# The Optimization of Initial Treatment of Seaweed *Ulva reticulata* Using CEM Synthesizer Method for Bioethanol Production

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**Abstract**: Research has been carried out on the optimization of initial treatment and hydrolysis using CEM microwave synthesizer and the production of bioethanol from *Ulva reticulata* seaweed. Optimization in the initial treatment was carried out by varying the concentration of HCl and $H_2SO_4$ (each in 1; 3; 5; and 7%), variations in time (30; 40; 50; and 60 minutes), temperature (100; 150; 200 and 250 °C), and electrical power (100; 150; 200; and 250 W). Fermentation was carried out anaerobically at 10% inoculum concentration and a production time of 6 days. Characterization of reducing sugar using DNS method and characterization of ethanol using GC-FID and HPLC. The results of the initial lignocellulosic analysis obtained the lignin content of 10.03%, cellulose 14.38% and hemicellulose 22.29%. After the initial treatment, the lignin content decreased to 3.86%, while cellulose increased to 24.50% and hemicellulose to 41.57%. The reducing sugar content produced using HCl is 97.10 g/L at optimum temperature 200 °C, for 60 minutes, using 7% concentration of HCl and 200 W of power, while the optimum reducing sugar content using $H_2SO_4$ is 76.40 g/L at optimum temperature 200 °C, time for 50 minutes, using 3% concentration of $H_2SO_4$ and 200 W of power. Production of bioethanol through fermentation and distillation processes obtained a bioethanol level of 43.89% (GC) or 18.89% (HPLC) for optimum conditions using $H_2SO_4$, whereas for optimum conditions using HCl, the bioethanol level is 44.29% (GC) or 18.09% (HPLC).

**Keywords:** *Ulva reticulata,* CEM microwave synthesizer, Hydrolysis, Fermentation, Bioethanol

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## 1. INTRODUCTION

Bioethanol can be produced through a fermentation process using raw materials that contain carbohydrates. The available raw materials are broken down into four main types. The first generation of bioethanol is mostly edible food crops such as rice, wheat, potatoes, corn, and sugar cane, so it competes with food consumption (1). Second generation bioethanol utilizes lignocellulosic biomass such as paper pulp, rice straw, corn cobs, and sugar cane bagasse. However, pretreatment is required which is expensive and difficult while producing large amounts of residue. Alternative raw materials with residues rich in monosaccharides or even polysaccharides other than lignin are needed. In this context, third generation bioethanol production from macroalgae is an alternative raw material solution to replace vegetable starch and lignocellulosic biomass. This is mainly due to the fast growth rate of macroalgae, the absence of competition with agricultural land, the high carbohydrate content and the relatively simple processing steps compared to lignocellulosic biomass (2).

Ulva macroalgae is considered to be a third generation renewable energy source because it contains carbohydrates and lipids (3). In contrast to the high starch or sugar content found in first-generation feedstocks, second-generation bioethanol typically utilizes non-edible feedstocks (4), such as lignocellulosic materials and agricultural forest residues (e.g., macroalgae) (5). Yu-Qing et al. (2016) state that *Ulva* seaweed contains carbohydrates in the form of heteropolysaccharides of glucose, arabinose, rhamnose and xylose which are very abundant (6). This type of seaweed is widely spread in the East Timor Sea (East Nusa Tenggara). However, *Ulva Reticulata* has not been utilized by the people on Timor Island so it has become garbage that reduces the aesthetics of the coast. On the other hand, seaweed is not used as food, so it does not compete with food.

*Ulva reticulata* conversion is carried out through initial treatment, hydrolysis, fermentation, and distillation (purification) (7). The use of acid catalysts during hydrolysis is very influential on the production of reducing sugar. The effectiveness of the HCl type of catalyst is higher producing glucose at the same temperature, concentration and time compared to $H_2SO_4$. This is because the nature of HCl is stronger with higher reactivity compared to $H_2SO_4$. In addition, one alternative tool in the initial treatment (delignification and hydrolysis) is the use of CEM microwave synthesizer which has many advantages, such as a short hydrolysis time compared to conventional methods, a rate of starch hydrolysis reactions to glucose that increases 50-100 times, is cost-effective, and is environmentally friendly because the acid concentration used is lower (4).

Kolo et al. (2021) reported that hydrolysis of *Ulva reticulata* seaweed with 2% $H_2SO_4$ for 50 minutes could maximize reducing sugar production of 33.4 g/L and a bioethanol concentration of 5.02% at an inoculum concentration of 10% for 6 days (8). Research by Kolo et al. (2022) also reported that the hydrolysis of *Ulva reticulata* macroalga through variations in temperature and reaction time using $H_2SO_4$ catalyst obtained a sugar content of 27.79 g/L and a bioethanol content of 7.76% (9). Furthermore, research by Kolo et al. (2023) reported that hydrolysis of Ulva reticulata using the $HNO_3$ catalyst obtained a sugar content of 86.5 g/L and a bioethanol content of 37.2% (10). The three previous studies that used the macroalga *Ulva reticulata* had not optimized the fermentation process, so the researchers tried to optimize the fermentation process to increase bioethanol levels.

The main problem in the fermentation process in an effort to find alternative energy is finding the right fermentation time and inoculum concentration so as to get the highest bioethanol concentration. The novelty value of this research lies in optimizing the initial treatment converting Ulva reticulata into bioethanol using the CEM synthesizer, and refining fermentation time and inoculum concentration to obtain the highest bioethanol content. This research aims to determine the optimum reducing sugar concentration using HCl and $H_2SO_4$ catalysts through variations in time, temperature, and concentration and to obtain the highest levels of bioethanol through variations in fermentation time and inoculum concentration. According to Febriani et al. (2020) using an inoculum concentration that is too high (>15%) can cause a decrease in cell viability. Apart from that, too high levels of bioethanol resulting from long fermentation will be toxic to cells so that cells die and their viability decreases (11). This research also provides information for industry to increase the economic value of *Ulva reticulata* macroalga as a renewable energy source in the future.

## 2. EXPERIMENTAL SECTION

### 2.1. Material and Tools

Materials: $H_2SO_4$ (Merck), NaOH (technical), HCl (technical), glucose (Merck), ethanol (Merck), acetic acid (Merck). Inoculum media consisted of *Saccharomyces cerevisiae* inoculum media (yeast extract 5 g/L; peptone 5 g/L; glucose 20 g/L as carbon source), fermentation media consisted of yeast extract 5 g/L; peptone 5 g/L; $KH_2PO_4$ 5 g/L; $MgSO_4.7H_2O$ 0.4 g/L; $NH_4SO_4$ 0.5 g/L; glucose hydrolysate.

Tools: Glassware, analytical balance, pH meter, autoclave, 37 °C temperature incubator, water bath, CEM Microwave Synthesizer, GC-FID, HPLC, UV-Vis Spectrophotometer, fermenter and SEM equipment, magnetic stirrer, loop wire, falcon tube.

### 2.2. *Ulva reticulata* Macroalga Preparation

The preparation of *Ulva reticulata* sp. involve two steps: drying and milling. The product is, then sieved using ± 100 mesh sieve to obtain *Ulva reticulata* sp. powder. The powder of *Ulva reticulata* sp. was analysed for lignocellulosic content using Surajit Method (4).

### 2.3. *Ulva reticulata* Macroalga Powder Saccharification

The saccharification stage of *Ulva reticulata* powder aims to hydrolyze cellulose into monosaccharides such as glucose. Saccharification was carried out with various concentrations of HCl and $H_2SO_4$, temperature, time, and power using a CEM microwave synthesizer (**Table 1**).

Acid hydrolysis was carried out in the following way: 10 g of *Ulva reticulata* powder and 100 ml of HCl or H2SO4 1; 3; 5; and 7% (v/v) were put into a 250 ml heating flask, then sterilized. The medium is then cooled to room temperature. The heating results are then filtered, and the glucose content analysis is carried out using UV-Vis, while the hydrolysis residues are surface analyzed using SEM (12). The optimum results in the hydrolysis process are then used to produce bioethanol.

**Table 1**: Optimization of *Ulva reticulata* sp. powder hydrolysis with HCl or $H_2SO_4$ as catalysts.

| Treatment | Optimization (HCl or $H_2SO_4$) | | | |
|---|---|---|---|---|
| | % (v/v) | Time (minute) | Temperature (°C) | Power (Watt) |
| 1. | 1; 3; 5; 7 | 40 | 100 | 100 |
| 2. | Result 1 | 30; 40; 50; 60 | 100 | 100 |
| 3. | Result 1 | Result 2 | 100; 150; 200; 250 | 100 |
| 4. | Result 1 | Result 2 | Result 3 | 100; 150; 200; 250 |
| Bioethanol Production with Optimum Concentration, Time, Temperature, and Power | | | | |

**2.4. The making of Fermentation Medium**

The fermentation medium used was 100 ml of hydrolysates in a 250 ml Erlenmeyer for each research treatment. The first step in making the starter was to inoculate the yeast culture of *S. cerevisiae* from the liquid culture into 100 ml of the fermentation medium, which was then incubated at room temperature for 24 hours (13).

**2.5. Bioethanol Production**

Fermentation was carried out using *S. cerevisiae*. The fermentation volume for the hydrolysate medium is 200 mL. The hydrolysate of *Ulva reticulata* powder was put in the fermentation medium and sterilized at 121 °C for 15 minutes. The fermentation medium used demineralized water as a solvent. Fermentation is set at pH 4.5 and a temperature of 30 °C. The inoculum concentration was 10% (v/v), and the fermentation time was 6 days (14).

**2.6. Analysis of the Chemical Content of Lignocellulose *Ulva reticulata***

A total of 1 g of *Ulva reticulata* powder was suspended in 150 mL $H_2O$ and then refluxed for 2 hours at 100 °C. The heating results are then filtered to separate the residue and filtrate. The dried residue was then refluxed again for 2 hours with 150 mL of 0.5 M $H_2SO_4$ at 100 °C. The dried sample residue was immersed in 10 mL of 72% (v/v) $H_2SO_4$ solution at room temperature for 4 hours, then diluted to 0.5 M $H_2SO_4$, refluxed at 100 °C for 2 hours, and dried. The residue was filtered and washed with demineralized water until neutral. The residue was then dried in an oven at a temperature of 105 °C until the weight was constant and counted as weight (d). After that, the residue was smoked into ash and weighed (e). The composition of the lignocellulosic components of *Ulva reticulata* sample was determined by the following calculations:

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

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

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

Note: a is the initial weight of dry powder of *Ulva reticulata* sample; b is the weight of the dry sample residue after refluxing with hot water; c is the residual weight of the sample after refluxing with 0.5 M $H_2SO_4$; and d is the residual weight of the sample after being treated with 72% (v/v) $H_2SO_4$ solution (4).

**2.7. Surface Texture Analysis of *Ulva reticulata* Powder**

The results of the hydrolysis are filtered and then neutralized for further processing. The solid fraction was examined for its surface texture by SEM, while the liquid fraction was analyzed for reducing sugar content using the DNS method.

**2.8. Reducing Sugar Analysis (3.5-dinitrosalicylic acid (DNS) method)**

The hydrolysis solution of *Ulva reticulata* powders were analyzed for reducing sugar content using the DNS method. The analysis of reducing sugars was determined using a UV-VIS spectrophotometer (9), with the following steps:

a. Standard glucose solutions were made with concentrations of 1000, 2000, 3000, 4000, and 5000 ppm.
b. Take 1 mL of each solution, and then add 1.75 mL of DNS reagent.
c. The solutions were homogenized and heated in boiling water for 5 minutes.
d. The cold solution was diluted 5 times and homogenized again.
e. The absorbance was measured using a spectrophotometer at a wavelength of 540 nm, then a standard curve was made to obtain a linear regression equation.
f. The measurement of the reducing sugar content in the sample is carried out in the same way at points b to e.

**2.9. Bioethanol Product Analysis**

Ethanol analysis was carried out using Gas Chromatography-Flame Ionization Detector (GC-FID) and High Performance Liquid Chromatography (HPLC) with detector temperature 40 °C, column temperature 60 °C, mobile phase flow rate 0.6 mL/minute, Phenomenex ROA Organic Acid column and RI detector (Refractive Index).

**3. RESULTS AND DISCUSSION**

**3.1. Ulva reticulata Powder Morphology before and after Pretreatment**

*Ulva reticulata* macroalga powder used as raw material in this study contains cellulose, hemicellulose, and lignin as the main components (4). However, before carrying out the initial treatment process, it is necessary to know the composition of the lignocellulosic constituents. It is necessary to develop an effective method for converting cellulose components into simple sugars and also as a basis for selecting suitable microorganisms to convert sugars into bioethanol. Therefore, the structural carbohydrate content of *Ulva reticulata* powder was determined before and after the initial treatment both by analyzing the surface morphology and by measuring the chemical content of *Ulva reticulata* powder. After initial treatment using CEM microwave synthesizer, the mixture was then filtered to separate the filtrate and *Ulva reticulata* residue. The filtrate was used for analysis of reducing sugar content, while the residue was washed until neutral pH and then dried for analysis of powder surface texture using SEM. The results of the morphological characterization of *Ulva reticulata* powder are shown in **Figure 1**.

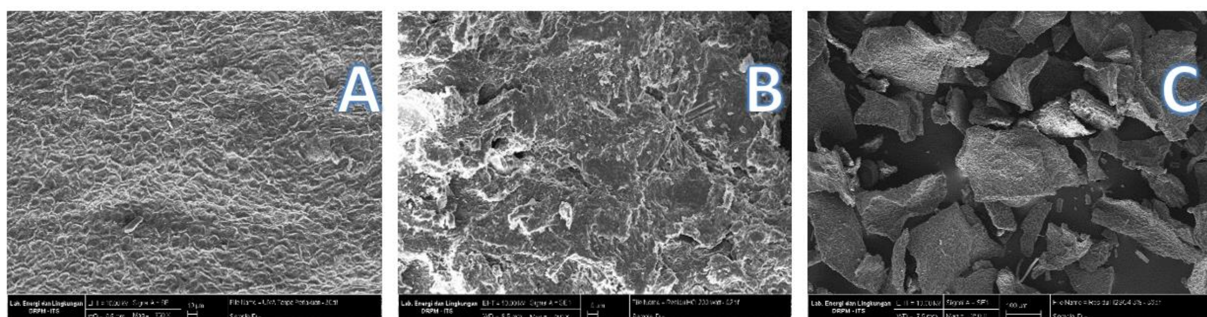


**Figure 1:** The morphology of *Ulva reticulata* powder: A. Without initial treatment; B. After CEM Synthesizer (HCl), and C. After CEM Synthesizer ($H_2SO_4$).

The surface morphology of *Ulva reticulata* macroalga showed that the powder surface was dense and stiff before initial treatment and acid hydrolysis (**Figure 1**(A)). The initial treatment process removes acetyl and other acid substitutions in lignin, and hemicellulose protecting the cellulose. The SEM results revealed that the macroalga powder was broken and suffered significant damage after the initial treatment using HCl and $H_2SO_4$ (**Figure 1** (B and C)). These results indicate that both treatments succeeded in degrading the lignin portion of the lignocellulosic part of *Ulva reticulata* powder.

The lignin is not a sugar polymer, so it cannot be used as a substrate for bioethanol production through microbial fermentation. Lignin will also inhibit microbial growth during the fermentation process (15). Lignin is broken down and released from the structure of cellulose and hemicellulose due to initial treatment with dilute acid combined with the help of microwave (CEM Synthesizer synthesizer). Acid hydrolysis is required to degrade the -1,4-glycosidic bonds of linear glucan-cellulose chains or -1,4-D-pyranosyl linkage of heterogeneous hemicellulose polysaccharides into sugar monomers, such as glucose, xylose, galactose, arabinose, and mannose (4). The initial treatment process combined with the CEM Synthesizer synthesizer helps to degrade and release lignin from cellulose or hemicellulose materials. Lignin is not a sugar polymer, cannot be used as a raw material for bioethanol production and inhibits microbial growth during fermentation (15).

### 3.2. Chemical Ingredients of *Ulva reticulata* Macroalga Powder

The results of the analysis of the lignocellulose content of *Ulva reticulata* showed that the cellulose content after initial treatment using microwave-assisted techniques increased from 14.38% to 24.50%, hemicellulose increased from 22.29% to 41.57%, while the lignin content decreased from 10, 03% to 3.86% (**Table 2**).

The use of CEM synthesizer in the alkaline delignification process increased the cellulose content obtained in this study. This indicates that the alkaline delignification process with microwave-assisted treatment has succeeded in breaking the structural bonds between lignin and polysaccharides, thereby releasing free cellulose into the solution. Kolo et al., (2020), also reported that the cellulose and hemicellulose content decreased after acid hydrolysis, because this process would also break down the glycosidic bonds of cellulose or the -1,4-D-pyranosyl bonds of hemicellulose, resulting in monosaccharides (4).

**Table 2:** Lignocellulose content of *Ulva reticulata* powder.

| Process | Water content (%) | Hemicellulose (%) | Cellulose (%) | Lignin (%) |
|---|---|---|---|---|
| Pre-treatment | 53.15 | 22.29 | 14.38 | 10.03 |
| Treatment (*CEM Synthesizer*) | 29.95 | 41.57 | 24.50 | 3.86 |

Sari et al. (2014) reported that the treatment of 100.0 g of *Sargasum duplicatum* macroalga yielded 15.08 g of cellulose (16). Meanwhile, in our study, 100.0 g of *Ulva reticulata* macroalga yielded 24.50 g of cellulose. Adini et al., (2015) also reported that 100 g of *Gracilaria* sp. macroalga contains 19.7 g of cellulose (17). Wadi et al. (2019) used alkaline delignification (NaOH) for the pretreatment of *Eucheuma cottonii*. They reported that 50.2 g of cellulose was converted to 6.1% bioethanol using the SSF method (18). The use of microwave irradiation can increase the rate of hydrolysis of starch into glucose by 100 times (9).

### 3.3. Reducing Sugar Content in *Ulva reticulata* Powder

The reducing sugar content in the sample was analyzed using a UV-Vis spectrophotometer at a wavelength of 540 nm with 3,5-dinitrosalicylic acid (DNS) reagent. The hydrolyzed filtrate was determined based on the formation of a brownish red reduced product when the sugar in the sample was reduced from 3,5-dinitrosalicylate to 3-amino-5-nitrosalicylic acid during heating. The brownish red color will be absorbed maximally at a wavelength of 540 nm. The reaction of glucose with DNS reagent will produce absorbance values that can be measured spectrophotometrically (19).

The initial treatment process in this study was combined with microwave irradiation techniques using a CEM synthesizer. The use of this technique is to increase the efficiency of the hydrolysis reaction to obtain a higher reducing sugar content. The results of the hydrolysis of *Ulva reticulata* powder using CEM synthesizer are presented in **Table 3**. The optimum

yield of reducing sugar using HCl was influenced by microwave irradiation, as indicated by the concentration of reducing sugar increasing with increasing acid concentration, temperature, time, and microwave irradiation power (**Table 3**).

### 3.4. Variation of Temperature, Time, Power, and Concentration of HCl

The optimization results of the initial treatment are presented in **Table 3**, showing the analysis of reducing sugars from the hydrolysate of *Ulva reticulata* powder using HCl.

**Table 3**: Results of *Ulva reticulata* powder reducing sugar using HCl.

| Temp (°C) | GP (g/L) | WR (Min) | GP (g/L) | HCl (% v/v) | GP (g/L) | DI (Watt) | GP (g/L) |
|---|---|---|---|---|---|---|---|
| 100 | 19.5 | 30 | 24.7 | 1 | 46.4 | 100 | 79.7 |
| 150 | 75.1 | 40 | 43.0 | 3 | 56.4 | 150 | 86.1 |
| 200 | 79.6 | 50 | 56.3 | 5 | 68.3 | 200 | 97.1 |
| 250 | 74.0 | 60 | 84.7 | 7 | 84.7 | 250 | 90.9 |

**Abbreviations**: HCl: Hydrochloric Acid; GP: Reducing Sugar; WR: Reaction Time; Temp: Temperature; DI: Irradiation Power.

The results of the hydrolysis in **Table 3** show an increase in reducing sugars with an increase in the concentration of hydrochloric acid used and the hydrolysis time. However, it was different for the hydrolysis temperature, which increased from 100 °C to 200 °C and then decreased at 250 °C. Likewise, the irradiation power increases from 100 to 200 W and then decreases at 250 W of power. This condition is caused by an increase in temperature and excess power, so sugar products will be converted into secondary compounds such as furfural and hydroxymethylfurfural (HMF) (20). The most optimal conditions for hydrolysis of *Ulva reticulata* macroalga using hydrochloric acid (HCl) combined with CEM synthesizer are an acid concentration of 7% (v/v) with an irradiation power of 200 watts for 60 minutes at 200 °C, which produces reducing sugars of 97.10 g/L. This result is higher than the study conducted by Kolo et al., (2021), which produced a reducing sugar content of 33.4 g/L obtained at a concentration of 2% $H_2SO_4$ at a temperature of 150 °C with a reaction time of 50 minutes using a Kirin type household microwave.

### 3.5. Variation of Temperature, Time, Power and Concentration of $H_2SO_4$

The results of the optimization of the initial treatment, obtained by analyzing the reducing sugars from the hydrolysate of *Ulva reticulata* powder using $H_2SO_4$ are shown in **Table 4**. The results of the hydrolysis in **Table 4** show an increase in reducing sugars with the increase in the concentration of acid used, hydrolysis time, temperature, and irradiation power of CEM synthesizer. Kolo et al., (2022) reported that hydrolysis of acid ($H_2SO_4$) in *Ulva reticulata* using a Kirin type microwave with a combination of delignification and hydrolysis through variations in hydrolysis time and temperature obtained an optimum reducing sugar of 27.97 g/L at 150 °C, a concentration of 2% $H_2SO_4$, and a hydrolysis time of 50 minutes. These results are still low compared to those obtained in this study. Optimum conditions for hydrolysis of *Ulva reticulata* macroalga using sulfuric acid ($H_2SO_4$) combined with CEM synthesizer at an acid concentration of 5% (v/v) with an irradiation power of 200 W for 50 minutes at 200 °C, produce reducing sugars of 76.40 g/L.

Based on the variation of reaction time and hydrolysis temperature, it was seen that there was a color change in the Ulva reticulata hydrolysate. The longer the heating time at high temperatures, the darker the color of the hydrolysate. This indicates that there has been a complete degradation of hemicellulose and cellulose into glucose (21), but if the hydrolysis process is continued at high temperatures, charcoal will form on the flask wall (**Figure 2**). This proves that the resulting glucose is damaged or burned, and the caramelization is formed (Kolo et al., 2022). In addition, the longer reaction time causes the formation of secondary compounds such as hydroxymethylfurfural (HMF), which then reacts to form formic acid (22). The use of CEM synthesizer is considered to be more advantageous than the standard reflux method and simple microwave due to the shorter reaction time (in minutes), less solvent, and higher reducing sugar product.

**Table 4**: Results of Ulva reticulata powder reducing sugar using $H_2SO_4$

| Temp (°C) | GP (g/L) | WR (Min) | GP (g/L) | $H_2SO_4$ (% v/v) | GP (g/L) | DI (Watt) | GP (g/L) |
|---|---|---|---|---|---|---|---|
| 100 | 7.3 | 30 | 3.1 | 1 | 63.0 | 100 | 56.8 |
| 150 | 12.0 | 40 | 19.2 | 3 | 64.7 | 150 | 60.0 |
| 200 | 23.7 | 50 | 33.4 | 5 | 61.3 | 200 | 76.4 |
| 250 | 17.1 | 60 | 28.0 | 7 | 59.7 | 250 | 69.9 |

**Abbreviations**: $H_2SO_4$: Sulfuric Acid; GP: Reducing Sugar; WR: Reaction Time; Temp: Temperature; ID: Irradiation Power.

**Figure 2:** Hydrolysis results using CEM synthesizer (personal documentation).

### 3.6. *Ulva reticulata* Powder Hydrolysate Fermentation

The result sugar is then utilized in the fermentation process by selecting the optimum conditions of the two catalysts used, namely HCl and $H_2SO_4$. The optimum condition of glucose hydrolysate used during the fermentation process was 3% $H_2SO_4$ treatment at a temperature of 150 °C for 50 minutes. Meanwhile, the glucose hydrolysate in the HCl treatment had 7% HCl concentration at a temperature of 150 °C and a reaction time of 50 minutes. The fermentation process uses *Ulva reticulata* powder hydrolysate as a substrate using the yeast *Saccharomyces cerevisiae*. *S. cerevisiae* was chosen because it has various advantages, including a high survival rate and the ability to produce alcohol in sufficient quantities (23). Before proceeding with the gas chromatography and HPLC testing, we performed a qualitative analysis using potassium dichromate ($K_2CrO_7$) to ensure that the sample from the graded distillation yielded ethanol. The results of the analysis are presented in **Table 5**. The results of purification after fermentation are shown in **Table 6**.

The results of the qualitative test showed that there was a color change from orange to bluish green in both standard ethanol and fermented samples. According to Kolo et al., (2023), stated that a positive test for the presence of ethanol was indicated by a change in the color of potassium dichromate from orange to bluish green. So, it can be concluded that in the fermented sample there has been a change in glucose to ethanol, which is marked by a change in the color of the potassium dichromate solution from orange to bluish green when the fermented sample is added (10).

**Table 5:** Qualitative test results using $K_2Cr_2O_7$.

| Sample | Test Result | Picture |
|---|---|---|
| Pure ethanol | + | |
| Bioethanol of *Ulva reticulata* powder | + | |

### 3.7. Ethanol Analysis Using Gas Chromatography

The quantitative analysis of bioethanol content in *Ulva reticulata* powder samples was carried out using a gas chromatography instrument. Analysis using GC was carried out to determine the presence of ethanol produced from the fermentation process. The compound used as an internal standard is toluene to create a perfect separation between the sample peaks and the measurement of compound levels is not influenced by other compounds. Toluene was chosen as the internal standard because it has a molecular formula similar to that of ethanol. With this similarity, the solubility between the two solutions

is easy to know based on the principle of like dissolved like (24).

The GC chromatogram in **Figure 3** shows that the fermented sample contains 3 peaks with different retention times, namely at retention times of 2.950, 3.216, and 4.288 minutes where the compound that comes out or evaporates as a peak is hexane, then followed by ethanol and toluene. Sample chromatograms were confirmed using standard ethanol chromatograms which were detected at a retention time of 3.214 minutes. The peak of the hexane compound comes out first because hexane has the lowest boiling point (68.7 °C) compared to ethanol (78.3 °C) and toluene (110.6 °C) (8). This is because the components of the mixture in the sample will separate or come out according to their boiling point. The component that has a lower boiling point will evaporate first, so it will come out as the first peak on the chromatogram. In this study, hexane was used as a solvent to dissolve ethanol and toluene before being injected into the GC. Hexane is used as a solvent because it is a non-polar compound and is a good organic solvent because it has a low boiling point (volatile), harmless, non-toxic, not explosive or flammable, inexpensive and inert (does not react with solutes) (24).

The chromatogram obtained can be used to calculate the concentration of bioethanol in the sample by

comparing the area of the ethanol peak with the area of the standard. The obtained calculation show that the optimum concentration of ethanol, at an inoculum concentration of 10% using glucose hydrolysate from optimization of $H_2SO_4$ was 43.8% and 44.2% from optimization using HCl. The low concentration of bioethanol produced in this study was caused by several factors, such as the fact that not all glucose molecules were converted into ethanol, and also because during the hydrolysis process using acid, hydroxymethylfurfural (HMF) compounds were usually formed, where this compound was an inhibitor compound that could inhibit the growth of microorganisms in the fermentation process. In addition, it is also caused by the slow fermentation process due to the small amount of nutrients in the medium. This situation is also due to the fact that ethanol has been oxidized to acetic acid either during the filtering process or when transferring the distillate sample from the distillation flask to the reagent bottle. According to Azizah et al., (2012), the products of the fermentation process include, in addition to ethanol, acetic acid, fussel oil, and acetaldehyde (25). Another factor that causes low concentrations of bioethanol is the presence of contaminants such as lactic acid bacteria, and acetic acid bacteria which are capable of being inhibitors in the fermentation process (26). Another influencing factor is the presence of residual water content in the distillation sample.

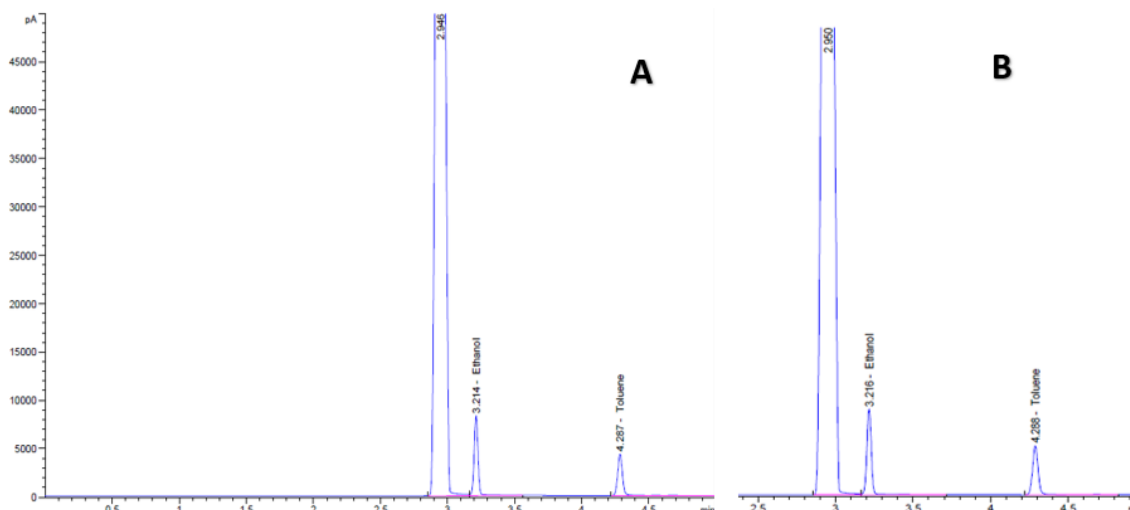


**Figure 3:** Chromatogram (GC): A. Ethanol standard, B. Bioethanol sample.

### 3.8. Ethanol Analysis Using High Performance Liquid Chromatography (HPLC)

The production of bioethanol is carried out through a fermentation process using an inoculum concentration of 10% for 6 days. The results of HPLC analysis of the content of ethanol and acetic acid in the product after fermentation are presented in **Figures 4** and **5**. Ethanol and acetic acid in the product after fermentation were confirmed by comparing the retention times of standard ethanol and acetic acid with the fermentation samples. Concentrations of ethanol and acetic acid were calculated using HPLC chromatogram analysis data. The area data of each ethanol and acetic acid were entered into the standard regression equation of ethanol and acetic acid.

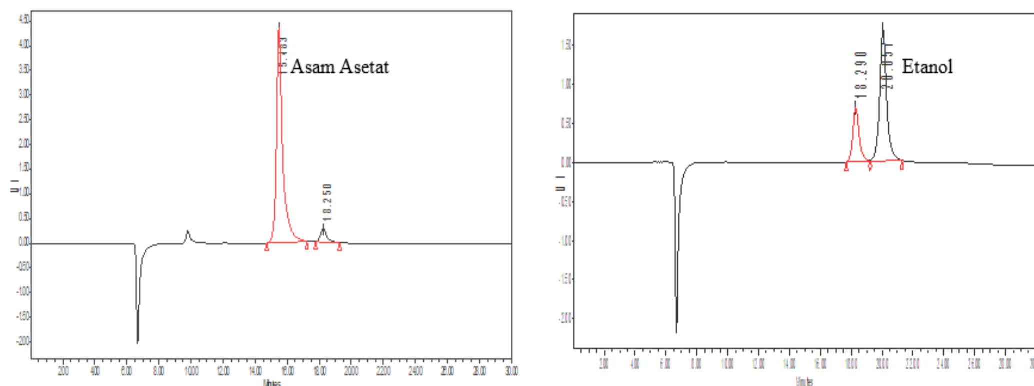


**Figure 4:** Standard Chromatogram of Acetic acid and Ethanol.

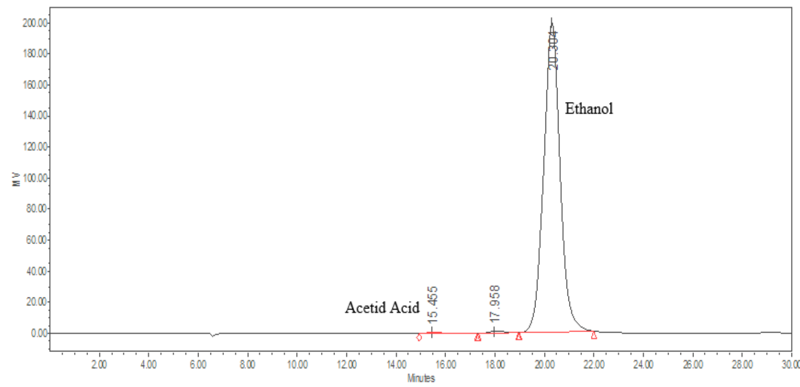


**Figure 5:** Chromatogram of Acetic acid and Ethanol in Fermentation Products.

The peak area of the ethanol product in the HPLC analysis chromatogram was then entered into the standard regression equation of ethanol and acetic acid as a variable (y) to obtain the concentration (x), as follows:

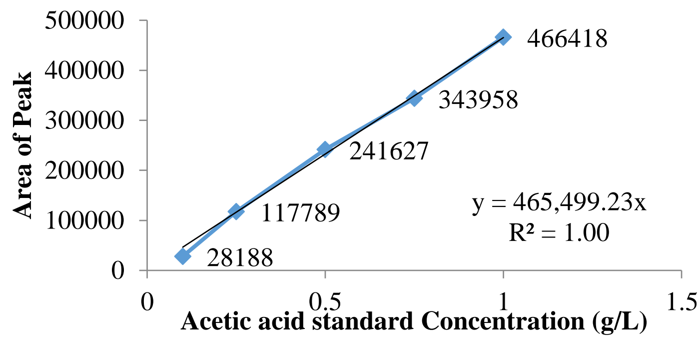


**Figure 6:** Linear plot between area in chromatogram and concentration of acetic acid.

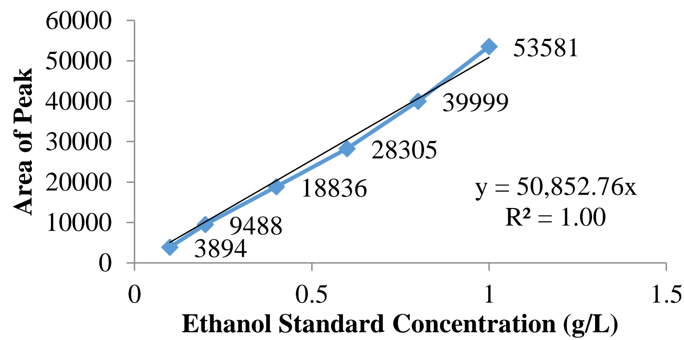


**Figure 7:** Linear plot between area in chromatogram and ethanol concentration.

Ethanol concentration (x): $\frac{y-b}{a}$
Ethanol concentration (g/L): Ethanol conc. (%) x ρ x 10 (correction factor) (4)

The initial concentration of glucose, obtained directly from the initial treatment process of *Ulva reticulata* macroalga was 97.1 g/L. The hydrolyzate at the optimum conditions of $H_2SO_4$ was then fermented and obtained optimal concentrations of bioethanol and acetic acid of 18.8% and 0.013%, respectively, by using a 10% inoculum concentration for 6 days of fermentation. Under these conditions, the bioethanol yield was 23%, with the highest productivity rate of 0.2 g/L per day. Fermentation efficiency reaches a level of 34.41% and substrate conversion efficiency is 56.38%.

**Table 6:** Bioethanol yield from *Ulva reticulata.*

| Optimum Hydrolysis | Inoculum (%) | Fermentation time (days) | Ethanol content (%) | | Acetic Acid content (%) |
|---|---|---|---|---|---|
| | | | GC | HPLC | |
| $H_2SO_4$ 3%, 150 °C, 50 min | 10 | 6 | 43,8 | 18.8 | 0.013 |
| HCl 7%, 150 °C, 50 min | 10 | 6 | 44,2 | 18.0 | 0.012 |

Yadav et al. (2011) reported the results of research on the topic of bioethanol production from rice straw hydrolyzate using co-cultures, namely *Sacharomyces cerevisiae* and *Pichia stipitis*, and obtained an ethanol concentration of 1.2% and an ethanol productivity rate of 0.33 g/L a day (14). Kolo et al. (2020) reported a research on the topic of bioethanol production from Hydrolyzed Elephant Grass using 2 types of yeast, namely *Sacharomyces cerevisiae-Pichia stipitis,* and the resulting ethanol concentration was 10.97 g/L with a productivity rate of 0.45 g/L per hour and fermentation efficiency reaching 69.48%. Based on the results of ethanol and the parameters determined, it can be concluded that the highest ethanol concentration was obtained by fermentation using an inoculum concentration of 10% to produce 44.2% ethanol with a fermentation time of 6 days. The ethanol content was then used to calculate the fermentation efficiency, yield, productivity rate, and substrate conversion (**Table 7**). When viewed from the effectiveness and efficiency of the use of materials and time in this study, it is necessary to optimize the fermentation process to determine the best inoculum concentration and fermentation time so as to increase the yield and ethanol content obtained.

**Table 7:** Substrate parameters and fermentation products.

| Initial treatment | EF (%) | $Y_{p/s}$ (%) | Rate (g/L.day) | Ks (%) |
|---|---|---|---|---|
| $H_2SO_4$ 3%, 150 °C, 50 min | 23.68 | 24.59 | 0.13 | 40.68 |
| HCl 7%, 150 °C, 50 min | 19.35 | 19.35 | 0.13 | 53.68 |

**Description**: Ks = Total substrate conversion (%), Yp/s (%) = Yield or yield (ethanol/substrate), Theoretical yield = 51%, Rate(g/L,days) = ethanol productivity rate/day, EF= Fermentation efficiency(%).

## 4. CONCLUSION

In this study, the biomass content after pretreatment using the CEM synthesizer technique was 24.50% cellulose, 41.57% hemicellulose, and 3.86% lignin. The reducing sugar content obtained was 97.1 g/L at the optimum conditions of 5% HCl; 200 °C; 200 Watts of irradiation power; and a 60 minutes of hydrolysis time. Bioethanol production

through fermentation and distillation processes resulted in bioethanol levels of 43.89% (GC) or 18.89% (HPLC) under optimum conditions with $H_2SO_4$ and 44.29% (GC) or 18.09% (HPLC) under optimum conditions with HCl. The efficiency of fermentation is 23.68%, the optimal rate of ethanol productivity is 0.13 g/L/day and the substrate conversion is 53.68%.

## 5. CONFLICT OF INTEREST

The authors declare there is no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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