constituent component (glucose), glucose is degraded into unwanted inhibitory products in the ethanol fermentation process, 5-hydroxymethylfurfural (HMF) and levulinic acid (Amr and Elwany 2021; Wu et al. 2023). Single H_2SO_4 pre-treatment causes a decrease in lignin levels from 3.95%-9.35%. In this research, the most notable reduction in lignin, specifically 5.05%, was observed in the pre-treatment using H_2SO_4 at a concentration of 0.75 M. At concentrations exceeding 0.75 M of H_2SO_4, there was no notable decrease in lignin. This lack of reduction was attributed to excessively high H_2SO_4 concentrations triggering a condensation reaction (Larasati et al. 2019a). The main reaction between lignin and hemicellulose in 72% sulfuric acid is the condensation reaction to form C-glycosidic structures. This reaction was caused by the aromatic ring's reactivity within the lignin, leading to the formation of lignin-carbohydrate bonds. This affects the increase in lignin weight and its solubility level in the pre-treatment solution becomes low (Matsushita et al. 2004).

In addition to acid pre-treatment, there is an alkaline pre-treatment which aims to effectively remove lignin from biomass and increase cellulose content (Nalawade et al. 2023). Figure 5 shows the results of a single NaOH pre-treatment which caused a decrease in lignin content of 5.2% - 10.27%. At a concentration of 1.5 M NaOH, the most significant decrease in lignin content, reaching 4.13%, was recorded, alongside the highest increase in cellulose content, which amounted to 60.79%. Increased OH- ions attacking the lignin bonds facilitate the breakdown of these bonds, causing the lignin to dissolve in the pretreatment solution and consequently decreasing the lignin levels (Inggrid et al. 2011). The reduction in lignin content at a concentration of 2 M was not

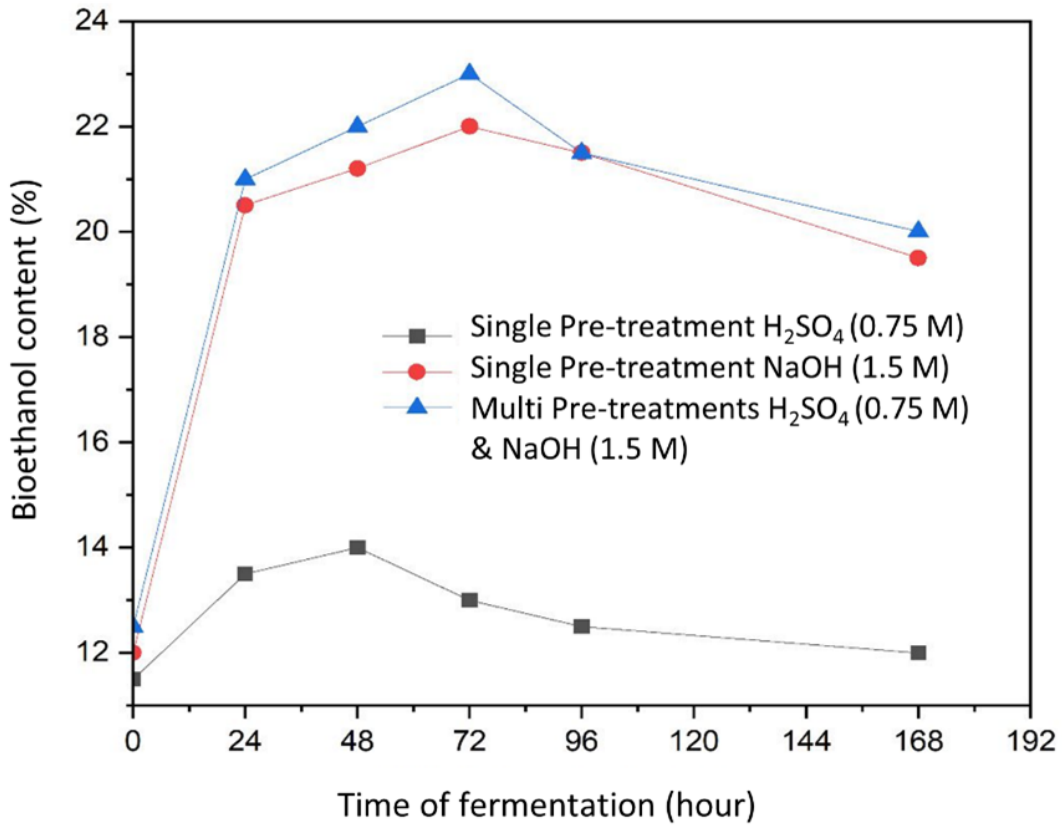


FIGURE 8. Bioethanol content during the SSF process.

more pronounced than at a concentration of 1.5 M. The observed increase in lignin content was attributed to the breakdown of the lignin structure, leading to partial condensation and precipitation of molecules. The lysis of the lignin structure causes the bonds between lignin units to break, resulting in some lignin molecules becoming more reactive and prone to condensation (Larasati et al. 2019b). Lignin condensation produces lignin molecules that are heavier molecularly due to the clumping of lignin molecules so that in the presence of condensed lignin molecules, affects the increase in lignin levels (Larasati et al. 2019b). The presence of lignin condensation influences the formation of new bonds, specifically methine, methylene, methyl, and carboxyl groups (Gosselink 2011).

Single NaOH pre-treatment caused an increase in cellulose by 25.4% -34.7%. At a concentration of 1.5 M NaOH, the most significant decrease in lignin content, reaching 4.13%, was recorded, alongside the highest increase in cellulose content, which amounted to 60.79%. This shows that the higher the concentration used, the higher the cellulose content. The cellulose content tends to decrease at more than 1.5 M NaOH concentration. This is thought to occur because the too-high NaOH concentration causes the cellulose chains to degrade. This affects the breakdown of crystalline regions in cellulose and makes cellulose easily dissolved in the pre-treatment solution (Sayakulu and Soloi 2022). The results of other studies showed a tendency for cellulose levels to decrease at concentrations of more than 4% after NaOH pre-treatment. This was due to the complex carbohydrate lignin reaction in rice straw. This reaction causes cellulose to dissolve in delignification solutions (Larasati et al. 2019b). Single NaOH pre-treatment led to a reduction in hemicellulose content of 6.4% -12.9%. The reduction in hemicellulose levels is because some of the xylene, which is a hemicellulose component, is solubilized with NaOH solution. NaOH solution can damage the chemical bonds that bind hemicellulose to cellulose fibers so that hemicellulose is separated from cellulose. As a result, some hemicellulose can break down into simpler components (Hashim et al. 2017). In addition, NaOH solution can also reduce the stability of bonds in the lignin structure of biomass so that it can increase the accessibility of enzymes used for the hydrolysis process of cellulose and hemicellulose (Brodeur et al. 2011).

Multi pre-treatments are a combination of H_2SO_4 and NaOH pre-treatment which is carried out using H_2SO_4 followed by NaOH which aims to improve delignification efficiency. The sequential application of H_2SO_4 followed by NaOH aims to initially break the lignocellulose bonds through acid pre-treatment, followed by the alkali method to separate lignin from cellulose and hemicellulose (Duque et al. 2021). Figure 6 shows the lignocellulosic content in each type of pre-treatment. The combination of multi-pre-treatments H_2SO_4 0.75 M - NaOH 1.5 M produced the highest cellulose content among other types of pre-treatments, reaching 68.14%. The multi pre-treatments process in this study led to enrichment of cellulose compared with the re-

spective single pre-treatments employed in several previous reports, in which cellulose contents of 40 to 65% were obtained from various pretreated biomasses using dilute acid, alkaline, and liquid hot water pretreatment (Chen et al. 2009, 2011; Imman et al. 2013; Wan et al. 2011; Weerasai et al. 2014). The multi-pre-treatment concentrations of 0.75 M H_2SO_4 and 1.5 M NaOH were obtained from the concentration of single pre-treatment H_2SO_4 and single pre-treatment NaOH which obtained the highest cellulose. This is thought to occur because by carrying out two stages in the multi pre-treatments, H_2SO_4 will dissolve some of the hemicelluloses so that the enzymes to be used in the hydrolysis process can reach the cellulose structure and NaOH will dissolve the lignin (Hidayat 2013). Based on Figure 6, the lignin content in the single pre-treatment H_2SO_4 (5.05%) is higher than that in the single pre-treatment NaOH (4.13%), indicating that the single pre-treatment NaOH is more effective in lignin solubilization. In Multi pre-treatments H_2SO_4 and NaOH, the lignin content is higher compared to other pre-treatments because the pre-treatment starts with acid first to dissolve some hemicellulose so that cellulase enzymes can access the cellulose structure, followed by NaOH base to dissolve lignin (Nalawade et al. 2023).

3.2 The effect of single and multiple pre-treatments of rice straw on bioethanol content

The process of saccharification and fermentation is carried out using the Simultaneous Saccharification and Fermentation (SSF) method, using saccharification microorganisms (cellulase enzymes) and fermentation microorganisms (*Saccharomyces cerevisiae*). In the SSF process, polysaccharides that are converted to monosaccharides are directly fermented into bioethanol and do not turn back into polysaccharides because the hydrolysis and fermentation processes take place simultaneously (Samsuri et al. 2010). Fermentation is the process of obtaining bioethanol which is converted from simple sugars and this process produces carbon dioxide as a by-product. One of the factors that affect fermentation is the substrate. The substrate is a raw material for fermentation that contains nutrients needed by microorganisms to grow and produce fermented products (Azizah et al. 2012). The substrate used in this study was rice straw containing reducing sugars resulting from the hydrolysis of cellulose. Reducing sugar is a source of nutrients needed by microorganisms for the growth and formation of bioethanol as a fermentation product (Ahmad et al. 2020). Data analysis of reducing sugar calculations during the SSF process is presented in Figure 7.

Figure 7 indicates that the multi-pre-treatment H_2SO_4 (0.75 M) and NaOH (1.5 M) resulted in the highest reducing sugar content of 131.69 mg/g at 24 hours. Meanwhile, the lowest reducing sugar content was 90.26 mg/g in single pre-treatment H_2SO_4 (0.75 M) at the same time. This is presumably because the single pre-treatment H_2SO_4 is less effective in removing lignin thus inhibiting the hydrolysis process of cellulose into simple sugars (Lismeri et al. 2018). The difference in reducing sugar levels in the single pre-treatment H_2SO_4, the single pre-treatment NaOH, and multi-pre-treatments H_2SO_4 and NaOH is due to the type of pre-treatment affecting the reducing sugar produced from rice straw samples. Pre-treatment can increase the formation of sugar and the ability to produce sugar because, without a pre-treatment process, cellulose will be difficult to hydrolyze. After all, it still has a structure and strong bonds (Sun and Cheng 2002). At 0-24 hours, reducing sugar levels increased in each type of pre-treatment. Reducing sugars that are formed during SSF come from the enzymatic hydrolysis of cellulose into simple sugars (Wilda and Pandebesie 2015). Enzymatic hydrolysis can activate other compounds specifically and can increase the speed of chemical reactions (Samsuri et al. 2010). The cellulase enzymes used in this study play a role in hydrolyzing β-1,4 glycosidic bonds in the amorphous regions of cellulose fibers which produce long-reduced oligosaccharides and polymers, such as Carboxy Methyl Cellulose (CMC) and cellobiose which will later produce glucose units. Reducing sugar levels at 24-168 hours tend to decrease because the reducing sugars contained in the fermentation medium are used as a carbon source for *Saccharomyces cerevisiae* for energy synthesis through the bioethanol fermentation process (Ahmad et al. 2020). In the single H_2SO_4 pre-treatment, the level of bioethanol produced was the lowest because the reducing sugar content was also low, 73.77 mg/g at 24 hours if compared to multi pre-treatment. So, the reducing sugar content will affect the work of *Saccharomyces cerevisiae* cells in the production of bioethanol during the SSF process. Reducing sugar levels that are too high or too low will affect microorganisms because they have an impact on decreased cell metabolism.

The largest reduction in reducing sugar content was seen in the multi-pre-treatments of H_2SO_4 and NaOH, which was 98.9 mg/g. The greater the reduction in reducing sugar levels, the higher the level of bioethanol formed. Bioethanol levels during the SSF process are shown in Figure 8. In the single pre-treatment H_2SO_4 (0.75 M), the bioethanol content increased at SSF 0-48 hours, by 2.5%. Whereas in the single pre-treatment NaOH, the bioethanol content increased at SSF 0-72 hours, by 10%. Bioethanol content with the highest increase, which increased by 10.5% for multi-pre-treatments H_2SO_4 and NaOH. Judging from the reduced sugar content, the multi-pre-treatment H_2SO_4 and NaOH also provided the highest reduction in sugar levels, 100.47 mg/g. So, the greater the reduction of glucose, the higher the concentration of bioethanol that is formed (Ahmad et al. 2020).

Based on the type of pre-treatment, multi-pre-treatments produced the highest levels of bioethanol compared to single pre-treatment. This happens because the multi-pre-treatments H_2SO_4 and NaOH can eliminate hemicellulose and lignin more efficiently and produce the highest cellulose content so that the resulting bioethanol content is also higher. Meanwhile, a decrease in bioethanol levels in each type of pre-treatment can occur because when the simple sugars used as nutrients start to decrease, the bioethanol produced during fermentation is used as a carbon source for cellular respiration (Gasmi et al. 2014).

The variation in ethanol concentration resulting from the fermentation of lignocellulosic biomass relies on several factors, such as the substrate's characteristics, pretreatment techniques, and the physical parameters of the fermentation processes. The bioethanol concentration achieved in this study was comparable to previous findings on the fermentation of rice straw subjected to sequential dilute acid/

alkaline process (Weerasai et al. 2014), dilute acid pretreatment (Karimi et al. 2006) and microwave/alkaline pretreatment (Zhu et al. 2005), falling within the range of 8.0 to 25.8 g/L of bioethanol.

4. CONCLUSIONS

Based on the research data, it can be concluded that the concentration of the pre-treatment solution that produced the highest cellulose content was a combination of multi-pretreatments H_2SO_4 0.75 M and NaOH 1.5 M, 68.14%, followed by a single pre-treatment NaOH 1.5 M, which was 60.79% and a single pre-treatment 0.75 M H_2SO_4 treatment of 44.89%. The type of pre-treatment of rice straw that produces the highest bioethanol content is multi pre-treatment of 0.75 M H2SO4 and 1.5 M NaOH of 23%.

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