# The Effect of Fermentation Time, pH and *Saccharomyces Cerevisiae* Concentration for Bioethanol Production from *Ulva Reticulata* Macroalgae

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## Abstract

Research has been carried out on the effect of pH, fermentation time and yeast concentration on bioethanol production through hydrolysis using a CEM (Ceramic Elekctromagnetic Microwave) synthesizer and bioethanol production from *Ulva reticulata* seaweed. *Ulva reticulata* seaweed contains carbohydrates in the form of heteropolysaccharides such as glucose, arabinose, ramnose and xylose which are very abundant and suitable for conversion into bioethanol because the people of Timor Island do not use them as food. The carbohydrate content of *Ulva reticulata* seaweed can be converted into hexose and pentose sugars (glucose, arabinose, ramnose and xylose) through hydrolysis using 3 types of acid catalysts, namely hydrochloric acid (HCl), sulfuric acid ($H_2SO_4$) and nitric acid ($HNO_3$). Fermentation was carried out with *S. cerevisiae* concentration variation of 6; 8; 10; 12 % (v/v) and fermentation time variation of 3; 5; 7; 9 days and a pH variation of 4; 4.5; 5; 5.5 at a temperature of 30 °C. Reducing sugar characterization used the Dinitrosalicylic acid (DNS) reagent, sample surface texture analysis was carried out using Scanning Electron Microscopy (SEM) and ethanol characterization used Gas Chromatography-Flame Ionization Detector (GC-FID). The results of surface texture analysis before and after hydrolysis experienced significant changes. Optimal conditions for hydrolysis of *Ulva reticulata* seaweed using sulfuric acid ($H_2SO_4$) combined with a CEM synthesizer at an acid concentration of 3 % (v/v) with irradiation power of 200 watts for 50 min at a temperature of 150 °C, produced reducing sugar of 97.06 g/L. The results of GC-FID analysis indicated that bioethanol concentration obtained at optimal conditions of pH 4.5 was 42.32 %, *S. cerevisiae* concentration was 12 % with a bioethanol content of 42.53 % at a fermentation time of 5 days. This research is expected to provide information for researchers and industry to overcome world energy problems and environmental pollution through *Ulva reticulata* waste.

**Keywords:** *Ulva reticulata*, Acid hydrolysis, CEM microwave, Fermentation time, Bioethanol

## Introduction

The content of carbohydrates and lipids in seaweed is considered a 4th generation renewable energy source [1]. Seaweed genus Ulva sp. (Cholorphyta, Ulvaceace) is generally referred to as sealettuce which consists of 3 species, namely Ulva fasciata, Ulva lactuca and Ulva reticulata. The Ulva is characterized by a bright green to light green color. The difference between the 3 Ulva species lies in their thallus sheets. Compared to other types of seaweed, Ulva reticulata has thalus in the form of small sheets measuring 2 mm wide forming clumps resembling a net, stiff and hard so it is not processed as food .Yu-Qing *et al.* [2] stated that Ulva seaweed contains quite abundant carbohydrates in the form of heteropolysaccharides such as glucose, arabinose, rhamnose and xylose. This type of macroalgae, Ulva reticulata, is widely distributed, especially in the waters of Bolok Beach, Timor Sea, East Nusa Tenggara with a species density value of 50.42 individuals/$m^2$ and the highest relative density of 50.42 %, so it has the potential to be produced on an industrial scale as a renewable energy source [3]. However, Ulva reticulata has not been utilized by the people of Timor Island to make food, so it does not compete with food when it becomes bioethanol.

Biomass conversion from seaweed is carried out through several stages, namely initial treatment, hydrolysis and fermentation and distillation (purification) [4]. The use of an acid catalyst during hydrolysis greatly influences the production of reducing sugars. The reducing sugar produced during treatment with

the hydrochloric acid (HCl) catalyst is higher than sulfuric acid ($H_2SO_4$) through variations in reaction temperature, acid concentration and the same reaction time. It occurs since HCl has stronger properties with higher reactivity compared to $H_2SO_4$ [5]. Apart from that, one of the alternative tools for initial treatment (deliginification and hydrolysis), namely the use of a CEM (Ceramic Elekctromagnetic Microwave) synthesizer, has many advantages, namely the hydrolysis time is relatively shorter compared to conventional methods, the rate of the starch to glucose hydrolysis reaction increases 50 - 100 times, saving costs. Apart from that, it is more environmentally friendly because the acid concentration used is lower [6]. Kolo *et al.* [7] reported that hydrolysis of Ulva reticulata seaweed with 2 % $H_2SO_4$ for 50 min can maximize reducing sugar production of 33.4 g/L and a bioethanol concentration of 5.02 % at an S. cerevisiae concentration of 10 % for 6 days. This result is not optimal according to SNI for industrial scale, namely 90 - 94 %, so it is necessary to optimize the time and inoculum concentration during fermentation. In addition, repeated distillation needs to be carried out so that the bioethanol purification process is optimal. The multistage distillation process is carried out by controlling the temperature at 78 °C and a heating time of 3 - 4 h. To the best of the writer's knowledge, the research on bioethanol production from Ulva reticulata was first carried out in 2021 by Kolo *et al.* [7] using $H_2SO_4$ solvent without the optimization of hydrolysis and fermentation processes thus it generated low bioethanol content.

The problem in seeking alternative energy from non-food carbohydrates is the pre-treatment before fermentation. Uncontrolled initial treatment will produce secondary compounds (inhibitors) during the fermentation process. The initial treatment process by optimizing hydrolysis time, hydrolysis temperature, and dilute acid concentration was carried out in the $1^{st}$ year. Meanwhile, in the $2^{nd}$ year, optimization was carried out during the fermentation process to find fermentation time, S. cerevisiae concentration, and optimal pH to obtain the highest bioethanol concentration. The purpose of this research is to determine the optimal bioethanol concentration through variations in fermentation time, S. cerevisiae concentration and fermentation pH using optimal conditions in the initial treatment. S. cerevisiae is superior to bacteria, other yeasts, and filamentous fungi in various physiological characteristics related to ethanol production in industrial contexts [3]. Compared with other types of microorganisms, yeast, especially S. cerevisiae, is a microbe commonly used in ethanol production because of its high ethanol productivity, high ethanol tolerance, and ability to ferment various kinds of sugars. However, there are several challenges in yeast fermentation that inhibit ethanol production such as high temperatures, high ethanol concentrations and the ability to ferment pentose sugars [8]. This is supported by research by Kolo *et al*. [6] who carried out co-fermentation using 2 yeasts, namely S. cerevisiae to convert glucose into ethanol and Pichia stipitis to convert xylose into ethanol. This research also provides information for industry to improve the economic value of Ulva reticulata seaweed as a renewable energy source in the future and as a reference for further research.

The novelty value of this research is the acquisition of a $3^{rd}$ generation bioethanol renewable energy source, namely Ulva reticulata seaweed through the innovation using CEM synthesizer in the pre-treatment process and variations in fermentation time, S. cerevisiae concentration, and fermentation pH in converting Ulva reticulata flour into bioethanol.

## Materials and methods

Research materials: $H_2SO_4$ (Merck) and NaOH (technical), neutralizing materials for the fermentation media, namely HCl (Merck), Glucose ($C_6H_{12}O_6$) (Merck), $H_2SO_4$ (Merck), Ethanol ($C_2H_5OH$) (Merck), acetic acid ($CH_3COOH$) (Merck). The inoculum medium consists of *Saccharomyces cerevisiae* inoculum media (5 g/L yeast extract (Sigma Aldrich); 5 g/L peptone (Sigma Aldrich); 20 g/L xylose (Sigma Aldrich) and glucose (Sigma Aldrich) as carbon sources), the fermentation medium consists of 5 g/L yeast extract; Peptone 5 g/L; 5 g/L $KH_2PO_4$ (Potassium dihydrogen phosphate), 0.4 g/L $MgSO_4.7H_2O$ (Magnesium sulphate heptahidrat) (Sigma Aldrich), 0.5 g/L $NH_4SO_4$ (Ammonium sulphate) (Sigma Aldrich); Glucose hydrolyzate. Research tools: Glassware, analytical scale (*Mettler ToledoAG 204, USA*), pH meter (*Thermo Orion Model* 710 A), autoclave (*Sturdy* Sa-232 X), incubator temperature of 37 °C (*FISHER model* 503), water bath (*Precision 280 series*), GC-FID (Agilent 6890), Ultra Violet Visible (Uv-Vis) Spectrophotometer (INNOVA), SEM (Zeiss), magnetic stirrer (*Jeio Tech TM-17R*), loop wire, and falcon tube.

### Sampling and preparation of *Ulva reticulata* seaweed

*Ulva reticulata* samples were taken in May - August 2023 on the coast of Bolok, Kupang Regency, East Nusa Tenggara Province. *Ulva reticulata* preparation consists of 2 steps, namely drying in the sun for 2×24 h then grinding using a blender, then filtering using a ± 100 *mesh* sieve to obtain *Ulva reticulata* flour.

### *Ulva reticulata* seaweed flour saccharification

The saccharification stage in *Ulva reticulata* flour aimed to hydrolyze cellulose into monosaccharides like glucose. Saccharification was carried out by treating the optimal acid concentration, temperature, time and power based on the optimization results as shown in **Table 1**.

**Table 1** Hydrolysis of *Ulva reticulata* powder with 3 catalysts.

| Catalyst | % (v/v) | Time (min) | Temperature (°C) | Power (watt) |
|----------|---------|------------|------------------|--------------|
| HCl | 7 | 60 | 200 | 200 |
| $H_2SO_4$ | 3 | 50 | 150 | 200 |
| $HNO_3$ | 7 | 60 | 200 | 250 |

### Acid hydrolysis was carried out in the following methods

Prepare 3 Erlenmeyer flasks measuring 500 mL then add 10 g of Ulva flour then add 250 mL of HCl, $H_2SO_4$ and $HNO_3$ solution into each Erlenmeyer flask. Further, this solution was heated using a *CEM synthesizer* based on the conditions in **Table 1**. The resulted hydrolysis solution was then filtered using filter paper to obtain the residue and filtrate. The filtrate was taken to analyze the reducing sugar using a DNS (Dinitrosalicylic acid) reagent. This analysis used a UV-Vis spectrophotometer while the residue was taken to observe its surface texture using SEM. The resulted reducing sugar for each catalyst was then used as a hydrolyzate in the fermentation process.

### Bioethanol production

The fermentation medium in use was 100 mL of hydrolyzate in a 250 mL erlenmeyer for each research treatment. The 1st step in making a starter was conducted by inoculating the regenerated *S. cerevisiae* yeast culture from liquid culture into 100 mL of fermentation medium, then incubated at room temperature until the middle of the logarithmic phase occurs [9]. Fermentation was performed using *S. cerevisiae* yeast. The fermentation volume for the hydrolyzate medium was 200 mL. The hydrolyzate of Ulva *reticulata sp* powder was first neutralized to analyze reducing sugars, then added with the fermentation medium and autoclaved at a temperature of 121 °C for 15 min. The fermentation medium used aquades as a solvent. The fermentation process was carried out at a temperature of 30 °C with a pH variations of 4; 4.5; 5; and 5.5 [10].

### Charaterization and analysis
#### *Powder surface texture analysis*

The hydrolysis results were filtered and then neutralized for the next process. The surface texture of the solid fraction was seen using SEM to see the morphology or surface texture of the powder at 250× magnification with a size of 100 μM, while the reducing sugar content of liquid fraction was analyzed with a DNS method using a UV-Vis spectrophotometer [11].

#### *Reducing sugar analysis*

The 1 mL of sample was added with 1 mL of DNS reagent (composition: Dinitrosalicylic acid, Rochelle salt and sodium hydroxide) then mixed and heated at 100 °C for 10 min. Reducing sugar measurement was conducted using a Uv-Vis spectrophotometer with a wavelength of 540 nm [7].

#### *Qualitative analysis of bioethanol*

Qualitative analysis steps ethanol namely:
1) 2 mL of 2 % $K_2Cr_2O_7$ was added to the tube reaction.
2) Add 5 drops of concentrated $H_2SO_4$.
3) Add 1 mL of the resulting ethanol sample distillation.
4) Shake it up and let it sit until it happens change from orange to green.

#### *Quantitative analysis of bioethanol*

Ethanol was analyzed using GC-FID instrument [12]. Prepare a sample with the composition is unknown and the standard solution has been is known. Performed tool running using FID detector with maximum conditions 200 °C. Measuring nanometer pressure on a tube of 3.5 kg/m. Setting the speed of the carrier gas (Helium) to the right or left by 300 mL/min. Inject a standard solution of at least 1 μL ethanol

and the ethanol peak appears at chromatogram. Written analysis results by the internal integrator RT (retention time) report form, AREA (peak area), TYPE (peak type), AREA% (percent of compound in solution). A sample solution of 1 µL of ethanol was injected and a chromatogram was made. The standard chromatogram is compared with sample chromatogram [13].

## Results and discussion

### Surface texture of *Ulva reticulata* powder before and after hydrolysis using CEM synthesizer

*Ulva reticulata* seaweed powder used as a raw material in this research contained cellulose, hemicellulose and lignin as the main components [6]. It was required to develop an effective method for converting cellulose component into simple sugar and also as a basis for selecting suitable microorganisms to convert sugar into bioethanol. Therefore, the structural carbohydrate content of *Ulva reticulata* seaweed powder was determined before and after pretreatment by analyzing its surface texture. After initial treatment using CEM Synthesizer, it was then filtered to separate the filtrate and *Ulva reticulata* residue. The filtrate was used to analyze reducing sugar content, while the residue was washed until neutral and then dried to analyze the powder surface texture using SEM. The result of *Ulva reticulata* powder characterization was shown in **Figure 1**.
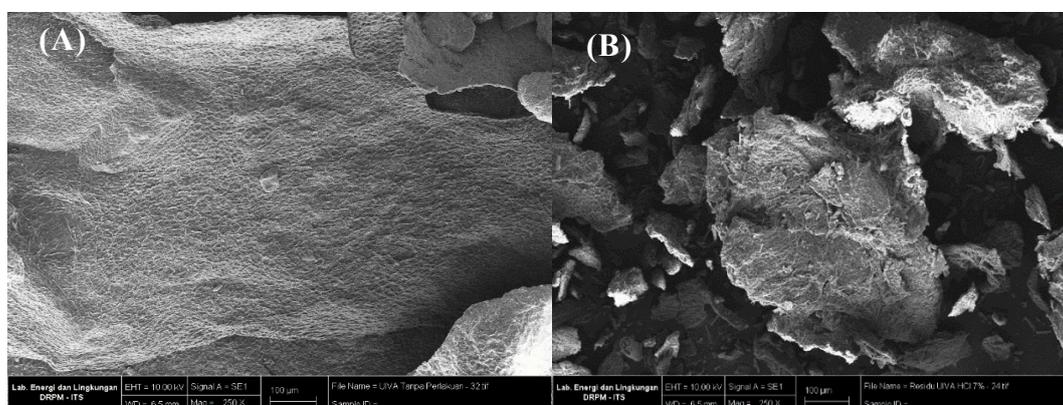


**Figure 1** *Ulva reticulata* powder morphology; (A) Without pre-treatment, (B) CEM microwave synthetizer.

The profile in **Figure 1**. A shows that the surface texture of *Ulva reticulata* seaweed before pre-treatment was dense and stiff. The pre-treatment process removed acetyl and other acid substitutes in lignin and hemicellulose that protected the cellulose. The SEM result revealed that the weed powder broke and suffered significant damage after pre-treatment with $HNO_3$ catalyst (**Figure 1(B)**). This result indicates that the treatment using CEM Microwave with dilute acid catalyst managed to degrade the lignin portion of lignocellulose in *Ulva reticulata* seaweed powder.

Wang *et al.* [14] explained that the lignin structure is not a sugar polymer as a substrate for bioethanol production through microbial fermentation thus it will inhibit microbial growth during the fermentation process. The use of a CEM synthesizer during hydrolysis using dilute acid will break down lignin and release it from the cellulose and hemicellulose structures. The-1,4-glycosidic bond of linear glucan cellulose chains or-1,4-D-pyranosyl linkage heterogeneous hemicellulose polysaccharides will be degraded through hydrolysis into sugar monomers, such as glucose, xylose, galactose, arabinose and mannose [6].

The pre-treatment process combined with CEM synthesizer helps degrade and release lignin from cellulose or hemicellulose materials. Lignin is not a sugar polymer and cannot be used as a raw material for bioethanol production and inhibits microbial growth during fermentation [14].

### Saccharification of flour of *Ulva reticulata* seaweed

The saccharification stage in *Ulva reticulata* flour aimed to hydrolyse cellulose into a monosaccharide form such as glucose. Saccharification was carried out by treating the optimum acid concentration, temperature, time and power. The sample's reducing sugar concentration was determined using a Uv-Vis Spectrophotometer at 540 nm and a 3.5-dinitrosalicylic acid (DNS) reagent. The hydrolysis filtrate was identified by the production of a brownish red-reduced product after heating when the sugar in the sample reduced 3.5-dinitrosalicylic acid to 3-amino-5-nitrosalicylicacid. The brownish red colour will be absorbed maximally at a wavelength of 540 nm. Glucose reaction with DNS reagent will produce absorbance value that can be spectrophotometrically measured [15].

The saccharification of *Ulva reticulata* powder was carried out using microwave irradiation technique using CEM synthesizer (Model Discovery 2.0, CEM Corporation, United Kingdom). This technique was used to improve the efficiency of the hydrolysis reaction in order to produce a higher reducing sugar concentration. In addition, the use of microwave irradiation can accelerate the glycosidic bond breaking reaction in cellulose molecules so that optimal glucose is obtained as a fermentation substrate. Rapid reaction in the hydrolysis process can reduce the formation of secondary compounds such as HMF (Hydroxymethylfurfural) and furfural [6]. The optimum reduction sugar was obtained using HCl, $H_2SO_4$ and $HNO_3$ catalysts, influenced by microwave irradiation along with increasing acid concentration, temperature, time and microwave irradiation power (**Table 2**).

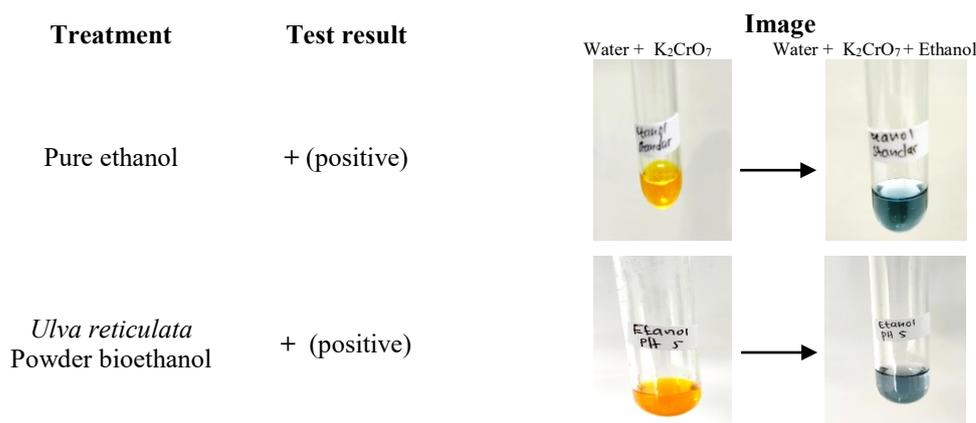**Table 2** Reducing sugar contents ($H_2SO_4$, $HNO_3$ catalysts).

| HCl catalyst | | $H_2SO_4$ catalyst | | $HNO_3$ catalyst | |
|---|---|---|---|---|---|
| CEM conditions | $C_{RS}$ (g/L) | CEM conditions | $C_{RS}$ (g/L) | CEM conditions | $C_{RS}$ (g/L) |
| t (60 min) | 84.67 | t (50 min) | 33.40 | t (60 min) | 35.20 |
| T (200 °C) | 79 - 60 % | T (150 °C) | 23.70 | T (150 °C) | 86.53 |
| $C_{Acid}$ (7 %) | 84.70 | $C_{Acid}$ (3 %) | 64.67 | $C_{Acid}$ (7 %) | 72.53 |
| P (200 watt) | 76.39 | P (200 watt) | 97.06 | P (250 watt) | 83.30 |

Description: t: Time; T: Temperature; $C_{acid}$: Acid concentration; P: Power; $C_{RS}$: Reducing Sugar Concentration

The hydrolysis results of the 3 catalysts in **Table 1** show that the $H_2SO_4$ catalyst was more effectively used in the saccharification process of *Ulva reticulata* powder because the highest reducing sugar content was obtained using faster time, lower catalyst temperature and concentration and the same CEM synthesizer irradiation power. The study of Kolo *et al.* [13] reported that the hydrolysis of *Ulva reticulata* with $H_2SO_4$ catalyst using microwave type Kirin with a combination of delignification and hydrolysis through variations in time and temperature of hydrolysis obtained optimum reducing sugar of 27.97 g/L at 150 °C, $H_2SO_4$ concentration of 2 % and hydrolysis time of 50 min. These results are still low compared to those obtained in this study. The optimum hydrolysis conditions of *Ulva reticulata* seaweed using sulfuric acid ($H_2SO_4$) combined with CEM synthesizer at 3 % acid concentration (v/v) with 200 watts irradiation power for 50 min at 150 °C, resulted in reducing sugar of 97.06 g/L. Phwan *et al.* [16] reported that low acid concentration with high temperature is preferred over high acid concentration because it will not cause the degradation of sugar into other unfavourable compounds that affect the hydrolysis results. The use of longer reaction times causes the formation of secondary compounds such as HMF and subsequently reacts to formic acid [17]. This can be seen in the optimization of reducing sugar results in HCl and $HNO_3$ catalysts, which when the time, temperature and concentration are higher, the reducing sugar results decrease compared to $H_2SO_4$ catalyst. The use of CEM synthesizers is considered more advantageous than standard and simple microwave reflux method due to shorter reaction times (min), fewer solvents and higher reducing sugar products. High acid concentrations also have a tendency to damage the experimental equipment. Dilute acid hydrolysis has been acknowledged as a promising way to produce bioethanol from biomass of carbohydrate-rich microalgae since it is more economical. Each catalyst's optimal reducing sugar production was subsequently utilized in the fermentation process as hydrolysate.

**Hydrolysed fermentation of *Ulva reticulata* powder**
The optimum conditions for reducing sugar hydrolysate were fermented using *Ulva reticulata* powder hydrolysate as substrate using *S. cerevisiae* yeast. *S. cerevisiae* yeast was chosen because it has various advantages, including high survival rate and the ability to produce alcohol in sufficient quantities [18]. Fermentation was carried out with variations in pH, fermentation time and *S. cerevisiae* concentration.

| Treatment | Test result | Image |
|---|---|---|
| Pure ethanol | + (positive) |  |
| *Ulva reticulata* Powder bioethanol | + (positive) |  |

**Figure 2** Qualitative test results using $K_2Cr_2O_7$ compound.

Qualitative analysis of bioethanol was carried out using potassium dichromate ($K_2CrO_7$) to ensure that the sample resulting from multilevel distillation produced ethanol. The results of the analysis are presented in **Figure 2**. Qualitative test results show that there is a color change from orange to bluish green in both pure ethanol (standard) and bioethanol (fermentation sample). According to Kolo *et al.* [19] stated that a positive test for the presence of ethanol is indicated by a change in the color of potassium dichromate from orange to bluish green. So it can be concluded that in the fermentation sample there has been a change in glucose to ethanol.

**Ethanol analysis using gas chromatography**

Quantitative analysis of *Ulva reticulata* bioethanol contents used gas chromatography (GC) tool. GC analysis was carried out to determine the presence of ethanol produced during fermentation. GC analysis requires toluene as an internal standard. This was performed for a complete separation between the sample peaks so that 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 the molecular formula of ethanol. The existence of these similarities makes it easy to know the solubility between the 2 solutions based on the principle of like dissolve like [20].
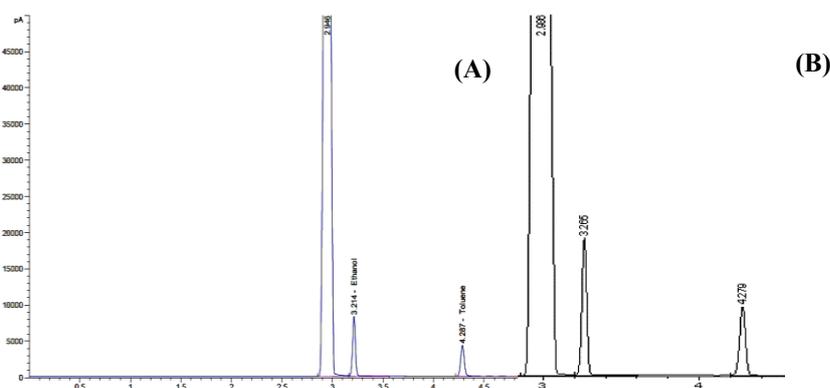


**Figure 2** Chromatogram (GC): (A) Standard ethanol and (B) Bioethanol sample.

The chromatogram of gas chromatography results in **Figure 2** shows that the fermentation sample contains 3 peaks with different retention times, namely at a retention time of 2.995, 3.266 and 4.279 min where the compounds that came out or evaporated as peaks first at a retention time of 2.995 min, namely hexane compounds followed by ethanol (3.266 min) and toluene (4.279 min). The peak of the hexane compound came out first because hexane had the lowest boiling point (68.7 °C) compared to ethanol (78.3 °C) and toluene (110.6 °C) [21]. This is because the components of the mixture in the sample will separate or come out according to the boiling point they have. Components that have a lower boiling point will evaporate first so that they will come out as the 1st peak on the chromatogram. Compounds used as internal standards in research this is toluene. Toluene was chosen as the internal standard because it has a molecular

formula similar to molecular formula of ethanol. Due to these similarities, the solubility between the 2 solutions is easy to determine based on the principle of like dissolves like [20]. The chromatogram area of the ethanol peak with the area of the standard was compared to calculate the bioethanol concentration in the sample. Ethanol contents through pH variations in the fermentation process using the initial treatment of $H_2SO_4$ and $HNO_3$ catalysts are shown in **Table 2**.

The results of the calculation obtained optimum ethanol concentration at pH 4.5 variation using glucose hydrolysate from $H_2SO_4$ catalyst of 41.16 %. Meanwhile, in the $HNO_3$ catalyst, the optimum ethanol concentration at pH 5 was 42.48 %. HCl catalyst obtained optimum ethanol concentration at pH 4.5 of 42.32 %. **Table 3** explains that ethanol contents tend to decrease along with the increase in pH value. This is due to the condition of the medium that leads to neutral pH. According to Frazier and Westhoff (1978) pH will affect the fermentation rate, the optimal pH for yeast growth is 4.0 - 4.5. The ethanol content produced at pH 4 and pH 5 is also very small so that with a high pH, *S. cerevisiae* cannot produce ethanol anymore, and the ethanol that has been formed is then reduced due to further fermentation into acetic acid and other organic acids [22].

**Table 2** Bioethanol contents of pH variations ($H_2SO_4$, $HNO_3$, HCl catalysts).

| pH | $H_2SO_4$ catalyst | | $HNO_3$ catalyst | | HCl catalyst | |
|---|---|---|---|---|---|---|
| | Ethanol content (%) | SD | Ethanol content (%) | SD | Ethanol content (%) | SD |
| 4,0 | 25.78 | 0.11 | 34.96 | 0.97 | 23.18 | 0.09 |
| 4,5 | 41.16 | 1.91 | 38.51 | 0.31 | 42.32 | 0.31 |
| 5,0 | 34.17 | 0.04 | 42.48 | 0.13 | 22.92 | 0.06 |
| 5,5 | 22.41 | 0.17 | 32.29 | 2.15 | 14.04 | 0.51 |

Description: SD: Standard deviation

Based on the ethanol results in **Table 2**, the fermentation process was continued utilising glucose hydrolysate from HCl catalysts, with variations in *S. cerevisiae* concentrations (6, 8, 10 and 12 %) and fermentation lengths (3, 5, 7 and 9 days). The ethanol results after being analyzed using GC-FID are shown in **Table 4**.

**Table 3** Bioethanol contents in *S. cerevisiae* concentration and time variations (HCl catalyst).

| *S. cerevisiae* Concentration, % | Bioethanol concentration, % | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | FT (3 days) | SD | FT (5 days) | SD | FT (7 days) | SD | FT (9 days) | SD |
| 6 | 19.44 | 0.42 | 15,09 | 0.02 | 28.91 | 0.91 | 20.78 | 0.11 |
| 8 | 20,08 | 0.10 | 30.42 | 0,01 | 30.07 | 1.03 | 23.18 | 0.09 |
| 10 | 23.50 | 0.19 | 37.22 | 0.30 | 27/31 | 0.33 | 25.08 | 0.28 |
| 12 | 38.92 | 1.12 | 42.53 | 0.44 | 43.40 | 0.79 | 22.92 | 0.06 |

Description: SD: Standard deviation; FT: Fermentation time

The ethanol results in **Table 3** show that the 12 % *S. cerevisiae* concentration produced higher fermented ethanol on day 3, 5 and 7 except for day 9, there was a decrease in ethanol concentration. The bioethanol content under the condition of 7-day fermentation operation was 43.40 % higher when the fermentation was continued for up to 9 days which decreased to 22.92 %. Rinastiti *et al.* [23] reported that the bioethanol content increases along with the longer fermentation time and high concentration of yeast. Yeast can develop and grow on a fermented solution medium so that it can develop to convert glucose into bioethanol. Thus, a high concentration of yeast will increase the contents of bioethanol formed in the fermentation process. However, the fermentation time has a maximum limit of 7 days, when the fermentation time exceeds the maximum amount it does not affect the increase in bioethanol content because the yeast undergoes a death phase so that the activity of the yeast in converting glucose to bioethanol decreases. Siregar *et al.* [24] also reported that if there are too many microbes, bioethanol will be formed and at the next time fermentation will no longer occur on the substrate so that the microbes will be in the death phase until the beginning, then bioethanol will be reduced due to secondary reactions.

According to Phwan *et al.* [16] reported, pre-treated samples through acid hydrolysis are essential before the fermentation process to increase ethanol productivity. According to Azizah *et al.* [25] mentioned that in addition to ethanol, other products of the fermentation process include acetic acid, fusel oil, and acetaldehyde. This is evidenced by research by Kolo *et al.* [6] which proves the presence of acetic acid after fermentation accompanied by a decrease in ethanol during the analysis using HPLC. The presence of impurities like lactic acid bacteria and acetic acid bacteria, which can act as inhibitors in the fermentation process, is another cause of low bioethanol concentration [26]. The factor that also affects is that there is still water content in the distilled sample. The low concentration of bioethanol produced in this study was due to several factors such as not all glucose molecules being converted into ethanol. In addition, a long reaction process caused glucose to form hydroxymethylfurfural compounds (HMF), formic acid, levulinic acid where these compounds are inhibitory compounds that can inhibit the growth of microorganisms in the fermentation process. Additionally, it results from a delayed fermentation process brought on by the medium's low nutrient concentration. This problem is also brought on by the fact that ethanol was converted to acetic acid either during the filtration procedure or when the distilled samples were transferred from the distillation flask to the reagent bottle [6]. Preliminary research results of Danmaliki *et al.* [27] also indicated that high amounts of sugar after hydrolysis do not guarantee high ethanol production after fermentation. This is due to the accumulation of other toxic compounds or secondary metabolites such as phenolic compounds, organic acids, and furans produced during fermentation of acid-treated samples. These toxic compounds may have caused the death phase of *S. cerevisiae* at the beginning of microbial growth. In addition, our results confirm that bioethanol production is treatment dependent.

## Conclusions

In this study, the results of surface texture analysis before and after hydrolysis experienced significant changes. Optimum conditions for hydrolysis of *Ulva reticulata* seaweed using sulfuric acid ($H_2SO_4$) combined with a *CEM synthesizer* at an acid concentration of 3 % (v/v) with irradiation power of 200 watts for 50 min at a temperature of 150 °C, producing reducing sugar of 97.06 g /L. The results of the GC-FID analysis showed that the bioethanol concentration obtained at optimum conditions of pH 4.5 was 42.32 %, the inoculum concentration was 12 % with a bioethanol content of 42.53 % at a fermentation time of 5 days.

## Acknowledgements

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