The Effect of Cabbage Waste Pretreatment on Lignocellulose Content for Bioethanol Production

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Abstract

Cabbage waste represents a resource rich in lignocellulose, offering substantial potential as a raw material for bioethanol production. However, the nature of lignocellulosic complexes necessitates effective pretreatment strategies to enhance cellulose accessibility. This study aims to determine the effect of pretreatment on cabbage waste on changes in lignocellulosic content before and after pre-treatment to determine the influence of solution concentration and pretreatment duration of cabbage waste on the level of reducing sugars, which will subsequently affect bioethanol fermentation. In this research, cabbage waste was pretreated using H_2SO_4 1 M and alkaline NaOH 1 M for 1 hour to determine the pretreatment agent that produced the highest cellulose content. Furthermore, the concentration (1 M; 1.5 M; 2 M) and the pretreatment time (1 hour; 1.5 hours; 2 hours) will be varied to produce the highest reducing sugar content, which will be continued to the Simultaneous Saccharification and Fermentation (SSF). The results showed that pretreatment using H_2SO_4 produced the highest change in cellulose content. Pre-treatment using H_2SO_4 at a concentration of 1.5 M for 1.5 hours produced the highest reducing sugar concentration of 0.32 g/L. The highest bioethanol content was obtained during fermentation for 72 hours at 22.25%.

Keywords: Bioethanol; Cabbage waste; Lignocellulose content; Pre-treatment

1. Introduction

The problem of fuel oil is in the public spotlight on rising prices and its supply is running low. This condition encourages various alternative energy discoveries to overcome dependence on fuel oil. One alternative that is being developed is biofuel in the form of bioethanol. The bioethanol fermentation process generally in Indonesia uses raw materials in the form of cassava, starch, bagasse, or materials with sugar content called first-generation bioethanol [1]. In the second generation, biomass with lignocellulose content can be a raw material for bioethanol production, for example such as corn cobs, banana fronds, pineapple peels, and cabbage waste. Lignocellulose materials are intensively developed because they are abundant, cheap, and have no impact on the transfer of food staples for bioethanol production [2], [3]. Nevertheless, the advancement of bioethanol faces obstacles, particularly the inability to achieve optimal bioethanol yields because of the existence of lignin constituents within the lignocellulosic matrix, impeding cellulose accessibility [4].

Wastes from agriculture and plantations contain abundant lignocellulosic biomass. These wastes have environmental impacts, for example: agricultural waste being improperly disposed of in rivers. Piles of cabbage vegetables in rivers create odor and pollution. Cabbages are discarded because they easily spoil and rot, making them unfit for sale. Cabbage (*Brassica oleracea*) is the most commonly cultivated vegetable in Indonesia and thrives in highland areas. As a lignocellulosic biomass, cabbage comprises lignocellulosic components, with cellulose accounting for 7.87%, hemicellulose for 3.09%, and lignin for 1.70% [4]. The utilization of cabbage as a feedstock for bioethanol fermentation can be classified as second-generation bioethanol (G2), wherein the content of hemicellulose and cellulose in cabbage can be converted into bioethanol [5]. The selection of cabbage as a raw material for bioethanol fermentation considers its relatively low cost and abundant availability in the East Java, Indonesia. The lignin component in the lignocellulose complex blocks access in cellulose conversion. Therefore, to produce reduced sugar from lignocellulose biomass, a pretreatment is needed that is able to break down the lignocellulose structure so as to produce cellulose fibers and hemicellulose fibers released from lignocellulose complex bonds [6]. Pretreatment can generally be carried out either physically or chemically, however, each method has limitations in technical and economic aspects [7]. The chemical pretreatment was employed using acid and alkali solutions [3]. The acid solution used was H_2SO_4, while the alkali solution used was NaOH [8]. The commonly used compound in acid pre-treatment is H_2SO_4 because the use of H_2SO_4 is better than other acid solutions based on the results of enzymatic hydrolysis [9]. 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 [9]. Because of this, NaOH solution can decompose lignin from cellulose [10].

Research conducted by Genemo shows that high bioethanol levels are obtained in cabbage waste that is pretreated in the form of H_2SO_4 solution [4]. Meanwhile, in research on sugarcane bagasse fermentation by *Saccharomyces cerevisiae,* pretreatment with NaOH was used [11], [12]. Both pretreatment of H_2SO_4 and NaOH can be used as variations and comparisons of results in this study. Based on Maharani's research, it was concluded that the variables of time and concentration of NaOH have a real influence on cellulose content [13]. Similar to Rilek's research, H_2SO_4 pretreatment is influenced by concentration and time [14]. The aim of this research is to investigate the effect of cabbage waste pretreatment using acid (H_2SO_4) and alkali (NaOH) on the change in cellulose content before and after pretreatment. It also aims to determine the influence of solution concentration and pretreatment duration of cabbage waste on the level of reducing sugars, which will subsequently affect bioethanol fermentation.

2. Method

2.1. Preparation of Raw Material

Cabbage waste from the Kediri (East Java) market is washed using clean water. The selected cabbage waste is in a condition that is still intact and slightly wilted. Cabbage was cut and dried in a vacuum oven at a temperature of 105ºC and a pressure of 25 mmHg for 2 hours. The dried cabbage was weighed until the mass was constant, and a water content of up to 10% was obtained. Dried cabbage is ground using a blender until it becomes a powder measuring 80 mesh. Then, the cabbage waste was tested using the Chesson-Datta method to determine the levels of cellulose and hemicellulose before pretreatment.

2.2. Preparation of Pretreatment Solutions

NaOH pretreatment solution was made by mixing 4 grams of NaOH (technical grade 98%) with distilled water until it had a concentration of 1 M; 1.5 M; and 2 M. The H_2SO_4 pretreatment solution was made by dissolving 5.56 mL of H_2SO_4 (technical grade 98%) then diluting it to a solution concentration of 1 M; 1.5 M; and 2 M.

2.3. Cabbage Waste Pretreatment

A total of 10 grams of dried cabbage waste was dissolved in 100 ml of pretreatment solution. The initial pretreatment process compared the use of NaOH and H_2SO_4 with a concentration of 1 M for 1 hour. The pretreatment results were tested to determine the highest cellulose content. The use of pretreatment solutions that produce the highest cellulose varies in concentration and soaking time. Pretreatment variations take place at the concentration of the pretreatment solution and with immersion at a time variation of 1; 1.5; and 2 hours. Substrate separation is carried out by filtering the solution. The pH of the residue was measured until it had a pH of 5. Next, the drying process was carried out using a vacuum oven. The results of the variations in concentration and pretreatment time were not tested for cellulose content but rather for the content of reducing sugars (reducing sugars represent the cellulose content in cabbage waste). The variables of concentration and pretreatment time with the highest reducing sugar concentration will be fermented to produce bioethanol.

2.4. Simultaneous Saccharification and Fermentation (SSF)

A total of 7 grams of cabbage waste, resulting from the pretreatment with the highest cellulose content, is introduced into the fermenter. In the simultaneous saccharification and fermentation (SSF) process, cellulase enzyme (Novozymes®) and *Saccharomyces cerevisiae* starter from Food Microbiology Laboratory, Faculty of Agricultural Technology, Universitas Brawijaya are added. The amount of cellulase enzyme and *Saccharomyces cerevisiae* starter added is 10% of the volume of the fermentation medium of 100 mL. Stirring is conducted at a speed of 150 rpm at room temperature for 96 hours.

2.5. Determination of Lignocellulose Content with Chesson-Datta Methods

The contents of cellulose, hemicellulose, and lignin in the sample can be calculated in the following way [15]:

$$Hemicellulose\ content\ (\%) = \frac{b-c}{a} x100\% \quad (1)$$

$$Cellulose\ content\ (\%) = \frac{c-d}{a} x100\% \quad (2)$$

$$Lignin\ content\ (\%) = \frac{d-e}{a} x100\% \quad (3)$$

Description:

a : Initial mass of lignocellulose biomass sample (gr)
b : Residual mass of the sample with hot water
c : Residual mass of the sample after H_2SO_4 treatment 0.5 M
d : Residual mass of the sample after H_2SO_4 treatment 72%
e : Sample ash mass after furnace

2.6. Determination of Reducing Sugar with Nelson-Somogyi Methods

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 Nelson A & B reagents and Arsenomolibdat reagents (analytical grade). 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 in 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 103 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 [16].

2.7. Determination of Bioethanol Content

The fermentation bioethanol test procedure was carried out every day by sampling 10 ml of a fermented substrate with an 18G syringe needle. The sample was then transferred into a centrifugation tube and centrifuged at a speed of 4000g 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 refractometer 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 Cabbage Waste Pretreatment on Lignocellulose Content

Cabbage waste contains lignocellulose, which can serve as a raw material for bioethanol production. Lignocellulose is a polymer component composed of lignin, hemicellulose, and cellulose [3], [17]. Pretreatment of cabbage waste is necessary to break down the lignocellulose structure, thereby enhancing the accessibility of cellulose during the conversion process from polysaccharides to simple sugars [13]. The pretreatment scheme for cabbage waste can be illustrated in Figure 1, where the complex lignocellulose structure, consisting of both crystalline and amorphous components, is broken down into cellulose, hemicellulose, and lignin compounds [18].

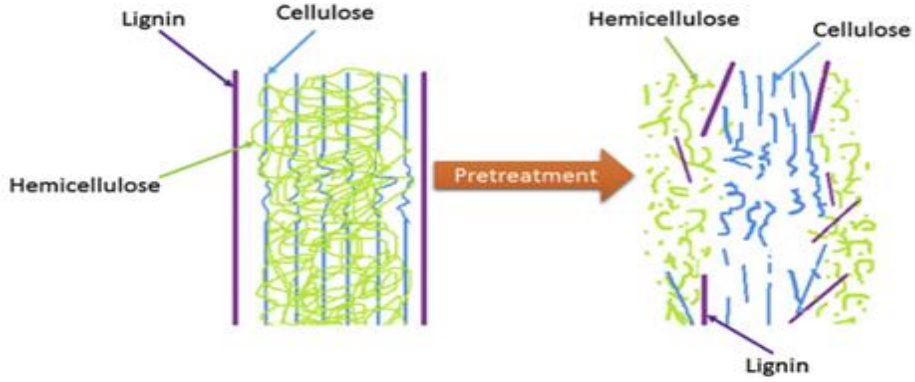

Figure 1. The Pretreatment Scheme for Lignocellulosic Biomass [18]

The lignocellulose content before and after pretreatment is presented in Figure 2. Both pretreatments, with H_2SO_4 and NaOH, were conducted at the same concentration and duration, which is 1 M concentration for 1 hour to determine

the highest cellulose change. Based on Figure 2, there are alterations in cellulose and lignin content. The cellulose content increased from 18.11% to 46.42% in the H_2SO_4 pretreatment and 28.83% in the NaOH pretreatment. Meanwhile, the lignin content decreased from 14.25% to 11.14% in the H_2SO_4 pretreatment and 9.60% in the NaOH pretreatment.

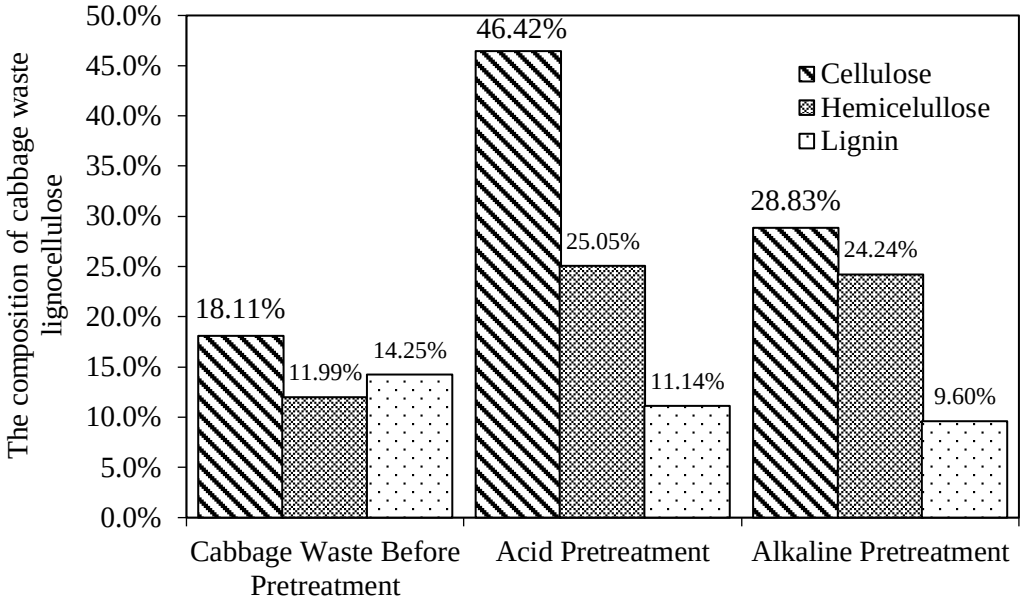

Figure 2. The Lignocellulose Content in Cabbage Waste Before and After Pretreatment

The changes in cellulose and lignin content in cabbage waste with pretreatment occur due to the process of breaking down the lignocellulose complex into cellulose, hemicellulose, and lignin. The increase in cellulose content is a result of the delignification process. During pretreatment, there is a modification in the crystalline structure of lignocellulose by degrading lignin, allowing the breakdown of the lignocellulose complex bonds [18]. The pretreatment process can reduce cellulose crystallinity, enhance cellulose porosity, and expand the mass transfer area for further depolymerization.

The reduction of lignin in cabbage waste through H_2SO_4 pretreatment occurs due to lignin cleavage reactions, as depicted in Figure 3. Under acidic conditions, hydrolysis reactions take place, forming a benzyl cation intermediate. This benzyl cation intermediate reacts to form enol ether compounds, releasing HCHO and H^+ from the γ position. Subsequently, hydrolysis continues to yield Hibbert Ketone, marked by its pungent odor and the presence of free phenolics [19].

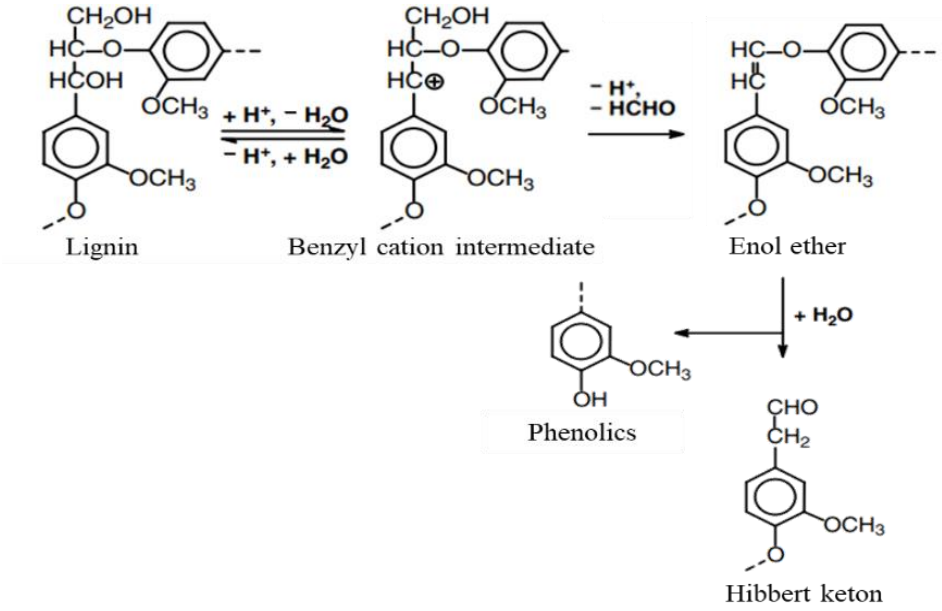

Figure 3. Lignin Degradation Reactions Under Acidic Conditions [19]

Figure 4 illustrates lignin cleavage reactions under alkaline or basic conditions. Lignin degradation is initiated by the hydroxide ion (OH) from NaOH, which breaks the hydrogen atom bond with the phenolic OH group. The hydrogen atom, being a strong acid, has its electrons pulled by the more electronegative oxygen atom, rendering it partially positively charged and readily releasing into an H^+ ion. Resonance effects of alkyl groups in the para position can also influence the increased acidity of the phenolic group's hydrogen atom. Additionally, there is a cleavage of the basic lignin structure by the hydroxide ion (OH^-) from NaOH, resulting in the formation of lignin bonds with sodium ions (Na^+), creating Sodium phenolate. Sodium phenolate is characterized by its solubility in water due to its polar nature [20].

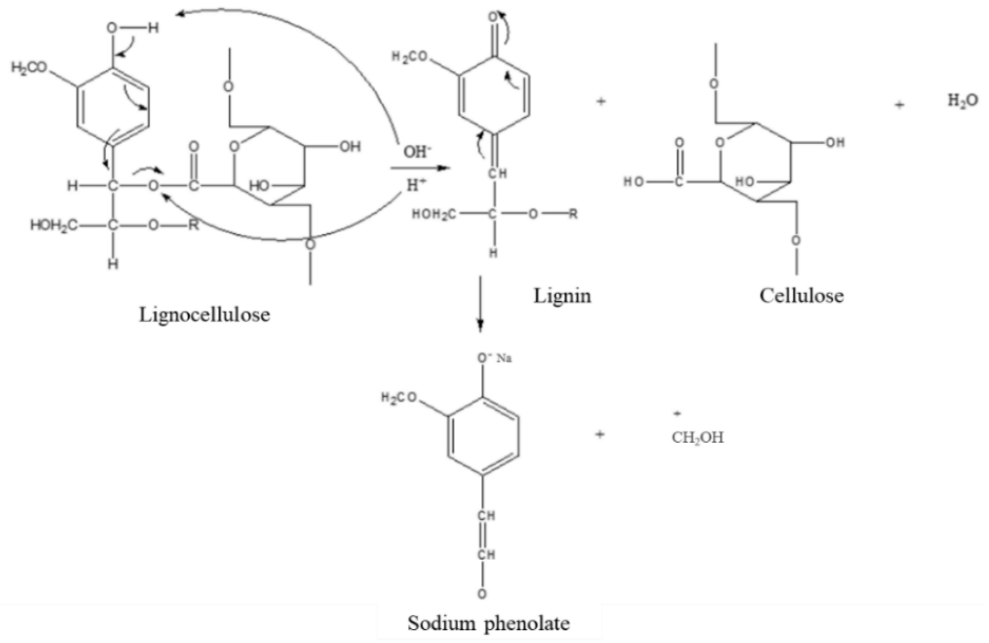

Figure 4. Lignin Degradation Reactions Under Alkaline Conditions [21]

The reduction in lignin content is more significant in NaOH pretreatment compared to H_2SO_4. This is because NaOH pretreatment is more effective in lignin solubilization by forming Sodium phenolate compounds, as indicated by the formation of a black-colored solution in the sample referred to as "black liquor" [9]. In contrast, H_2SO_4, being polar, can only precipitate the products of acid hydrolysis reactions [22].

The cellulose content change is more significant in H_2SO_4 pretreatment, while the lignin reduction is more pronounced in NaOH pretreatment. The greater increase in cellulose content under acidic conditions is consistent with research conducted by Maftucha (2022), which indicates that acidic conditions have the ability to yield high cellulose content but are less effective in lignin removal [23]. This can be observed by the higher viscosity of the samples in H_2SO_4 pretreatment, resembling cellulose fiber pulp. Therefore, the use of H_2SO_4 solution is preferred as the pretreatment solution for cabbage waste, as it can produce the highest cellulose content in the bioethanol production process.

3.2. The Effect of Concentration and Pretreatment Time of H_2SO_4 on Cabbage Waste on Reducing Sugar Content

The pretreatment process can increase the porosity of cellulose and expand the mass transfer area for further depolymerization [24]. Under acidic conditions, the hydrolysis of cellulose chains occurs, leading to the formation of disaccharide cellobiose and subsequently yielding reducing sugars [25]. Hence, high cellulose content has the potential to contain abundant reducing sugar molecules. The content of reducing sugars can represent the cellulose content in cabbage waste after pretreatment. Reducing sugars are a category of sugars capable of reducing electron-accepting compounds. Examples include all monosaccharides (glucose, fructose, galactose) and disaccharides (lactose, maltose), excluding sucrose and starch (polysaccharides), which are not considered reducing sugars [26]. Based on Rilek's research (2017), H_2SO_4 pretreatment can be influenced by several factors, such as concentration and time [14].

Therefore, it is essential to investigate the effects of concentration and pretreatment time on cabbage waste to break down lignocellulose and achieve optimal bioethanol production.

Figure 5 represents a graph illustrating the influence of concentration and time in H_2SO_4 pretreatment on cabbage waste regarding reducing sugar concentration. Based on the results of the reducing sugar tests conducted on cabbage waste after H_2SO_4 pretreatment, where concentration and time were varied, the values of reducing sugar content in descending order are as follows: sample L (2 M; 3 hours) had a concentration of 0.06±0.003 g/L; sample J (1 M; 3 hours) had a concentration of 0.07±0.006 g/L; sample G (2 M; 1 hour) had a concentration of 0.09±0.009 g/L; sample A (1 M; 1 hour) had a concentration of 0.12±0.002 g/L; sample B (2 M; 1.5 hours) had a concentration of 0.13±0.008 g/L; sample D (1.5 M; 1 hour) had a concentration of 0.16±0.009 g/L; sample F (1.5 M; 2 hours) had a concentration of 0.21±0.009 g/L; sample H (2 M; 1.5 hours) had a concentration of 0.23±0.007 g/L; sample I (2 M; 2 hours) had a concentration of 0.25±0.008 g/L; sample C (1 M; 2 hours) had a concentration of 0.26±0.006 g/L; and sample E (1.5 M; 1.5 hours) had the highest reducing sugar content at 0.32±0.003 g/L. The highest reducing sugar content in cabbage waste was found in sample E, which underwent H_2SO_4 pretreatment with a concentration of 1.5 M for 1.5 hours.

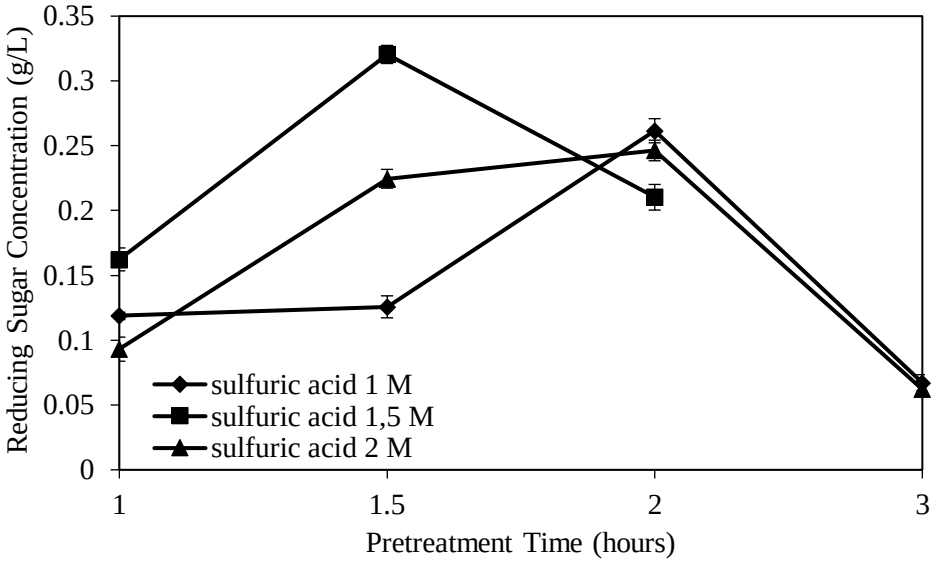

Figure 5. The Influence of Sulfuric Acid (H_2SO_4) Concentration and Pretreatment Time on Cabbage Waste on Reducing Sugar Concentration

At a 1 M concentration, there is a decrease of 0.01 g/L within 1.5 hours, indicating the suboptimal nature of the pretreatment process at a relatively low concentration with a 0.5-hour time difference, thus resulting in no significant increase in reducing sugar content. Subsequently, at the 1 M concentration, there is an increase at the 2-hour mark but a decrease at the 3-hour mark. For the 1.5 M concentration, there is an increase at the 1.5-hour mark but a decrease at the 2-hour mark, while the 2 M concentration experiences an increase up to the 2-hour mark but decreases at the 3-hour mark. The increase in reducing sugar concentration is attributed to the addition of H_2SO_4 solution concentration, which releases more H^+ protons that interact with the 1,4 glycosidic bonds, forming numerous free radical groups [27]. H_2SO_4 concentration is linked to the breakdown of lignocellulosic structures and the quality of reducing sugar. This occurs because H+ ions from strong acids can break glycosidic bonds in cellulose [28].

The decrease in the amount of reducing sugar is due to the fact that a higher concentration of H_2SO_4 solution results in a reduced amount of water in the pretreatment solution, causing a decrease in the number of OH groups available as free radical binders. This, in turn, hinders the balance between the number of free radical groups [27]. Additionally, the increase in H_2SO_4 concentration leads to the degradation of already formed glucose into inhibitors (such as furfural, 5-hydroxymethylfurfural, levulinic acid, acetic acid, formic acid, and others) during the bioethanol formation process through fermentation [27]. The properties of inhibitors are contrary to catalysts, which are intended to accelerate reaction rates. In the case of substrates, inhibitors are compounds that can inhibit or decrease the rate of reaction for an enzyme. Inhibitors work by binding to the enzyme, causing it to become damaged or incompatible with its substrate [29].

Based on the research conducted by Rilek (2017), there is a relationship between pretreatment time and the content of reducing sugar in the time range of 60-100 minutes. The reducing sugar content increases with the increasing time, and samples with a 100-minute pretreatment result in the highest amount of reducing sugar [14]. The increase in reducing sugar concentration occurs at concentrations of 1 M and 2 M from the 1st hour to the 2nd hour, and at a concentration of 1.5 M, it increases from the 1st hour to the 1.5-hour mark. The duration of pretreatment time affects the number of reactant molecules colliding, leading to increased breakdown of lignocellulose into cellulose and degradation into reducing sugars [30].

Between the 2nd and 3rd hour of pretreatment, there is also a decrease in reducing sugar concentration with an increasing H_2SO_4 concentration. This is because an extended time in acidic pretreatment leads to the degradation of monosaccharides, resulting in the production of furfural as an inhibitor that can later inhibit the fermentation process [31]. Side products like furfural will form if the pretreatment time is prolonged, potentially disrupting enzyme activity in converting cellulose into simple sugars [32]. The pretreatment time is proportional to the amount of reducing sugar formed, but at a certain time limit, the reducing sugar content may not exhibit significant differences due to very low conversion rates [30].

3.3. The Effect of H_2SO_4 Pretreatment on Cabbage Waste on Bioethanol Yield

Cabbage waste pretreated with H_2SO_4, with the highest reducing sugar content, which is sample E (1.5 M; 1.5 hours), is then subjected to fermentation to produce bioethanol. The fermentation process is carried out for 96 hours using the Simultaneous Saccharification and Fermentation (SSF) method, with the involvement of *Saccharomyces cerevisiae* and cellulase enzymes. Several factors influence bioethanol fermentation, including substrate, temperature, pH, initial sugar concentration, supplementation of an external nitrogen source, and the inoculum size [9]. The fermentation process begins by adding 7 grams of cabbage waste, which contains a reducing sugar concentration of 0.32 g/L, along with a suspension of *Saccharomyces cerevisiae* that has been incubated for 48 hours with a culture size of 0.638 g/L.

Saccharomyces cerevisiae can produce enzymes such as zymase and invertase, where zymase can break down sucrose into glucose and fructose. However, *Saccharomyces cerevisiae* is incapable of breaking down cellulose into reducing sugars [33]. Therefore, the assistance of cellulase enzymes is required to break down the cellulose chains into simpler components. Cellulase enzymes primarily focus on breaking down cellulose into reducing sugars and consist of three main parts: endoglucanase, exoglycanase, and beta-glucosidase. Endoglucanase attacks the crystalline part of cellulose, exoglycanase cuts the cellulose's main chain, producing several cellobiose units, which are then further degraded into smaller units, namely reducing sugars, by beta-glucosidase [27]. Cellulase enzymes work optimally in a pH range of approximately 4.5 to 6.5 and a temperature range of 20 to 50°C [34], [35].

Hydrolysis results in the production of reducing sugars, which are subsequently processed in bioethanol fermentation. Reducing sugars are sugars that, under basic conditions, have the potential to reduce transition metal ions such as Cu^{2+} due to the presence of aldehyde or free ketone groups at the ends of monosaccharides. This forms the basis for sugar content tests based on the reactivity of aldehydes in reduction, as exemplified in the Fehling's test, where Cu^{2+} ions are reduced to Cu^{+} and aldehydes are oxidized into carboxylic acids [36]. These reducing sugars include glucose and fructose [37].

In Figure 6, it shows the concentration of reducing sugars and the bioethanol content over the course of fermentation. At 0 hours, the reducing sugar content is 0.62 g/L, and it increases to 3.69 g/L at 24 hours. This increase is due to the hydrolysis process catalysed by cellulase enzymes, which results in the production of reducing sugars during fermentation. The increase in reducing sugar concentration is also influenced by the reaction rate, which is affected by the amount of cellulose formed after pretreatment [38].

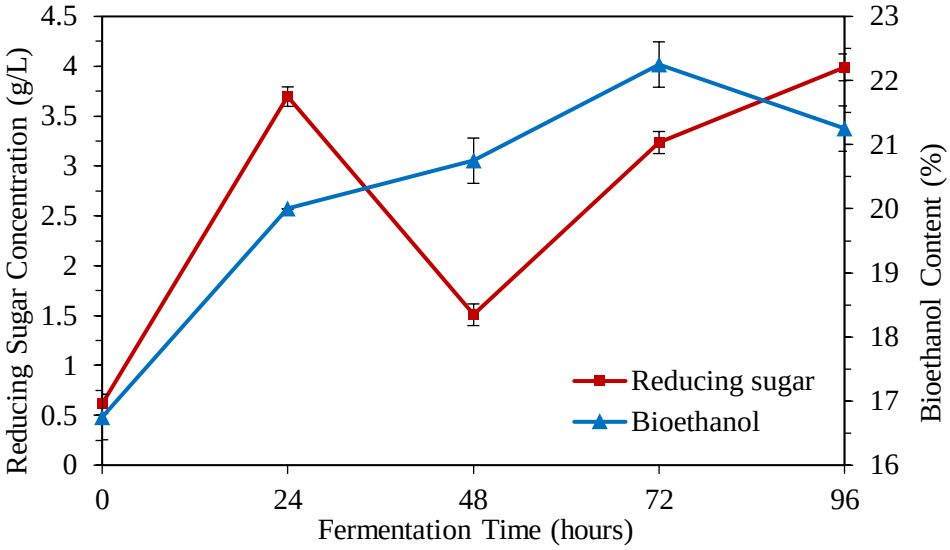

Figure 6. The Concentration of Reducing Sugars and the Bioethanol Content

A decrease in reducing sugar concentration occurs at 48 hours, reducing it to 1.51 g/L. This reduction in reducing sugar content is because the sugar is consumed by *Saccharomyces cerevisiae* as a carbon source and converted into bioethanol [39]. At 72 hours, there is still an increase in reducing sugar concentration, reaching 3.24 g/L at 96 hours. This is attributed to the ongoing hydrolysis process in Simultaneous Saccharification and Fermentation (SSF), breaking down cellulose into reducing sugars [40].

In the fermentation process using *Saccharomyces cerevisiae*, the breakdown of reducing sugars will result in the production of ethanol and a byproduct in the form of carbon dioxide [41]. The ethanol content represents the product generated from anaerobic fermentation. In Figure 6, the ethanol content at 0 hours is 16.75%; at 24 hours, it's 20%; at 48 hours, it's 20.75%; at 72 hours, it's 22.25%; and at 96 hours, it's 21.25%. The highest ethanol content, 22.25%, is achieved at the 72-hour, and a decline in ethanol content begins at the 96-hour. At 0 hours, bioethanol has been detected because it is possible to use previously active starters, thus accelerating the beginning of the fermentation process. Additionally, it is supported by the presence of sufficient sugar sources (reducing sugar at 0 hours was 0.62 g/L) to be consumed by microorganisms, enabling bioethanol detection at the beginning of the fermentation process.

The activity of yeast consuming carbon sources from reducing sugars can increase the ethanol content [42]. The ethanol content continues to rise until it reaches its peak at 72 hours. However, the decrease in ethanol content at 96 hours is suspected to be due to the conversion of reducing sugars into glycerol by *Saccharomyces cerevisiae*, reducing ethanol production [43]. A decrease in the number of *Saccharomyces cerevisiae* and the continuous increase in reducing sugar content led to high osmotic pressure within the suspension. The synthesis and intracellular accumulation of glycerol are *Saccharomyces cerevisiae*'s responses to balance the osmotic pressure of the cell membrane. However, *Saccharomyces cerevisiae* is not capable of accommodating a large amount of intracellular glycerol molecules, causing yeast to rupture and release glycerol [44].

4. Conclusions

Pretreatment using H_2SO_4 and NaOH can change lignocellulose levels in cabbage waste. The hemicellulose content in the pretreatment using H_2SO_4 increased by 13.06%, cellulose increased by 28.31%, and lignin decreased by 3.11%. Whereas in the pretreatment using NaOH the hemicellulose content increased to 12.25%, cellulose increased to 16.84%, and lignin decreased by 4.65%. Pretreatment using H_2SO_4 at a concentration of 1.5 M for 1.5 hours produced the highest reducing sugar concentration of 0.32 g/L. The highest bioethanol content was obtained during fermentation for 72 hours at 22.25%.

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