Alkaline Pre-Treatment of *Gelidium Latifolium* and *Caulerpa Racemosa* for Bioethanol Production

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Abstract. Raw material pre-treatment becomes an important step in the bioethanol production from macroalgae. It’s help to breakdown sulfated groups and lignin, facilitate the hydrolysis process to increase reducing sugars. *Gelidium latifolium* and *Caulerpa racemosa* are red and green algae which used for bioethanol production in this study. The carbohydrate content was 23.81% and 30.54% respectively. The study aims to evaluate the effect of alkaline pre-treatment to the chemical characteristics of macroalgae, productivity of sugar and bioethanol. The alkaline used was sodium hydroxide (NaOH) with a concentration of 0.05 and 0.1 N, soaking time for 3 days at room temperature. From the result, it was known that water and alkaline pre-treatment increased cellulose content for both macroalgae, as well as yield of reducing sugars. The highest reducing sugar for *G. latifolium* was 5.26% w/v obtained from 0.1 N NaOH pre-treatment. While for *C. racemosa* it was 7.67% w/v obtained from the one which submerged on water. *G. latifolium* treated with 0.05 N NaOH has the highest ethanol content of 6.3 ml/L with 21.94% fermentation efficiency and 37.30% substrate efficiency. On the other hand, the highest ethanol production for *C. racemosa* obtained from water immersion of 7 ml/L with 17.26% fermentation efficiency and 15.41% substrate efficiency.

1. Introduction

Macroalgae is a potential source of biomass to produce bioethanol because it’s high content of carbohydrate. Moreover, coastal area potential for macroalgae cultivation in Indonesia is abundance which is 1.1 million ha, while until 2003 the utilization was only 222,180 ha or about 20% [1]. Macroalgae also has a short life cycles, it only takes 45 days to be harvested without requiring fertilizer or nitrogen-based additives [2].

Polysaccharides of macroalgae have different characteristics compare to terrestrial plants. It has a crosslinked polysaccharide with sulfate, lignin and various types of salt. To facilitate the hydrolysis process of polysaccharides, a pre-treatment technique that can be used is submersion in alkaline solutions.

Seaweed submersion in a NaOH solution can remove impurities that solubles in alkaline [3]. This technique is capable of dissolve lignin, so that the material structures become more amorphous after lignin separated from cellulose and hemicellulose. *Sargassum sp*. which was submersed in 0.5% NaOH solution can increase the yield of reducing sugars up to 46% [4]. Similarly, alkaline submersion of *Gracilaria sp.* can increase galactose content from 39% to 56.4% [5]. The increase of reducing sugar means that more sugar available for fermentation by yeast for bioethanol production. Acid hydrolysis of *Euchema cottonii* resulted on high reducing sugars (11.34%, w/v) and low hydroxymethyl furfural.
content, while ethanol yield was 2.49% (v/v) [6]. Hereinafter, hydroxymethyl furfural abbreviated as HMF.

G. latifolium is red alga and C. racemossa is green algae, both have a high carbohydrate content of 67.85% and 23.63%, respectively, so that they could be the potential raw materials for bioethanol production. Sulfuric acid is more suitable for hydrolysis of G. latifolium than hydrochloric acid [7]. As explained previously, the polysaccharides of seaweed is different from terrestrial plant, so it requires special techniques to degrade its chemical linkage of monosaccharides into fermenting sugar. Therefore, this research aimed to evaluate the effect of alkaline pre-treatment on the characteristics of hydrolysates and the yield of reducing sugar after hydrolysis of G. latifolium and C. racemosa in order to increase bioethanol production.

2. Materials and Methods

2.1. Materials
G. latifolium and C. racemossa collected from Pari Island-Thousands Islands, Indonesia. AL IX for ethanol fermentation is a yeast of S. cereviceae (IPBCC) which slowly adapted on seaweed hydrolysates. Chemicals for treatment and analysis were HCl, NaOH, NPK, urea, Yeast-Malt-Peptone Glucose medium abbreviated as YMGP medium, PDA (Potato Dextrose Agar) media, DNS (3,5 dinitrosalisilic acid) reagent, fenolftalein, Luff Schoorl reagent, KI, H$_2$SO$_4$, 5% fenol solutions, 0.5% starch solutions and Na$_2$S$_2$O$_7$.

2.2. Alkaline Pre-Treatment
A hundred grams of each type of macroalgae was submerged in 5 liters of water, 0.05 N and 0.1 N NaOH solutions, respectively. Submersion was done for three days. Then, seaweeds were washed, cut ±1 cm long, and dried for two days. Seaweeds from alkaline pre-treatment were analyzed their proximate parameters include water, ash, carbohydrate, crude fiber, fat, and protein content. Fiber composition did by Van Soest method.

2.3. Acid Hydrolysis
Fifteen grams of each macroalgae were added by 100 ml of 1% H$_2$SO$_4$ solution. Acid hydrolysis was carried out on autoclave at a temperature of 121ºC for 45 minutes. Neutralization was performed by 10% NaOH solution until pH 5-6. Hydrolysates were then filtered using a vacuum pump with filter paper Whatman 41 to obtain neutralized acid hydrolysates. Hydrolysates was analysed on reducing sugars with DNS reagent [8] and total sugars with Phenol-Sulfur reagent [9].

2.4. Culture Preparation
Two loops of S. cereviceae IPBCC AL IX isolate grown on PDA medium for 3 days was inoculated into 10 ml of YMGP medium, then incubated for 24 hours at 30ºC. This culture was referred as working culture or starter for fermentation process.

2.5. Bioethanol Fermentation
Ninety milliliters of acid hydrolysates was put into 250 ml erlenmeyer flask, and then pasteurized at 70-80ºC for 15 minutes. After hydrolysate was cool, urea, NPK and culture starter were added aseptically. Fermentation process lasted for 4 days at room temperature. Distillation was done to separate bioethanol from fermentation broth. Distillate was measured its density by Density Meter Anton Paar DMA 4500M and calculated for bioethanol concentration.
3. Result and Discussion

3.1. Raw Material Characterization

Alkaline pre-treatment was conducted to break sulfated groups bound on monosaccharides chain. Release of sulfates was expected to increase the yield of fermentable sugar. This technique also did to breakdown the lignin (delignification) and remove impurities generally found in macroalgae. In addition, alkaline treatment caused swelling on macroalgae that can increase the surface area of acid absorption.

Alkaline treatment on *G. latifolium* led to an increase of water content, due to swelling during the submersion process. Water was absorbed and trapped in the structure of polysaccharides chain, so it was difficult to evaporate. Similarly, the crude fiber content was increased in materials after alkaline treatment caused by a release of soluble components into water during submersion. In contrast to the moisture content and crude fiber, submersion techniques actually reduced salt or other minerals which were calculated as ash content. Submersion also caused a decrease in carbohydrate content of *G. latifolium* which could be caused by partially released of soluble polysaccharides into alkaline solution.

Salts, other minerals, polysaccharides and impurities which dissolved during the immersion process caused the lower yield of macroalgae (Table 1).

### Table 1. Chemical composition of *G. latifolium*

| Chemical Compositions | Pre-treatment with water | Pre-treatment with 0.05 N NaOH | Pre-treatment with 0.1 N NaOH | Without Treatment [10] |
|-----------------------|-------------------------|-------------------------------|-------------------------------|------------------------|
| Yield (%db)           | 85.80                   | 82.20                         | 83.20                         | 100                    |
| Water (%)             | 8.90±0.12               | 9.90±0.16                     | 11.77±0.26                    | 9.66±0.02              |
| Ash (%db)             | 8.74±0.99               | 9.07±0.10                     | 11.54±1.95                    | 11.91±1.07             |
| Fat (%db)             | 0.53±0.02               | 0.09±0.001                    | 0.69±0.007                    | 0.13±0.02              |
| Protein (%db)         | 9.00±0.06               | 8.73±0.37                     | 6.45±0.39                     | 9.32±0.25              |
| Carbohydrates (%db)   | 21.95±0.64              | 19.59±0.76                    | 19.65±0.39                    | 23.81±1.08             |
| Crude Fiber (%db)     | 26.49±1.27              | 28.29±1.16                    | 27.29±1.46                    | 16.34±0.10             |

For *C. racemosa*, immersion technique also led to an increase of water content and crude fiber. Ash content was significantly declines with the water immersion which causes the lower yield than control without submersion, that is 79.80% db. The best alkaline treatment for *C. racemosa* obtained from submersion with 0.1 N NaOH solutions with 38.97% carbohydrate content (Table 2). The addition of Na⁺ from NaOH will increase the mineral content of the material which is counted as ash content.

### Table 2. Chemical composition of *C. racemosa*

| Chemical Compositions | Without Treatment | Pre-treatment with water | Pre-treatment with 0.05 N NaOH | Pre-treatment with 0.1 N NaOH |
|-----------------------|-------------------|--------------------------|-------------------------------|-----------------------------|
| Yield (%)             | 100               | 79.80                    | 79.90                         | 78.00                       |
| Water (%)             | 12.35 ± 0.01      | 15.69 ± 0.16             | 12.39 ± 0.10                  | 12.90 ± 0.08               |
| Ash (%db)             | 16.83 ± 0.02      | 10.57 ± 0.01             | 12.81 ± 0.13                  | 15.73 ± 0.48               |
| Fat (%db)             | 11.71 ± 0.05      | 10.22 ± 0.23             | 8.50 ± 0.65                   | 9.31 ± 0.34                |
| Protein (%db)         | 0.75 ± 0.003      | 0.61 ± 0.06              | 0.30 ± 0.32                   | 0.27 ± 0.07                |
| Carbohydrates (%db)   | 30.45 ± 4.39      | 36.69 ± 0.40             | 36.12 ± 0.79                  | 38.97 ± 0.00               |
| Crude Fiber (%db)     | 39.88 ± 0.04      | 40.87 ± 0.32             | 44.50 ± 2.42                  | 46.73 ± 2.82               |

3.2. Fiber Composition of *G. latifolium*

Analysis of fiber composition by Van Soest method show that the NDF and ADF value of materials which submerged with 0.1 N NaOH solutions was lower than other treatments (Table 3). Neutral Detergent Fiber (NDF) is the biggest part of plant cell walls consist of cellulose, lignin, silica,
hemicellulose, and some proteins. While Acid Detergent Fiber (ADF) is part of the cell wall which is insoluble and composed of cellulose, lignin, and silica. Difference between the NDF and ADF are known as hemicellulose. ADF digested in \( \text{H}_2\text{SO}_4 \) at 15°C for three hours and then filtered. Solids were filtered weighed as cellulose. The residue remained after washing and drying were weighed, then made into powder. The powder showed silica content, while the reduced weight during the formation of the silica powder calculated as insoluble lignin in acid solutions [11].

Submersion with a higher concentration of alkaline will dissolve hemicellulose and causing lower NDF values. Due to the low value of hemicellulose, lignin and cellulose content rose because both components were more resistant to alkaline. Seaweed which submerged with water and 0.05 N NaOH had higher levels of hemicellulose, lignin, and cellulose compared to controls without submersion. This could be due to seaweed without alkaline treatment contains other components or impurities which could reduce the percentage of NDF and ADF values.

Cellulose is the structure of all plant cell walls [12]. The cellulose content in the cell walls of higher plants is about 35–50% db. The lowest cellulose content derived from control without alkaline treatment of 20.10%.

### Table 3. Crude fiber composition of G. latifolium

| Fiber Compositions | Pre-treatment with Water | Pre-treatment with NaOH 0.05 N | Pre-treatment with NaOH 0.1 N | Control Without Treatment [12] |
|--------------------|--------------------------|-------------------------------|-------------------------------|-------------------------------|
| NDF (%db)          | 82.42                    | 86.10                         | 73.64                         |                               |
| ADF (%db)          | 30.64                    | 31.69                         | 59.27                         |                               |
| Cellulose (%db)    | 23.03                    | 24.87                         | 37.20                         | 20.10                         |
| Lignin (%db)       | 7.29                     | 6.43                          | 25.29                         | 4.41                          |
| Hemicellulose (%db)| 51.78                    | 54.41                         | 14.37                         | 43.96                         |

*Gelidium sp.* is a marine biomass which has low lignin content, so that the pretreatment steps can be relatively easier compared to cellulosic biomass [13]. Lignin has a complex, irregular, and random stucture. Therefore it is difficult to be broken. Alkaline submersion expected to break down lignin compounds (delignification) so that the hydrolytic enzyme can reached hemicellulose and cellulose. Thus, more sugar is formed and can be used by yeast during the fermentation process. Delignification performed with NaOH because it can damage the lignin structure and separate lignin with cellulose and hemicellulose [14].

The lowest lignin content was obtained from treatment without submersion (4.41%) compared to water submersion (7.29%), 0.05N NaOH (6.43%) and 0.1N NaOH (25.29%). This can be due to the presence of other non-lignin carbohydrate and cutin which was count as lignin. Delignification need a proper alkaline concentration to be able to breakdown the lignin structure [15].

Hemicellulose stucture consist of D-glucose, D-galactose, D-mannose, D-xylose, and L-arabinose. It was similar to cellulose which could be hydrolyzed by dilute acid into mannose and galactose. But, hemicellulose dissolves easier in acid and alkaline, so that the lowest levels of hemicellulose was obtained from macroalgae submerged in 0.1 N NaOH (14.37% db).

### 3.3. Fiber Composition of C. racemossa

The highest content of hemicellulose obtained from treatment of water submersion (71.64%), while the lowest was from submersion with 0.1 N NaOH (10.10%) (Table 4). These results indicated alkaline treatment caused a decrease in hemicellulose content because this compound easily dissolved in alkaline solution. Hemicellulose molecules were easily absorb water, more flexibles, and had a larger contact surface between molecules compared to cellulose [16].
### Table 4. Crude fiber composition of *C. racemosa*

| Fiber Compositions      | Control Without Treatment | Pre-treatment with water | Pre-treatment with NaOH 0.05 N | Pre-treatment with NaOH 0.1 N |
|-------------------------|---------------------------|--------------------------|--------------------------------|--------------------------------|
| NDF (%db)               | 80.80                     | 83.58                    | 80.78                          | 77.05                          |
| ADF (%db)               | 72.49                     | 11.94                    | 36.68                          | 66.95                          |
| Hemicellulose (%db)     | 8.31                      | 71.64                    | 42.10                          | 10.10                          |
| Cellulose (%db)         | 47.31                     | 3.78                     | 19.99                          | 43.46                          |
| Lignin (%db)            | 24.18                     | 7.86                     | 18.66                          | 19.34                          |

3.4. **Acid Hydrolysis of *G. latifolium***

Acid hydrolysis aimed to break down macroalgae polysaccharides into monosaccharides that can be used by yeast in bioethanol fermentation. Gelidium sp. polysaccharides consists of a complex fibrin (cellulose) and agar (galactans) consisting of glucose and galactose as a monomer. Agar structure consists of two main components, which is agarose and agaropektin in varying amounts [17].

Acid hydrolysis of Gelidium sp. in a batch system with an autoclave will produce D-galactose, 3,6-anhydro-L-galactose (3,6-AHG), and D-glucose as the main product. Among these products, galactose and glucose classified as monosugars that can be fermented by yeast while 3,6-AHG as sugar that can not be fermented [18].

![Figure 1. Reducing sugar of acid hydrolisates of rinsed *G. latifolium* after alkaline submersion](image)

The highest reducing sugars obtained from *G. latifolium* submerged with 0.1 N NaOH (5.26% w/v) compared to 0.05 NaOH (4.05% w/v), water (3.05% w/v) and control without submersion (2.20% w/v). *G. latifolium* which was submerged with 0.1 N NaOH solutions had the lowest hemicellulose but the highest reducing sugar. Immersion treatment with high concentration of alkaline was able to break down the sulfated groups of macroalgae polysaccharides, thus resulted in lower hemicellulose content. Lignin decomposition made polysaccharides easier to hydrolysis. Therefore the reducing sugar content was higher than other treatment. Control without submersion and water submersion having a high hemicellulose content but low on reducing sugars because the sulfated groups can become an obstacles in acid hydrolysis. Thus it can be concluded that the alkaline submersion can increase reducing sugars on acid hydrolyse of macroalgae.

Rinsing with clean water after alkaline treatment of macroalgae was very affected to the yield of reducing sugar. Reducing sugar content of acid hydrolysates from *G. latifolium* which was rinsed after alkaline pretreatment was higher than without rinsing (Fig 1 and 2). Rinsing the macroalgae with water can dissolve the remains of alkaline material, so that when the hydrolysis took place, acid solutions were not neutralized by alkali. Thus, the acid hydrolysis run effectively and raised the yield of reducing sugars.
Total sugar was the overall sugar content in the form of monosaccharides or oligosaccharides and this number didn’t linearly related with the ethanol content from hydrolysates after fermentation. This was because the sugar content which was measured not only reducing sugars but also non-reducing sugar.

Seaweed hydrolysates treated with alkaline submersion had higher total sugar content than controls (Fig 3), but lower than water submersion. This maybe because there were polysaccharides components that decomposed and dissolved in alkaline, such as hemicellulose. Total sugar was a mixture of reducing and non reducing sugars produced from seaweed hydrolysis.

3.5. Acid Hydrolysis of C. racemosa
As G. latifolium, acid hydrolysis of C. racemosa showed the lowest reducing sugars present in the C. racemosa without submersion while the highest reducing sugar obtained from submersion in water (Fig 4). Submersion treatments using NaOH and water capable to break down the bond between hemicellulose, cellulose and lignin and increase the surface area of hydrolysis by acid solutions.

Although the carbohydrate content raised with increase of NaOH concentration, the reducing sugars level of the macroalgae which were submerged with water was the highest (7.67%, w/v). This was due to the alkaline material still remained on macroalgae and slightly neutralized the acid solutions in the hydrolysis process. The amount of impurities and minerals that were still present in the control without submersion alleged to inhibit hydrolysis process so that the sugar yield from the controls was the lowest one (4.70% w/w).
Rinsing with clean water after alkaline treatment also affected the yield of reducing sugar. The highest reducing sugars obtained from macroalgae which were rinsed after water submersion (Fig 5). Alkaline submersion without rinsing will leave the remains of alkaline in the material and lead to higher pH value. The addition of 1% H$_2$SO$_4$ solutions during hydrolysis will neutralize the sample before break down seaweed polysaccharides.

In addition to rinsing step after submersion, acid concentration and the amount of macroalgae also affected the yield of reducing sugar after hydrolysis. The higher solid concentration for acid hydrolysis mean more polysaccharides available to converted into monosugars. However, increase of macroalgae concentrations should be folowed by an increase in acid concentration. Sulfuric acid concentration of 2% (v/v) was the optimum concentration for macroalgae hydrolysis at 15% (w/v) total solid concentration.

**3.6. Fermentation of G. latifolium Acid Hydrolysate**

One of the parameter observed during fermentation process was the changes in reducing sugar level. It already known that reducing sugar level of hydrolysates will decrease at the end of fermentation which showed the sugars consumption by yeasts. The highest sugar consumption found at submersion treatment with 0.1 N NaOH solutions, while the lowest one was control or without submersion (Fig 6).

Galactose was the dominant monosaccharides produced from the hydrolysis of macroalgae polysaccharides. This type of monosaccharide was a non-conventional nutrition for yeast. Galactose metabolism in yeast was done with the help of specific enzymes in the Leloir pathway, namely galactose mutarotase, galactokinase, galactose-1-phosphate uridiltransferase, UDP 4-epimerase and

**Figure 4.** Reducing sugar of *C. racemosa* acid hydrolysates

**Figure 5.** Reducing sugar content of *C. racemossa* acid hydrolysates with different washing treatment, substrate and acid concentration
phosphoglucomutase. Most organisms including Saccharomyces cerevisiae has the special enzyme. When galactose is available in the media, these enzymes would change the D-galactose into D-glucose 6-phosphate [19].

![Figure 6](image_url)

**Figure 6.** Reducing sugar levels of *G. latifolium* hydrolyzate before and after fermentation

Ethanol content referred in this study (% v/v) was the volumes of ethanol contained in the fermentation broth. The highest ethanol content found from submersion treatment with 0.05 N NaOH solutions, this was 0.63% v/v. Control which has the lowest sugar consumptions also had the lowest ethanol content of 0.30% v/v (Fig 7).

Efficiency of substrates described the amount of sugar consumed by yeast during fermentation. As can be seen on Fig 8, alkaline treatment with 0.1 N NaOH has the highest efficiency of substrate (50.82%), but had the lowest ethanol content (0.43% v/v). One of monosaccharide compound in red algae was 3,6-anhydro-L-galactose. Although it has similar structure to D-galactose, this sugar can not be metabolized by *S. cerevisiae* due to the lack of enzymes in yeast cells that capable to consume this sugar. The 3,6-anhydro-L-galactose can be act as a growth medium, but can not be converted into an ethanol product [20]. This lead to low ethanol content although high levels of sugar was being consumed.

![Figure 7](image_url)

**Figure 7.** Ethanol content of fermented *G. latifolium* hydrolyzate from various alkaline pre-treatment

Efficiency of fermentation shows a comparison between the actual ethanol production and theoretical ethanol which was calculated based on the amount of reducing sugars in the hydrolysate and the coefficient of sugar conversion into ethanol (0.51). Submersion with water has the highest efficiency of fermentation of 22.75%, followed by 0.05 N NaOH (21.94%), control without alkaline treatment (18.89%) and 0.1 N NaOH (12.04%). Submersion with 0.1 N NaOH has the lowest efficiency of
fermentation due to lower ethanol production although it has high sugar consumption. High salt content in the hydrolyzate were allegedly become an obstacle in the bioethanol production.

Figure 8. Efficiency of fermentation and efficiency of substrate of G. Latifolium

3.7. Fermentation of C. racemosa Acid Hydrolysate
Reducing sugar analysis of C. racemosa hydrolysate by DNS method showed decreased on level of reducing sugars after fermentation. The highest sugar consumption derived from alkaline treatment with 0.05 N NaOH is about 1.81% w/v (Fig 9). While the highest ethanol content obtained from water submersion was about 0.70% (v/v) (Fig 10) with efficiency of fermentation of 17.26% and efficiency of substrate of 15.41% (Fig 11).

C. racemosa without submersion treatments had the highest efficiency of fermentation (39.67%) compared to another treatment (Fig 13). This is due to the macroalgae with NaOH solutions had twice additions of alkaline, that was when submersion treatment and neutralization after acid hydrolysis. Neutralization of macroalgae acid hydrolysates with 10% NaOH solution cause the formation of salts which can increases the salinity of hydrolyzates and potentially inhibits the bioethanol production by microorganisms [6]. Therefore, more alkaline compounds are added, more salt could formed and inhibit the performance of yeast and cause lower ethanol content.

The highest efficiency of substrate was obtained from 0.05 N NaOH submersion treatment (28.60%) (Fig 11). Nevertheless, the ethanol content was lower than the submersion with water. This might be because not all sugar were consumed by yeast converted into ethanol. Allegedly, sugar consumption used by yeast to adapt with salt and toxic compounds in the hydrolyzate before being used for ethanol production.

Figure 9. Reducing sugar levels of C. racemosa hydrolyzate before and after fermentation
Figure 10. Ethanol content of fermented C. racemosa hydrolyzate from various alkaline pre-treatment

Figure 11. Efficiency of fermentation and efficiency of substrate of C. Racemosa

4. Conclusion
Submersion technique with water and alkaline decreased carbohydrate and ash content of G latifolium. However, alkaline treatments for C. racemosa increased ash content. Submersion treatment with 0.05 and 0.1 N NaOH solutions were able to dissolve hemicellulose but didn’t not work for lignin content in both types of seaweed. Alkaline pre-treatments raised the yield of reducing sugar on hydrolysate of G latifolium significantly but not with C. racemosa. Rinsing with clean water after alkaline submersion affected on the yield of reducing sugar. Increase of substrate concentration should be followed by an increase on acid concentration at hydrolysis process. The highest ethanol content of G. latifolium obtained from immersion treatment with 0.05 N NaOH (0.63% v/v), which has substrates efficiency of 37.30% and fermentation efficiency of 21.94%. While for C. racemosa, the highest ethanol content obtained from water submersion (0.70% v/v) with efficiency of substrate was 15.41% and efficiency of fermentation was 17.26%. Submersion of G latifolium with water or alkaline solution was able to raised reducing sugar content in the hydrolyzate and lead to a higher ethanol conversion and efficiency of fermentation. But the use of higher alkaline concentrations will increase salt content in the hydrolyzate and disrupt yeast activity in the bioethanol production.
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