# Effect of Alkali Pretreatment on Enzymatic Hydrolysis of *Gracilaria* sp.

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**Abstract.** Pretreatment of seaweed biomass for bioethanol production. This study aimed to determine the effect of biomass pretreatment with 5% NaOH alkali on enzymatic hydrolysis in the bioethanol production process using *Gracilaria* sp. as raw material. Pretreatment with 5% NaOH alkali was carried out with different time variations, namely 60, 90, and 120 minutes at a temperature of 50ºC. After pretreatment, the enzymatic hydrolysis process was carried out using cellulase enzymes on a shaker incubator at a temperature of 50ºC, 150 rpm for 72 hours, then the fermentation process for 72 hours using *Saccharomices cerevisiae* mold. The results showed that 5% NaOH alkali pretreatment could improve the quality of *Gracilaria* sp. biomass. The lignin content produced was 10.43 ± 0.15%.

## 1 Introduction

Rapid growth in industry, transportation, urbanization and population leads to a significant increase in energy demand [1]. Fuels are essential for modern life, from essentials such as cooking to the use of heavy equipment. The increasing need for energy has raised concerns about the sustainability of fossil fuel use. The rapid decline in the availability of fossil fuels has prompted governments, scientists and researchers to look for alternative energy sources. This drive has also been influenced by rising crude oil prices, environmental damage and global climate change. Increased greenhouse gas emissions are caused by the burning of fossil fuels (natural gas, coal and petroleum), as well as the impact of uncontrolled forest burning. The increase in the content of air pollutants such as carbon dioxide, methane, and nitrous oxide is the main factor causing temperature rise [2]. The use of non-renewable

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energy in fuel production results in increased $CO_2$ emissions and is not environmentally friendly [3]

Research results show that until 2030, a significant increase in energy demand is expected, and by 2060, it is estimated that humans will no longer depend on fossil fuels as a source of energy [1]. Increasing public interest in environmental issues is driving the exploration of alternative energy sources that are environmentally friendly, but economically prospective. Biofuels can reduce the effects of greenhouse gases such as carbon monoxide and volatile organic compounds are hazards to human health and the environment. Biofuels from renewable resources have been generally regarded as one of the most sustainable options to replace fossil fuels, as well as a method that is environmentally friendly and supports economic sustainability [4]. One area of intense research is the production of bioethanol. It increases energy security and variety in energy supply and can contribute to increased employment and boost economic growth [5].

Bioethanol is a single-chain alcohol with an octane number of 108, difficult to evaporate, low in calories, and flammable with the chemical formula of bioethanol is $C_2H_5O$ [6]. Bioethanol is used as a useful substitute for renewable energy sources to meet national fuel and energy demand. Bioethanol can help reduce the impact of climate change as it produces lower carbon emissions than fossil fuels [2]. The use of bioethanol as a substitute for reformulated gasoline E95 reduces carbon dioxide emissions by 90%, which can help reduce the impact of climate change. Ethanol was first used as a motor fuel in 1897 by Nikolas Otto for internal combustion engines. Ethanol became popular as an alternative fuel after the crisis in the 1970s. Many countries started initiatives to learn and produce economical fuels from easily accessible raw materials such as Brazil, India and the United States [7].

Various potential feedstock sources from agriculture, waste, fisheries, and marine for bioethanol production. The use of biomass such as rice, wheat and sugarcane in the manufacture of bioethanol is considered a potential source of human food, which could pose a risk of food scarcity and discontent globally, especially in developing countries [4]. One of the prospective feedstocks to be developed on a large scale is algae biomass. Algae or seaweed has rapid growth of 30-45 days, land use efficiency, does not compete with food crops, and reduces environmental impact through $CO_2$ sequestration from the atmosphere [8]. Research trends are exploring the potential of various types of algae as raw materials for bioethanol production, for example *Sargassum, Hydropuntia*, *Gracilaria* and *Ulva* [9, 10, 11].

*Gracilaria* is a seaweed from the phylum Rhodophyta and a species of red algae. *Gracilaria* has cellulose-based lignocellulose that contains many complex polysaccharide compounds that can be broken down into simple sugars [12]. The hydrocolloid source of *Gracilaria* sp. can be used in the agar and carrageenan industry. The agar industry has grown in recent years resulting in increased production of *Gracilaria* sp. Agar production and bioethanol production using *Gracilaria* sp. will not be compromised if *Gracilaria* varieties with high agar content are selected for agar production. Good management in the supply of *Gracilaria* as raw material for the sustainability of agar or bioethanol production [13].

The cell wall consists of a mixture of neutral polymer agarose, pyruvate agarose, and sulfated galactans. Polysaccharides owned by *Gracilaria* can be processed into bioethanol. The breakdown of polysaccharides will produce monosaccharides that can serve as basic ingredients in the bioethanol fermentation process. Indonesia is one of the largest producers of *Gracilaria* sp. in the world. *Gracilaria* production continues to increase in 2021 at 1,916,185 tons with the highest production in South Sulawesi province at 1,106,996 tons [14]. Red seaweed produces a hydrocolloid called agar.

The bioethanol production process consists of pretreatment, hydrolysis and fermentation stages. The process of bioethanol production from seaweed is strongly

influenced by the choice of method, especially at the hydrolysis and fermentation stages, because it has a significant impact on the final ethanol yield [15]. This study used NaOH in the pretreatment process to reduce the levels of lignin present in seaweed. The use of NaOH can break the bonds of the basic structure of lignin, which makes lignin easily soluble [16]. Ethanol production is influenced by the amount of fermentable sugars liberated during the hydrolysis process. This study used cellulase and agarase enzymes.

The process of biomass hydrolysis through enzymatic saccharification with cellulase is more attractive than chemical conversion due to its benefits, including lower cost and or no pollution during the bio-process [17]. In fact, it is a more environmentally friendly way to treat saccharification chemical sulfuric acid than other methods. Agarase is an enzyme that plays a role in facilitating the hydrolysis process on agar or agarose. The enzyme has the ability to hydrolyze agar into oligoagar. The fermentation process involves microorganisms that aim to convert simple sugars into bioethanol. The fermentation process can be done by Separated Saccharification and Fermentation (SHF) or simultaneously with enzymatic hydrolysis Simultaneous Saccharification and Fermentation (SSF). The advantage of the SHF method is that enzymes and yeast can work at ideal temperatures. The SSF method can minimize the inhibitory substances that appear during the process of converting sugar into bioethanol because both methods work simultaneously [18]. This study aims to determine the length of NaOH alkali pretreatment time and the addition of agarase to the characteristics of biomass and hydrolysate in the process of making bioethanol.

## 2 Materials and methods

### 2.1 Tools and Materials

The tools used in this study were mixer, measuring cup (AGC Iwaki), erlenmeyer (HERMA), beaker (AGC Iwaki), centrifuge, vortex (DLAB MX-S), autoclave (Vacuum Drying 2321 VD), furnish (WiseTherm), oven (WiseVen Fuzzy Control System), shaker, hot plate (Cole Parmer VEIA), analytical balance (Sartorius), muslin cloth, filter paper, micropipette, tubes, tips, UV-Vis spectrophotometer (Hitachi U-2900, Illinois -USA), and Fourier transform infrared spectroscopy (PerkinElmer Spectrum One S4934, Ohio, USA), Scanning electron microscope (FE-SEM Apreo 2).

The material used was Gracilaria sp. seaweed from the waters of Brebes, Central Java, Indonesia, NaOH, $H_2SO_4$, distilled water, 0.05 M citrate buffer, sodium potassium tartrate, 3,5-Dinitrosalicylic acid (DNS), Cellic Ctech 2 and Htech 2 cellulase enzymes (Novozymes) and agarase enzymes.

### 2.2 Alkaline Pretreatment

The pretreatment process uses chemical pretreatment. The pretreatment process consisted of several stages and treatments, namely sample preparation, neutralization and drying. Pretreatment was carried out using NaOH with a concentration of 5% at 30$^{o}$C with time variations of 60, 90, and 120 minutes. To obtain pretreated biomass, the pretreated samples were washed, filtered, neutralized. Pretreated biomass samples were dried in an oven at 50°C for 3 days. Dried biomass was analyzed for moisture content, aiming for a maximum of 10%. The preferred process involves several stages: sample preparation, neutralization, and drying. During preparation, samples were treated with 5% NaOH at 30°C for varying durations of 60, 90, and 120 minutes. The samples were then washed, filtered, and neutralized to obtain the desired biomass. Subsequently, the samples were dried in an oven at 50°C until the

moisture content dropped below 10%. After drying, the moisture content was checked to ensure it did not exceed the 10% limit.

## 2.3 Hydrolysis

The hydrolysis process was carried out by preparing a pretreated sample of 2.5 g in an erlenmeyer. Adding 0.05 M citrate buffer with a total of 22.5 mL, and heated in an autoclave at 121°C for 30 minutes, let the sample cool down. The hydrolysis process was treated with the addition of 1% w/v agarase enzyme and no agarase after the sample was cooled and C-Tech, H-Tech and agarase enzymes were added to the sample with a total enzyme of 30 Filter Paper Unit (FPU) in the sample that was not given agarase enzyme. The total addition of C-Tech was 0.75 mL while the total addition of H-Tech enzyme was 0.15 mL. After 24 hours, the agarase- treated samples were added with 30 Filter Paper Unit (FPU) of C-Tech and H-Tech enzymes. The samples were again continuously shaken at 150 rpm for 3 days with a temperature of 50°C and sampled at 24, 48, 72 hours. Each sampling result was be measured for sugar content with DNS analysis.

## 2.4 Analysis

### *2.4.1 Proximate Analysis*

Proximate analysis was carried out according to the guidelines established by the AOAC 2005. This involved four parameters: ash, protein, fat and carbohydrate on a dry basis.

#### Moisture Content Analysis

The cold, dry porcelain cup is weighed to determine its weight. Samples as much as 1-2 g were weighed and put into a porcelain cup. The cup was placed in the oven for 3 hours at 105°C. After that, the samples were cooled in a desiccator before being weighed. Moisture content was calculated using the following formula:

$$\text{Moisture content (\%)} = \frac{((A+B)-C)}{B} x\ 100\% \quad (1)$$

Description :
A = Weight of empty cup (g)
B = Weight of cup (g)
C = Weight of cup with sample after drying (g)

#### Ash Content Analysis

The ash content analysis process was carried out by weighing as much as 3g of sample and put into a porcelain cup. The sample was then put into a furnace with a temperature of 550$^{o}$C with a time of 4 hours or until complete ignition, which is indicated by the color of the sample becoming grayish white ash. The next step is cooling the cup in a desiccator and weighing it until the weight stabilizes. The calculation of the percentage of ash content uses the following formula:

$$\text{Ash content (\%)} = \frac{(C-A)}{B} x\ 100\% \quad (2)$$

Description :
A = Weight of empty cup (g)

B = Weight of cup (g)
C = Weight of the cup with sample after annealing (g)

### Fat Content Analysis

The fat content in the sample is measured using the Soxhlet extraction technique with hexane as the solvent. The process begins by drying a 300 mL fat flask in an oven at 105°C for 30 minutes, cooling for 15 minutes, and then weighing it. The sample, weighing 1 g, is mixed with 15 mL of 25% HCl and 10 mL of distilled water in a glass cup, covered, and heated on a hot plate for 15 minutes until boiling. The mixture is filtered through hot gray filter paper and washed with hot distilled water. The residue is dried at 105°C for one hour. Next, the Soxhlet extractor is used to add hexane, with the fat flask filled halfway and the sample fully immersed. The extraction continues for three hours until the solvent runs clear. Afterward, the hexane is evaporated, and the remaining fat is dried, cooled, and weighed to determine the fat content using a specific formula.:

$$\text{Fat content (\%)} = \frac{(C-A)}{B} x\ 100\% \quad (3)$$

Description :
A = Weight of empty cup (g)
B = Weight of cup (g)
C = Weight of the cup with sample after heating (g)

### Protein Content Analysis

The Kjeldahl method is used to determine the protein content of a sample. The process starts by weighing 1 g of the sample and placing it in a 300 mL Kjeldahl tube. The KjelDigester is preheated to 420°C, then the tube is inserted for digestion at the same temperature for one hour to produce a clear solution. After digestion, the apparatus is turned off, and the tube is cooled to room temperature. The distillation unit is set up with an Erlenmeyer flask to collect the distillate, which is distilled for 4 minutes until the solution turns from red to bluish-green. The distillate is then titrated with 0.2 N HCl until the endpoint, indicated by a color change from green to pink, is reached. The protein content is calculated using a specific formula:

$$\text{Protein content (\%)} = \frac{(Vp-Vb)\times N\ HCl\ x\ 14{,}01 \times FK}{W}\ x\ 100\% \quad (4)$$

Description :
Vp = HCl volume used for sample titration (mL)
Vb = HCl volume used for blanko titration (mL
N HCl = Normality value of HCl solution used for titration (N)
14,01 = Atomic weight of nitrogen
FK = Dilution factor
W = Sample weight (mg)

### Carbohydrate Analysis

Analysis of total carbohydrate content using the by difference method was used, in which the percentages of water, protein, ash, and fat were reduced by one hundred percent. The calculation of carbohydrate content is as follows:

$$\text{Carbohydrate content (\%)} = 100\% - A \quad (5)$$

Description :
A = Total percentage of water content + fat content + ash content + protein content

### *2.4.2 Lignin Content*

The raw materials used in the form of seaweed before pretreatment were analyzed for chemical components. The analysis process began with weighing a sample of 0.3 g, and mixed into 3 mL of $H_2SO_4$ with a concentration of 72%. Next, it was put into a waterbath for 2 hours at 30°C, and every 30 minutes was done. Vortex on the sample. Then autoclave for 30 minutes at 121°C, followed by cooling the sample. The process continues with collecting 10 mL of the liquid filtrate in a test tube, to which 3 mL of 4% $H_2SO_4$ and 100 µL of the filtrate are added. The absorbance is then measured using a UV spectrophotometer at a wavelength of 205 nm.

$$\text{Soluble lignin (\%)} = \frac{\left(\frac{Absorbansi\ UV \times fp}{110}\right) \times \frac{87}{1000}}{Dry\ weight\ of\ biomass}\ x\ 100\% \quad (6)$$

$$\text{Insoluble lignin (\%)} = \frac{Dry\ weight\ of\ biomass\ sediment - ash}{Dry\ weight\ of\ biomass}\ x\ 100\% \quad (7)$$

$$\text{Lignin (\%)} = Soluble\ lignin + Insoluble\ lignin \quad (8)$$

### *2.4.3 Reducing Sugar Analysis*

The filtrate results that have been collected are then analyzed for reducing sugar content in the sample with DNS analysis. The sample was diluted with a 10x dilution, the results of the dilution were taken 0.5 mL and then added with 0.5 mL of DNS. Then heated for 5 minutes and cooled, after which 1.5 mL of distilled water was added. Calculate the total absorbance of the sample with a spectrophotometer with a wavelength of 540 nm [19].

### *2.4.4 Function Group Analysis*

Functional group analysis using Fourier transform infrared (FTIR) is one of the highly effective instruments in identifying functional groups, along with potential molecular bonds between chemical compounds within them. FTIR analysis was carried out by weighing the pretreated sampel as much as 0.5 g. The sample was pulverized into powder form, then the sample powder was mixed with potassium bromide (KBr) powder and pressed together under high pressure of about 12,000 psi for 1-2 minutes. The ratio between the sample powder and KBr is about 1:100, resulting in a very homogeneous KBr pellet. The formed KBr pellets can be placed in a holder on an FTIR spectrometer [20]

## 3 Result and discussion

### 3.1 Characteristics of *Gracilaria* sp.

*Gracilaria* sp. a type of red seaweed belonging to its genus, is one of the most valuable marine macrophytes in aquaculture and the global economy. *Gracilaria* is widely distributed in tropical and subtropical waters in various parts of the world, and has a fast growth rate [21]. The *Gracilaria* sp. used in the study contained impurities such as stones, mud, and shells of animals such as clams and snails. The seaweed was then cleaned and dried, then pulverized with a grinder. Figure 2 shows the visualization of the seaweed sample.


**Fig 1.** Visualization of *Gracilaria* sp., (a) fresh seaweed after harvesting and (b) dried seaweed

Figure 1 shows the visual of the *Gracilaria* sp. raw material after harvesting and after cleaning and then mashing using a grinder. The visual color after mashing has a lighter and fainter color than before harvesting. The initial stage before pretreatment is proximate testing. Proximate testing was carried out to determine the content contained in the sample. Chemical composition analysis consists of ash content, protein content, fat content, and carbohydrate content. The proximate and lignin can be seen in Table 1.

**Table 1.** Results of proximate analysis and lignin content of *Gracilaria* sp. raw materials

| Parameters | Value (%) |
|---|---|
| Water Content | 6.01 ± 0.07 |
| Ash Content | 8.92 ± 0.24 |
| Protein Content | 6.16 ± 0.16 |
| Fat Content | 0.65 ± 0.13 |
| Carbohydrate Content | 83.64 ± 1.45 |
| Lignin Content | 10.53 ± 0.15 |

This study found that the ash content was 8.92%, while the protein content of *Gracilaria sp.* seaweed was 6.61%. The fat content measured was 0.65%, and the carbohydrate content was 83.64%. Additionally, the lignin level was notably high, reaching 10.53%. The results of Sa'diyah and Anugerah's research [22] showed that the resulting content for ash content was 32.76%. The ash content was 5.83 ± 0.08 - 7.62 ± 0.20% [23]. harvest age can cause differences in ash content in seaweed [24]. The decrease in ash content occurs because the seaweed raw material used has a lower mineral content than that found in the research of Kawaroe [25], which reached 10.12%. Increased harvest time in seaweed can cause more minerals to be absorbed which results in increased ash content.

The red seaweed *Gracilaria* sp. is composed of dry matter containing 16.0% crude protein, 76.67% carbohydrates, 6.13% ash, 7.87% crude fiber, and 1.2% crude lipids [26]. According to research by Kawaroe [25], the protein content of *Gracilaria* sp. seaweed was found to be 4.43%, while the fat content was 0.82% [24]. Carbohydrate levels obtained from research by Chan and Matanjun [27] on *Gracilaria* changii amounted to 41.52% [27]. The results of the analysis showed lower carbohydrate levels because the seaweed analyzed came from cultivation rather than from the sea directly. Lignin levels resulting from this study were higher than the results of research by Sabili (2016), which amounted to 5.41% [28]. Lignin with a large amount will be more difficult for enzymes to convert cellulose into glucose. Lignin has a higher pH than cellulose and hemicellulose, which can change the pH of the hydrolysis process and can affect enzyme activity and hydrolysis efficiency [29].

### 3.2 Characteristics of *Gracilaria* sp. with Alkali Pretreatment

Pretreatment is the process of changing the chemical composition, macro- and microstructure of lignocellulose. It can also increase the susceptibility of lignocellulosic macromolecules to degradation by microorganisms. The pretreatment process is an important step required to

reduce cellulose crystallinity, remove lignin, and increase material porosity [30]. *Gracilaria* sp. seaweed is considered an important species in agricultural programs in Southeast Asia, particularly in South Sulawesi, Indonesia, as they produce the main raw material with cellulose content reaching 30-35% [31]. The apperance seaweed biomass after pretreatment using 5% NaOH can be seen in Figure 2.

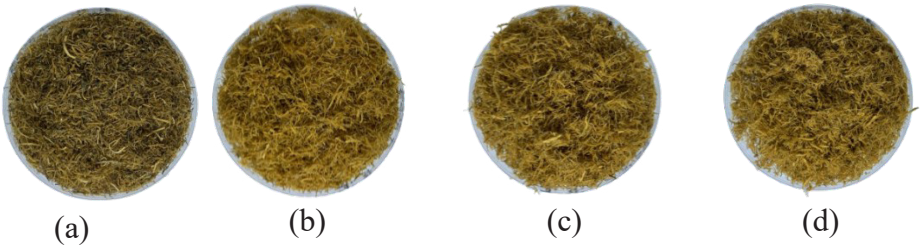


**Fig 2.** a) before pretreatment and after pretreatment with 5% NaOH for b) 60 min, c) 90 min d) 120 min.

The figure above shows the visual of *Gracilaria* sp. raw material after pretreatment with 5% NaOH for 60, 90 and 120 minutes. Changes in color and softer structure of the samples can be observed visually. The sample before pretreatment was darker in color compared to the pretreatment sample. The color change is caused by pretreatment with 5% NaOH alkaline solvent. This is in accordance with the research of Habibah *et al.,* (2017), which stated that using alkaline solutions during the extraction process can cause the color of the material to brown as a result of the browning reaction [32]. The lighter color of *Gracilaria* sp. indicates a decrease in the amount of lignin and hemicellulose components that bind to cellulose [33]. Pretreatment with NaOH with various times showed that the longer the pretreatment time, the lighter the color of the seaweed because the pigment is removed [34]. Another effect can occur due to the use of pH above 10 which causes seaweed to become brighter [35]. In addition to significant visual differences between the different time pretreatments, each sample showed differences in color parameters.

### 3.2.1 Functional Groups of *Gracilaria* sp.

FTIR was used to examine changes in the functional group structure of the samples before and after alkaline pretreatment. The functional group spectra of each sample are presented in Figure 3.

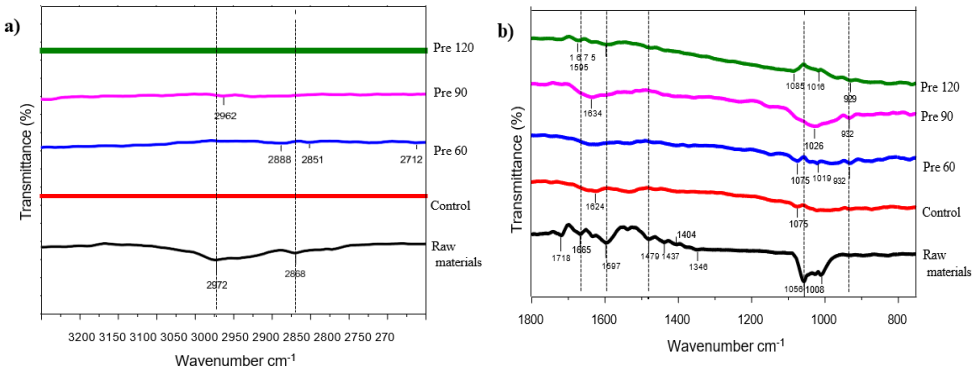


**Fig 3.** FTIR spectra of *Gracilaria* sp. seaweed without pretreatment and with 5% NaOH pretreatment for 60, 90, 120 minutes, a) wavenumber 2700-3200, b) wavenumber 800-1800

The FTIR spectrum patterns for *Gracilaria sp.* seaweed, with and without 5% NaOH pretreatment, are nearly identical, as shown in Figure 5. The peak at 932 $cm^{-1}$ indicates O-H stretching. According to Stevulova [36], peaks between 2800-3400 $cm^{-1}$ correspond to polysaccharides, with hemicellulose observed at 1026 and 1733 $cm^{-1}$, and lignin at 1500-1600 $cm^{-1}$ [36]. The 1300-1400 $cm^{-1}$ peak suggests the presence of cellulose, characterized by (-O-) linked to the carbon chain [37]. The C-O absorption band appears between 1000-1200 cm–1, and bands at 1056 $cm^{-1}$ indicate C-O stretching in primary and secondary alcohols of lignin, hemicellulose, cellulose, and extracts [38]. Peaks between 1058-1060 cm–1 suggest C-O and CH vibrations, indicating cellulose structures.

O-H and CH peaks serve as key absorption markers for carboxylic groups ($COO^-$), with similar intensity seen at 1400-1600 $cm^{-1}$. The absence of C=C aromatic lignin stretching at 1433-1513 cm–1 indicates the removal of lignin from the sample [39]. Lastly, the band at 3100-3700 $cm^{-1}$ is linked to O-H stretching vibrations in hydrogen-bonded hydroxyl (-OH) groups [40].

### 3.3 Hydrolysate Characteristics of *Gracilaria* sp

The hydrolysis process is the breaking of seaweed polymer chains into simple sugars by water. The hydrolysis process is the most important stage in the manufacture of bioethanol. The hydrolysis process serves to break the polysaccharide chain into monosaccharides. The hydrolysis method used is enzymatic. The hydrolysis process with the treatment of not given agarase and giving agarase to seaweed. The results of ydrolysis are presented in Figure 4.

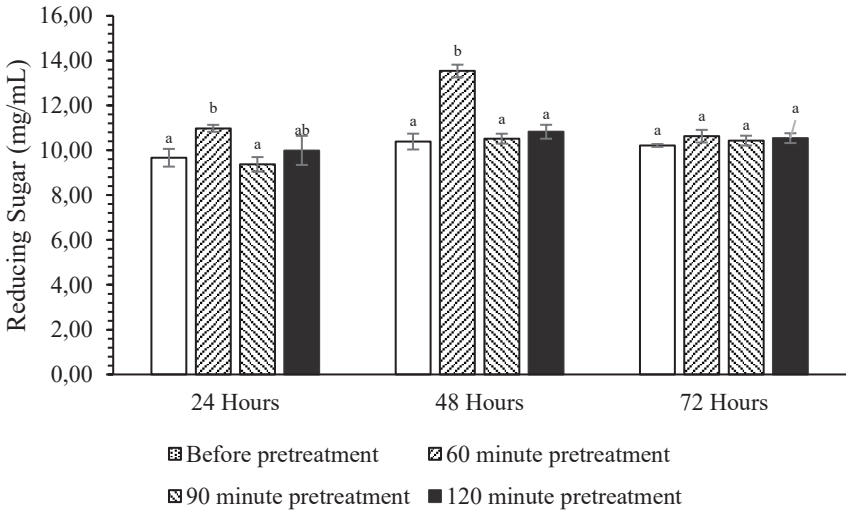


**Fig 4.** Reducing sugar content of hydrolysis process with agarase. Different letters in each parameter indicate significantly different statistically significant values ($p<0.05$).

Hydrolysis result of sample without agarose treatment becomes gel so that it cannot be tested for reducing sugar content. Hydrolysis with agarase enzyme, the highest value of sugar content is obtained in samples with NaOH pretreatment of 60 minutes, and enzymatic hydrolysis duration of 48 hours 13.54 mg/mL. The 5% NaOH pretreatment sample, treated for 90 minutes, had the lowest sugar content of 9.38 mg/mL after 72 hours. According to Nguyen [40] that *Gracilaria sp.* could release 21.17 mg of reducing sugar [40]. In the study by Wu [41], the sugar yield from *Gracilaria sp.* was found to be 27.7% for galactose and 31.5% for glucose [41]. A significant effect on the total reducing sugar produced was shown

by the length of incubation (24, 48, and 72 hours). A decrease was observed at 72 hours, likely due to factors such as the pH of the hydrolysis process and extended storage time before analysis, which can lead to reduced sugar content [42]. The content of lignin, hemicellulose, and cellulose can be affected by the length of pretreatment with NaOH, and shows that longer pretreatment can accelerate cellulose degradation and release soluble sugars from pretreated samples, which causes loss of glucose yield and increases the decrease in cellulose content [43]. An important process in bioethanol production is to produce reducing sugars that serve as a carbon source for the growth of *S. cerevisiae* [44].

Pretreatment is necessary for cellulose to be fully utilized, as lignin is a biopolymer that functions as a hemicellulose adhesive and protects cellulose. The main obstacle in hydrolyzing cellulose is the lignin and hemicellulose located around it. The chemical structure of lignin and hemicellulose forms hydrogen bonds, making hydrolysis more challenging. Incomplete hydrolysis can also produce disaccharides from cellulose, such as cellobiose, which cannot be fermented to produce ethanol [45]. Lignin has a higher pH than cellulose and hemicellulose, which can change the pH of the hydrolysis process and can affect enzyme activity and hydrolysis efficiency [29].

Alkali can cause swelling of cellulose, increase cellulose surface area, decrease the degree of polymerization and crystallinity of cellulose, destroy glycosidic and ester bonds in lignin, and serve as a partial solvent of hemicellulose [46]. Pretreatment using alkaline solution or NaOH can expand the amorphous area and release lignin from the coating [47]. Chemical pretreatment with acidic and alkaline compounds can reduce lignin and ash content allowing better enzymatic hydrolysis [48]. This process is effective in breaking down lignin, known as delignification, as well as removing impurities commonly found in seaweed [49]. When 7% NaOH was used for pretreatment, the gel strength increased by 152.5% (301-760 g/cm$^2$).

a) b)

**Fig 5**. The appearance of enzymatic hydrolysate at 72 hours, a) no agarase, b) agarase.

The enzymes used in this study are C-Tech 2 and H-Tech 2 enzymes which are a combination of cellulase and xylanase. These enzymes are responsible for breaking the β-1,4 bond on hemicellulose to produce xylooligosaccharides (XOS) and breaking the β-1,4 bond on cellulose to produce glucose [16]. Agarase hydrolyzes the agar still present in *Gracilaria* sp., while cellulase enzyme hydrolyzes cellulose. The optimum pH for agarase is around 7-8, while cellulase is at pH 4-5. When agarase and cellulase are combined, agarase is not at the right pH to hydrolyze agar, so the sugar content produced will be lower [50].

Enzymatic hydrolysis is much better than chemical hydrolysis, because hydrolysis using enzymes can reduce the use of acids which can reduce side effects on the environment. Enzymes can hydrolyze cellulose bonds faster and produce monosaccharides such as glucose [43]. Enzymes have high specificity, the right and appropriate substrate concentration can improve enzyme performance. Enzyme concentration affects thehydrolysis process can run optimally and hydrolysis can run faster [16]. The use of enzymes can increase fermentation efficiency and produce more bioethanol. These simple conditions do not cause the formation of harmful by-products. Enzyme type and pH are some important components that can affect

the saccharification process [39]. NaOH can increase cellulose content in seaweed biomass while decreasing the amount of lignin. A very high concentration of NaOH can make lignin more difficult to break down, which means the amount of cellulose produced will be lower [51].

## 4 Conclusion

Pretreatment of *Gracilaria* sp. seaweed at different times with 5% NaOH solvent affects the reducing sugar content. Lignin content was this raw material reached 10.53 ± 0.15%. The best method was found in pretreatment for 60 minutes with the highest reducing sugar content of enzymatic hydolysate at 13.54 mg/mL. Pretreatment also causes differences in wavelength intensity on FTIR.

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