Optimum Fermentation Process for Red Macroalgae *Gelidium latifolium* and *Gracillaria verrucosa*

Mujizat Kawaroe[1,*], Dahlia Wulan Sari[2], Junkwon Hwangbo[3] & Joko Santoso[4]

[1]Department of Marine Science and Technology, Faculty of Fisheries and Marine Science, Bogor Agricultural University, Darmaga Campus, Bogor 16680, Indonesia
[2]Surfactant and Bioenergy Research Centre, Bogor Agricultural University, Baranang Siang Campus, Bogor 16143, Indonesia
[3]Research Institute of Science and Technology POSCO, Kumho-dong, Gwangyang City, Jeollanam-do, South Korea
[4]Department of Fish Processing, Faculty of Fisheries and Marine Science, Bogor Agricultural University, Darmaga Campus, Bogor 16680, Indonesia
E-mail: mujizatk@gmail.com; mujizat@ipb.ac.id

Abstract. Red macroalgae have the potential to be processed into bioethanol due to their high carbohydrate and low lignin content. *Gelidium latifolium* and *Gracilaria verrucosa* are red macroalgae commonly found in Indonesian seas. Sometimes an over-supply of red macroalgae is rejected by the food industry, which opens up opportunities for others uses, e.g. for producing bioethanol. The objectives of this research were to analyze the influence of sulfuric acid concentration on hydrolysis of *G. latifolium* and *G. verrucosa* and to calculate the optimum fermentation process to produce bioethanol. *G. latifolium* and *G. verrucosa* were hydrolyzed using H_2SO_4 at concentrations of 1%, 2%, 3%, and 4%, at a temperature of 121 °C and a pressure of 1.5 bar for 45 minutes. The process of fermentation was done using *Saccharomyces cerevisiae* in anaerobic conditions for 4, 5, 6 and 7 days. The results show that the optimum H2SO4 concentrations to hydrolyze *G. latifolium* and *G. verrucosa* were 1% and 2% respectively. The number of *S. cerevisiae* cells in hydrolysate *G. latifolium* and *G. verrucosa* increased in the third adaptation. *S. cerevisiae* can convert sugar from *G. latifolium* and *G. verrucosa* into bioethanol through fermentation. The highest bioethanol yields were achieved on days five and six. Therefore, red macroalgae can be seen as a potential raw material for bioethanol production.

Keywords: *acid hidrolysis; anaerobic; bioethanol; fermentation; red macroalgae.*

1 Introduction

Efforts in developing renewable energy based on biomass still have various obstacles. For example, lignocellulose is an alternative potential raw material for biofuel production but its high level of lignin makes it hard to degrade the lignocellulose [1-4]. Macroalgae are another potential biomass source and have various advantages: 1) do not compete with food sources, 2) have a high level of sugar, 3) have a low level of lignin, 4) have high productivity [2-6].

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Macroalgae can also reduce the CO_2 level in the atmosphere and at the same time increase oceanic O_2 levels, while some species can also absorb heavy metals from water.

Research has shown that red macroalgae found in Japanese seas contain the highest amount of carbohydrate compared to green and brown macroalgae [7]. Several species of Rhodophyceae (red macroalgae), i.e. *G. amansi*, *G. latifolium*, *Gracilaria crasa*, *Glacilaria verrucosa* and *Euchema cottonii*, have a high carbohydrate content: 67.3% [6], 26-62% [8], 37.7% [9], 24-43% [10], 26,49% [11], respectively. In Indonesia, the most dominant and commonly found red macroalgae are *G. latifolium*, *G. verrucosa* and *E. cottonii*. Because their availability is abundant and they can be found throughout the Indonesian archipelago, it is worthwhile to conduct research on the fermentation process of *G. latifolium* and *G. verrucosa* using acid (H_2SO_4).

Generally, the stages of bioethanol production are: 1) hydrolysis of polysaccharide into monosaccahride; 2) monosaccahride fermentation into bioethanol; and 3) bioethanol purification. Hydrolysis, or saccharification, is needed to breakdown polysaccharide into monosaccharide compounds, which are then fermented into bioethanol. Hydrolysis of macroalgae can be done using enzymes or acid. Sulfuric acid can be used in hydrolysis (H_2SO_4) since it can produce high levels of sugar [12]. Polysaccharide in red macroalgae can be hydrolyzed using low-concentrate sulfuric acid (H_2SO_4) [5,13,14]).

Hydrolysis of *G. amansii* using H_2SO_4 at a concentration of 3% for 45 minutes at a temperature of 120 °C produces 49.32% and 12.62% monosaccharide galactose [7]. Fermentation of *E. cottonii* conducted using *Saccharomyces cerevisiae* that has been adapted to *E. cottonii* hydrolysate gives 2.20% ethanol [15]. Each type of macroalgae has a different H_2SO_4 concentration for hydrolysis and optimum fermentation period, thus further research is needed.

The objectives of this research were to analyze the influence of sulfuric acid concentration on the hydrolysis process of *G. latifolium* and *G. verrucosa* and to measure the optimum fermentation process for producing bioethanol.

2 Material and Method

Material Preparation. The chemical materials used in this research were: H2SO4 (Merck), NaOH (Merck), 3,5-Dinitrolisalicylic acid (Sigma-Aldrich), kNa tartarate, phenol, Na metabisulphite, HCl, pp indicator, potato dextrose agar (Himedia), yeast (BD), malt (BD), glucose (Gluco-VD) and peptone (BD). The macroalgae used in this research were: G. latifolium from Pari Island, Seribu Islands, DKI Jakarta, and G. verrucosa from a tambak (fish farm) in Indramayu, West Java. The G. verrucosa had been rejected by the food industry. The macroalgae were soaked and washed using freshwater to reduce salt and dirt. Then, the macroalgae were dried by sun exposure and cut into samples of ± 1 cm.

Proximate and Crude Fiber Analysis. The results of the proximate analysis, referring to AOAC [16], included water, ash, protein, fat and carbohydrate

content. Crude fiber was analyzed using the Van Soest method [17]. For determination of ash, carbohydrate, lipid and water content, 2 gram of each dried macroalgae species was used. Meanwhile, for determination of protein content, 0.1 gram of each dried macroalgae species was used. For determination of crude fiber content, 5 gram of each dried macroalgae species was used.

Acidic hydrolysis. Hydrolysis was conducted using a hydrolysis autoclave at a temperature of 121 °C for 45 minutes. Macroalgae substrate was 15% b/v (15 gram of dried macroalgae in 100 ml sulfuric acid solution) with sulfuric acid concentrations of 1%, 2%, 3%, and 4% as treatments. Afterwards, the sample was neutralized using natrium hydroxide (NaOH) 10%. Reducing sugar analysis was done by mixing 1 mL hydrolysate with 3 mL DNS solution and boiling for 5 minutes [18]. Color changes that appeared in the next solution were measured using a Thermo Scientific Spectronic Genesys Visible Spectrophotometer 20 at 550 nm wavelength. Monosaccharide characterization was done using an Aminex® HPX-87H high-performance liquid chromatograph at 0.008 N (the wavelength of mobile phase specification H2SO4), with 300 mm x 7.8 mm column, reactive index detector, flow rate 1 ml/min, injection volume 20 µl, column temperature 35°C.

Yeast Adaptation. The yeast used in this research was S. cerevisiae AL IX adapted by yeast [15] to the hydrolyzed medium of E. cottonii. The purpose of yeast adaptation is to prepare the yeast to be able to survive in galactose media instead of glucose. Swift adaptation was done by adding 10% (v/v) S. cerevisiae to the hydrolysate macroalgae and then incubating for 3 days at a temperature of 30°C. 10% (v/v) of the hydrolysate was then added to new hydrolysate medium. This process was repeated four times. The cells in the hydrolisate were counted using a haemacytometer and the sugar consumption value was calculated using the sugar remaining at the end of the hydrolysis process using the DNS method.

Fermentation. The S. cerevisiae used was the best adaptation product. The S. cerevisiae was aseptically inoculated on a petri dish using potato dextrose agar (PDA) medium with an ose and then incubated for 2 days at 30 °C. Preparation of inoculum (starter) was conducted in yeast malt peptone glucose (YMPG) liquid medium [3]. Sterile YMPG (10 mL) was added with ± 2 ose of S. cerevisiae inoculant from the PDA and then incubated for 24 hours at 30°C. Fermentation was done in anaerobic condition at 30°C. Starter (10 mL) was added to 90 mL of hydrolysate, and also 0.5% urea, 0.06% NPK from sugar as nutrient source [15]. The samples were then incubated for 4, 5, 6, and 7 days. The product of the fermentation was distilled to measure the bioethanol level using a DMA 4500 M density meter (Anton Paar), and was also analyzed using high performance liquid chromatography, column C18.

Data Analysis. The results are stated as average value ± standard deviation. This research used single-factor completely randomized sampling with different acid concentrations in the hydrolysis process and fermentation periods. The treatment effect toward the response factor was analyzed using analysis of variance. Treatments that gave a significant effect were tested further using the Duncan test with SPSS software, version 18.

3 Result and Discussion

Chemical and crude fiber composition of *G. latifolium* and *G. verrucosa*. *G.latifolium* mainly consists of carbohydrate (33.48%) and crude fiber (22.97%) (Table 1), which are polymers. It can be utilized for bioethanol production through fermentation. Carbohydrate in *G. latifolium* and *G. verrucosa* is in the form of polysaccharides, i.e. agar and cellulose [6]. Polysaccharide content in *Gelidium* sp. and *Glacilaria* sp. is mainly agar [19,6]. Agar is a complex linear polysaccharide that has a molecular weight of 120.000 dalton and consists of several types of polysaccharides: 3,6-anhydro-L-galactose, D-galactopiranosa, and a trace amount of methyl D-galactose [6].

Table 1 Chemical Composition of *G. latifolium* and *G. verrucosa*.

Chemical Composition	*G. latifolium*	*G. verrucosa*
Total Carbohydrate	40.15	32.27
- Carbohydrate* (%)	23.81±1.08	10.69±0.49
- Crude Fiber (%)	16.34±0.10	21.58±0.43
Ash (%)	11.91±1.07	48.68±0.55
Water (%)	9.66±0.02	15.62±0.94
Protein (%)	9.32±0.25	15.58±0.51
Fat (%)	0.13±0.02	0.1±0.01

* Conversion to starch

G. latifolium has approximately 26-62% carbohydrate [8]. The carbohydrate content in *G. verrucosa* (10.69%) is smaller than its crude fiber content (21.58%). Its carbohydrate content is lower compared to carbohydrate that is found in *G. verrucosa* along the Indian coast, which ranges from 41.83% to 43.53%, depending on the season [20]). Different macroalgae species, habitat, season and environmental conditions in which the macroalgae live can affect their chemical composition [8,21,22].

Crude fiber (in Table 1 = 22.97% and 21.58% for both macroalgae species) in the form of cellulose, hemicellulose and lignin, are difficult to hydrolyse. Lignin is a crude fiber component that exists in very small amounts in macroalgae, or even not at all. The lignin content was 6.44% in *G. latifolium* and 31.18% in *G. verrucosa*, the lowest crude fiber content compared to cellulose and hemicellulose (Table 2). The low amount of lignin is an advantage of marine macroalgae compared to terrestrial plants [5], as it makes macroalgae easier to degrade compared to terrestrial plants. The high lignin content found in terrestrial plants would prevent the hydrolysis process [23,2].

The cellulose in macroalgae can be degraded into glucose, which can then be fermented into ethanol [24,14]. Cellulose content in *G. latifolium* and *G. verrucosa* is 29.37% and 33.59% respectively. Hydrolysis using acid can also degrade hemicellulose into xylose [3].

Table 2 Crude fiber in *G. latifolium* and *G. verrucosa*.

Chemical composition	*G. latifolium*	*G. verrucosa*

Cellulose (%)	20.10±0.02	14.22±0.08
Lignin (%)	4.41±0.38	13.20±2.23
Hemicellulose (%)	43.96±0.33	14.92±0.90

Acidic Hydrolysis. Acidic hydrolysis of *G. latifolium* and *G. verrucosa* showed that the highest values of reducing sugar was obtained by the 2% H2SO4 treatment for 45 minutes. These values were 3.97±0.15% (b/v) and 1.99±0.0% (b/v), respectively (Figure 1). *G. latifolium* substrate hydrolyzed by 2% H2SO4 treatment was 37.52±4,83%, while for *G. verrucosa* it was 57.64±3.44% (Figure 1).

One of the indicators in determining the optimum acid concentration for hydrolysis is the amount of reducing sugar produced. The H_2SO_4 concentration used in the hydrolysis process had a significant impact toward the production of reducing sugar. The addition of 1% acid concentration to *G. latifolium* gave a high amount of reducing sugar. This was significantly different from 3% and 4% addition but not from 2% addition. The addition of 2% H_2SO_4 concentration to *G. verrucosa* produced a high amount of reducing sugar. This was significantly different when compared to the other concentrations. Based on these results, the optimum acid concentrations for *G. latifolium* and *G. verrucosa* hydrolysis are 1% and 2%, respectively.

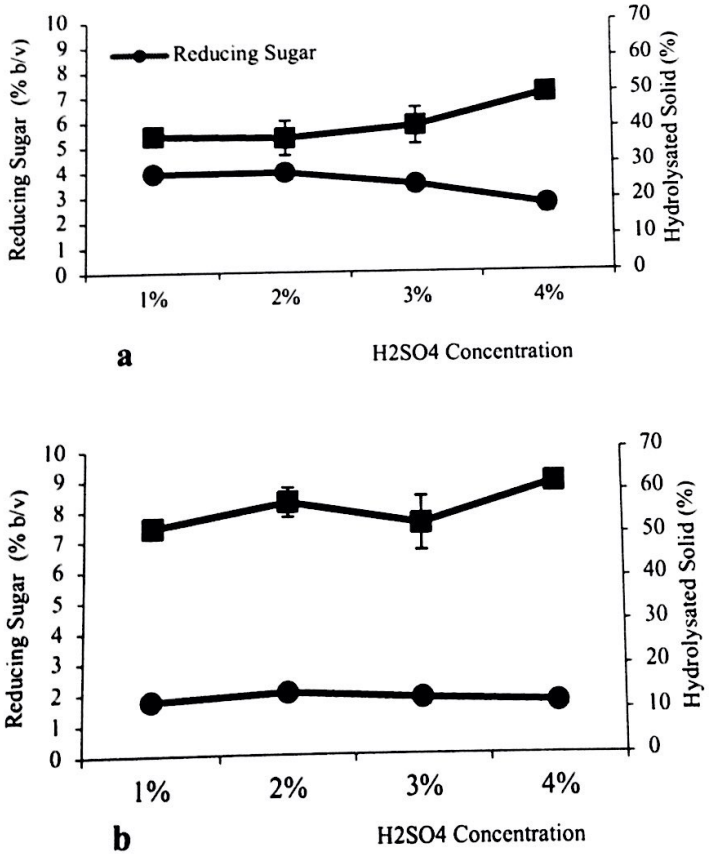


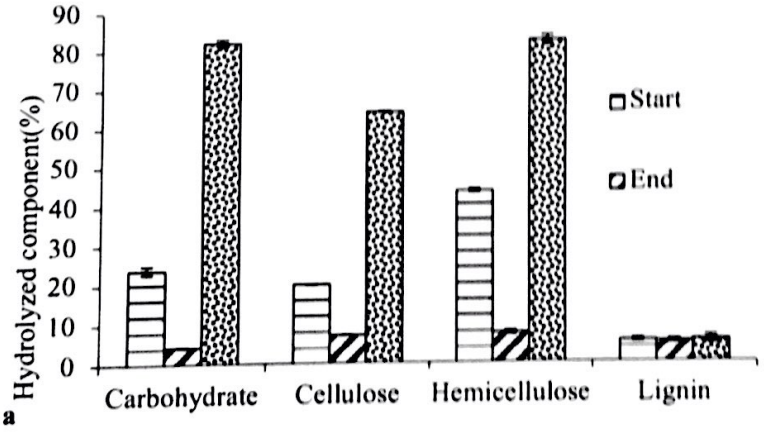
Figure 1 Reducing sugar as acidic hydrolysis product in: (a) *G. latifolium*, and (b) *G. verrucosa*.

Usage of higher concentrations of H_2SO_4 doesn't always produce a higher amount of reducing sugar. Sugar content will drop if the H_2SO_4 concentration exceeds 0,2 M. It will also cause monosaccharides, such as glucose and galactose that will degrade into hydroxymethylfurfural (HMF) and levulinic acid [5,12,25,]. HMF can also be degraded into levulinic acid if the acid concentration is above 1 M [12].

HMF and levulinic acid act as inhibitors in the fermentation process [5,12,13]. HMF and levulinic acid are known to impend cell growth and bioethanol production [12]. HMF has lower toxicity compared to levulinic acid [5]; [12] reported that 5 g/L of HMF and levulinic acid content will reduce cell growth as much as 27,2% and 63,6% respectively. The concentration limits of formic acid, levulinic acid and HMF in the fermentation process are: 0.5 g/L, 2.0 g/L and 10 g/L, respectively [6].

Hydrolysis could not degrade the entire polysaccharide content in *G. latifolium* or *G. verrucosa*. The remaining hydrolysate material still contained unhydrolyzed carbohydrate, cellulose, lignin and hemicellulose. The percentages of hydrolyzed components are presented in Figure 2.

Total carbohydrate was the most hydrolyzed component in *G. latifolium* and *G. verrucosa,* at 82.35±0.89% and 86.51±0.64%, respectively (Figure 2). Carbohydrates in these macroalgae species in the form of polysaccharides were agar and cellulose [6]. The polysaccharides in *Gelidium* sp. and *Glacilaria* sp. mainly consisted of agar [6,19].



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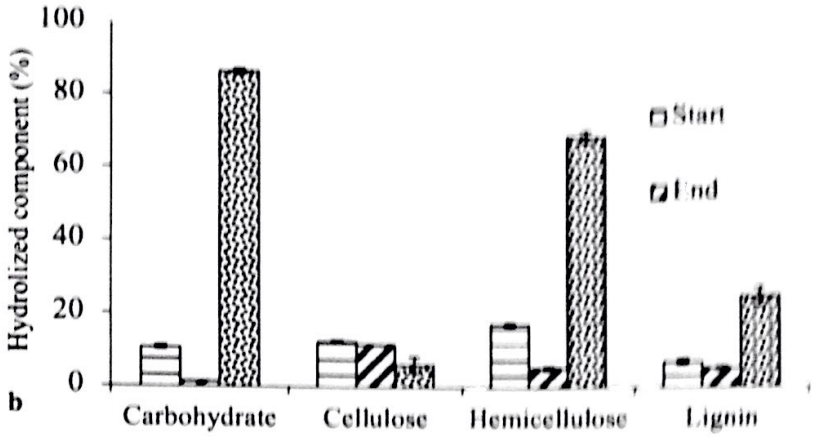

Figure 2 Hydrolyzed components (%) in: (a) *G. latifolium* and, and (b) *G. verrucosa*.

Agar's morphology is softer than that of cellulose, thus agar is easier to hydrolyze compared to cellulose [6]. It is suspected that the unhydrolyzed component is cellulose.

Lignin is the crude fiber component least found in macroalgae, in some species it is even non-existent [14]. Lignin is also a component of lignocellulose that is hard to degrade biologically [2] and it can also cause cellulose and hemicellulose to be difficult to hydrolyze [3]; [13] reports that cellulose can be hydrolyzed using 3% H_2SO_4 at a temperature of 190 °C for 3 minutes. Glucose starts forming at a temperature of 190 °C after 0.9 minutes and keeps increasing until the reaction period reaches 3 minutes. Monosaccharides formed in the hydrolysis of *G. latifolium* and *G. verrucosa* with H_2SO_4 concentrations of 1% and 2% are presented in Figure 3.

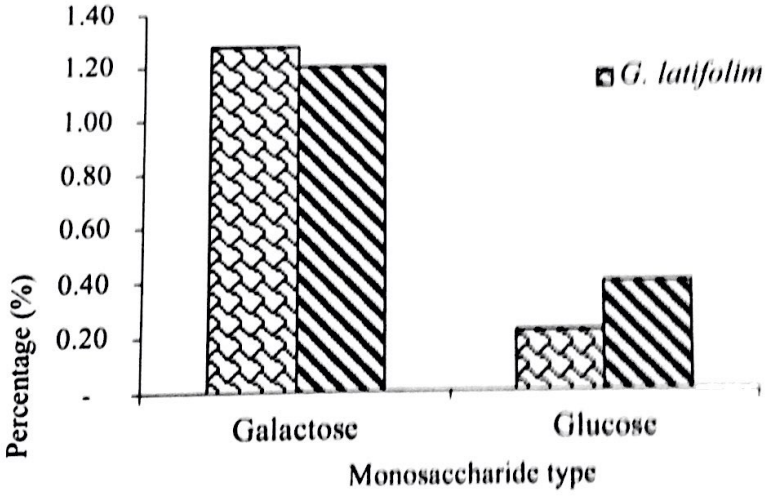

Figure 3 Types of monosaccharides resulting from acidic hydrolysis process.

Galactose is the largest monosaccharide component in the hydrolysis product of *G. latifolium* and *G. verrucosa*. Galactose is a sugar derivate of agar, which is the main polysaccharide in both macroalgae. The amount of galactose found in *G. latifolium* was higher (1.28%) compared to that in *G. verrucosa* (1.20%). Glucose is an agar derivate of cellulose [14,24]. The forming of glucose will

increase with the rise of the hydrolysis temperature in a short period of time. Meanwhile, at a low temperature, the glucose concentration will increase along with the period of hydrolysis. A high temperature for a long period of hydrolysis can lower the glucose concentration formed. This is possibly because glucose can be degraded into other chemical substances [25]. Glucose will start forming at a temperature of 190 °C after 0.9 minutes and keeps increasing until the reaction period reaches 3 minutes [13].

The formation of galactose increases at a temperature of 130°C [25]. Hydrolysis with a temperature exceeding 130 °C over a long period of time can cause the galactose to decompose into other chemical compounds, such as HMF or leuvulinic acid. A long reaction period along with a high temperature can drastically lower the galactose concentration. Galactose will be maximally formed in 45 minutes of hydrolysis with a 108.2 °C temperature and 3% acid concentration.

Yeast adaptation. Adaptation of *S. cerevisiae* was conducted in *G. latifolium* and *G. verrucosa* in order to prepare the yeast so it could convert sugar within the hydrolysate into ethanol. Several parameters measured in the adaptation process are presented in Figure 4.

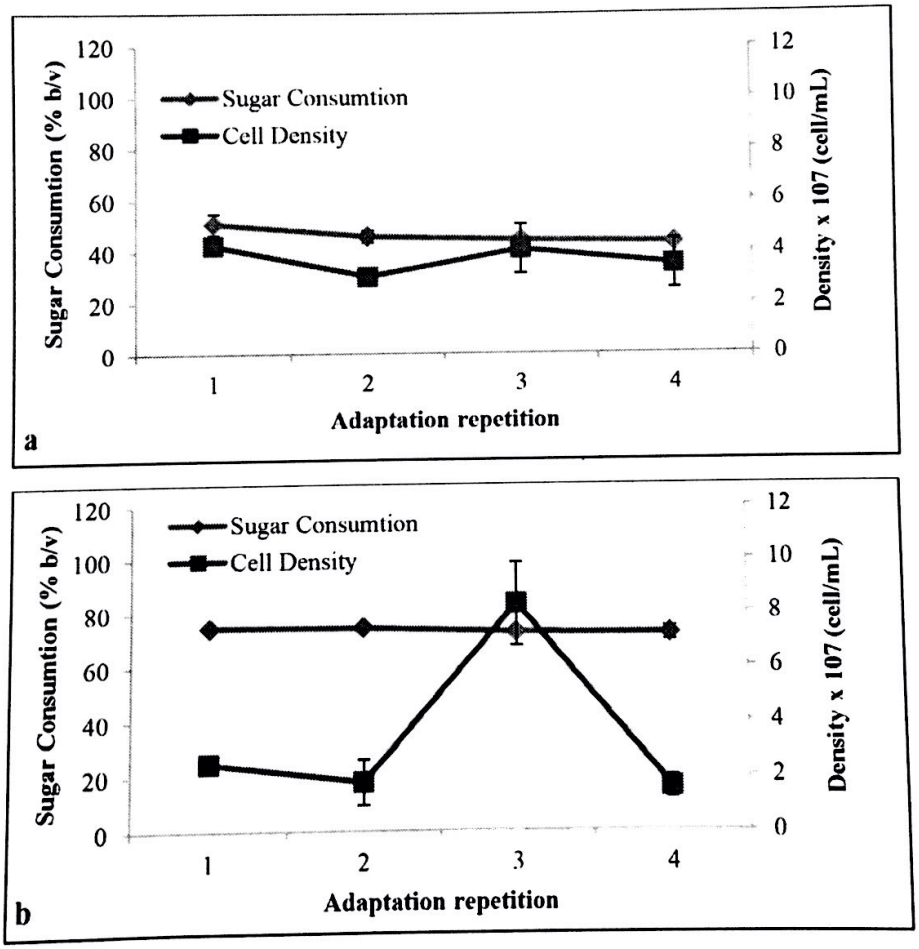

Figure 4 Cell density and sugar consumption in yeast adaptation in: (a) *G. latifolium*, and (b) *G. verrucosa*.

The yeast cell populations in *G. latifolium* and *G. verrucosa* increased in density in the third adaptation with values of 4.08 x 10^7 cell/mL and 8.30 x 10^7 cell/mL, respectively (Figure 4). This is higher compared to the rapid adaptation process of *S. cerevisiae* in the *E. cottonii* hydrolysate, which had a value of 3.15 x 10^5 cell/mL in the same adaptation process [15]. Sugar consumption in this adaptation process was relatively stable, which is in accordance with the research by Setyaningsih [15]. The decreased amount of reducing sugar at the end of the adaptation indicates sugar consumption by *S. cerevisiae* to support its metabolism. The adaptation process was stopped in the fourth cycle because the cell amount kept decreasing.

Fermentation. Fermentation was conducted using *S. cerevisiae* AL IX as yeast which had been adapted on G. *latifolium* and *G. verrucosa* hydrolysis medium for three times. The fermentation products in *G. latifolium* are shown in Figure 5.

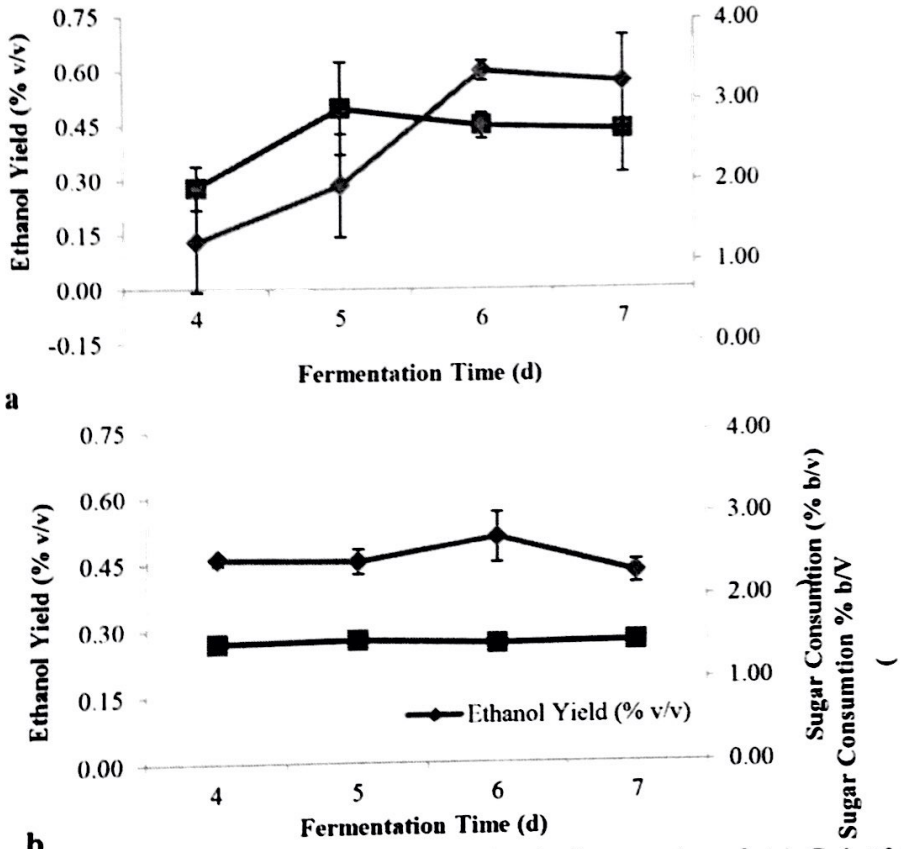

Figure 5 Ethanol yield and sugar consumption in fermentation of: (a) *G. latifolium*, and (b) *G. verrucosa*.

The highest ethanol yield from *G. latifolium* was obtained on the fifth day, with a value of 0.50±0.13% (v/v) and a sugar consumption of 1.95±0.64% (b/v) (Figure 5). The ethanol yield obtained on day five was significantly different from day four, but not so compared to day six and seven day. On the sixth day, fermentation sugar consumption by *S. cerevisiae* increased up to 3.38±0.12% (b/v), unfortunately it didn't give any change in the amount of ethanol produced. This was because of the sugar being used by *S. cerevisiae* to produce a variety of enzymes for galactose fermentation. Sugar consumption in the early stages of the

fermentation process was relatively small and gave a high amount of ethanol. Probably this happened because in the early stages the amount of glucose as the main substrate of *S. cerevisiae* was relatively high. *S. cerevisiae* would first consume the glucose, but when this ran out *S. cerevisiae* would start consuming other monosaccharides, such as galactose and xylose [26]. The glucose in *Sargassum sagamianum* was thoroughly consumed within 24 hours, while it took 96 hours for *S. sagamianum* to consume the xylose [26].

The highest fermentation product on *G. verrucosa* was obtained on day six with a value of 0.51±0.06% (v/v), with sugar consumption at 0.43±0.01% (b/v). The ethanol yield produced on day six was significantly different from day four, five and seven. Sugar consumption did not vary greatly since *S. cerevisiae* needs a relatively longer time to convert the sugar in the hydrolysate to ethanol.

The main content of monosaccharides in *G. latifolium* and *G. verrucosa* is galactose, which is a derivate from agar [24]. Galactose has a similar structure to glucose; the only difference is in the carbon stereochemical of its C4. *S. cerevisiae* is known to use galactose to produce ethanol [1]. *S. cerevisiae* will produce enzymes such as galactokinase, galactose-1-phosphate uridyltransferase and uridine diphosphoglucose-4-epimerase through the Leloir pathway [1,4]. After galactose is transformed into glucose-6-phosphate, it will be transformed into ethanol through the glycolitic pathway.

Glucose is the monosaccharide component that is mostly consumed during fermentation. Galactose and glucose in *G. latifolium* consumed by *S. cerevisiae* in five days of fermentation were 5.34% and 87.36%, respectively. After six days the glucose was thoroughly consumed by *S. cerevisiae* (Figure 6), while 97.92% galactose was consumed by S. *cerevisae.*

Glucose and galactose within *G. latifolium* and *G. Verrucosa* hydrolysate are sugars that can be converted to bioethanol. Glucose is a type of monosaccharide that is commonly consumed by *S. cerevisiae.* The glucose is not consumed by *S. cerevisiae* at the same time as the galactose. The galactose will be consumed when the glucose within hydrolisate has run out [3]. This explains how the glucose in *G. latifolium* and *G. verrucosa* was thoroughly or mostly consumed during fermentation in contrast to the galactose (Figure 6). The same phenomenon was also observed in [26], where after 24 hours of fermentation, the glucose was thoroughly consumed while the xylose was left untouched. The xylose was thoroughly consumed by the yeast after 96 hours of fermentation and the optimum period for *Sargassums agamianum* fermentation was 48 hours.

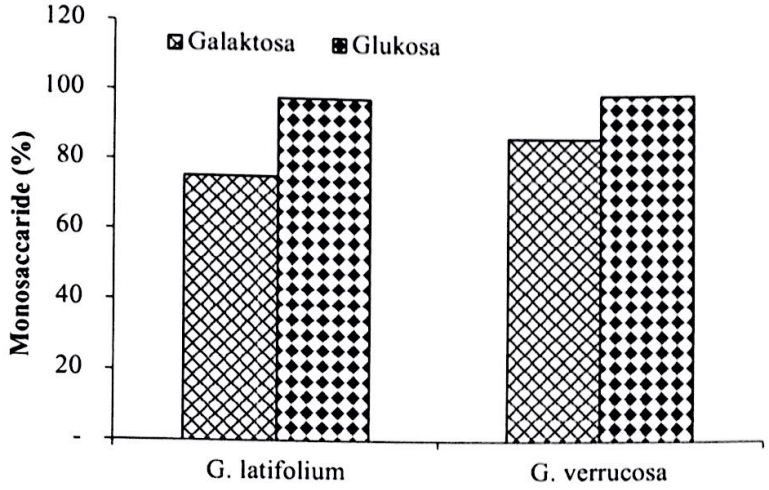

Figure 6 Monosaccharide (%) consumed by *S. cerevisiae*.

4 Conclusion

The optimum H_2SO_4 concentrations for hydrolysis of G. *latifolium* and *G. verrucosa* are 1% and 2%, respectively. The hydrolysis process produces monosaccharides in the form of galactose and glucose. The adaptation process on *G. latifolium* and *G. verrucosa* hydrolysate showed that *S. cerevisiae* can survive in the existing hydrolysate condition. The third adaptation showed an increase in yeast cell density. The highest ethanol content from *G. latifolium* and *G. verrucosa* was obtained at the fifth and sixth day of fermentation, i.e. 0.50% (v/v) and 0.51% (v/v), respectively. The glucose in *G. latifolium* and *G. verrucosa* (97.92% and 100%) was consumed better compared to the galactose (75.34% and 87.36%).

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