Hydrolysis of Microalgae *Spirulina platensis, Chlorella sp.*, and Macroalgae *Ulva lactuca* for Bioethanol Production

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Abstract – *Algal bioethanol is a renewable alternative fuel for gasoline that resulted in no disruption in food sources. This study investigated the effect of acid hydrolysis using H_2SO_4 on microalgae (Chlorella sp. and Spirulina platensis) and a macroalga (Ulva lactuca) with varying acid concentrations, temperatures, and hydrolysis time. The acid hydrolysis was carried out followed by separated hydrolysis and fermentation method (SHF). The hydrolysis process was used to break down the cell walls of algae and to convert complex carbohydrates from the cell wall into simple sugars. The Chlorella sp., Spirulina platensis, and Ulva lactuca were hydrolyzed with H_2SO_4 concentration of 0.5–2 N. The results showed that the highest total sugar concentration of Ulva lactuca biomass was 12.85% (v/v) when using hydrolysis of 2 N H_2SO_4. However, for Spirulina platensis and Chlorella sp resulted only 4% (v/v) and 10% (v/v), respectively. The results are in agreement with proximate carbohydrate analysis that showed the highest carbohydrate of 74.82% on Ulva lactuca was obtained as compare to that on Spirulina platensis and Chlorella sp. of 53.85% and 55.39%, respectively. Thus, Ulva lactuca was further investigated to determine the effects of hydrolysis time from 60 to 120 min at different temperatures of 40 –100°C. The maximum total sugar concentration (23.04%; v/v) was obtained using 2 N H_2SO_4 at 100°C for 60 min. The fermentation time on bioethanol production was also investigated for Ulva lactuca hydrolysate (2 N H_2SO_4 at 80°C for 60 min) at a different time of 24–72 h. The highest bioethanol concentration (1.45%; v/v) was obtained at a fermentation time of 72 h. This study indicated that acid hydrolysis is useful for rupturing the cell walls of Chlorella sp., Spirulina platensis, and Ulva lactuca for fermentative bioethanol production.*

Keywords – *acid hydrolysis, bioethanol, Chlorella sp., Spirulina platensis, Ulva lactuca.*

1. INTRODUCTION

In the industrial era, energy sources, such as coal, natural gas, and petroleum oil, have become vital components [1]. In 2018, primary energy consumption in the world rapidly grew by 2.9%, which was almost doubled that obtained in the previous 10 years, with an average of 1.5%. A significant increase in energy consumption is driven by an increase in a population dominated by average fossil fuel demand above 1.4 million (b/d) [2]. Increased energy demand must be balanced by availability. However, the availability of energy sources especially those from fossils has been estimated to decrease every year and will be exhausted within the next 70–150 years [3]. Researchers continually offer solutions to overcome the problem of energy scarcity. One of these solutions is the use of renewable fuels commonly called biofuels [4]. Several types of biofuels, such as biodiesel and bioethanol, are potential materials to replace fossil fuels. Bioethanol is an additive or gasoline substitute derived from vegetable materials [5]. The advantages of bioethanol include raw materials that originate from renewable biomass. Although bioethanol features a high excess octane value of 108, its initial ignition antiknock prohibits the knock-on engines [6].

Based on the raw materials used, bioethanols are grouped into several generations. The first generation of bioethanol comes from cereal grains, such as corns, canes, or soybeans, whereas the second generation includes lignocelluloses, such as bagasse or potato peel [7]-[9]. The first-generation raw materials are considered less effective because they will conflict with food sources; the second-generation bioethanol is developed from non-food raw materials, such as forestry or agricultural waste [10]. Biofuel based on the first and second generation is unsustainable due to the competition in agricultural land used for food crops [11]. The third-generation bioethanol is developed and derived from algae, which are interesting raw materials. The advantages of third-generation bioethanol include a rapid growth rate, the capability to fix CO_2, growth on unproductive land, and non-competition with food crops [12].

Based on morphology and size, algae are grouped into microalgae and macroalgae. Microalgae are single-celled, whereas macroalgae are multicellular [13]. In general, microalgae called phytoplankton float above the water surface. Several types of microalgae can be used as raw materials for bioethanol production because of their high carbohydrate content (20–40%) [14]. Algal

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species, such as *Chlorella vulgaris* [15], *Scenedesmus dimorphus* [16], and *Chloroccum sp.* [17] have been used for bioethanol production. In addition, the other microalgal types, including *Chlorella* [18] and *Arthrospira (Spirulina) platensis* [19] possess a carbohydrate content of more than 40% of their dry weight (DW). Carbohydrates in microalgae occur mostly in the form of cellulose and starch, where starches are bound in rigid cell walls composed of cellulose, xylan, mannan, and glycated sulfate [20]. The cell wall in microalgae comprises an outer layer and an inner layer. The outer layer is composed of pectin, agar, or alginate. The inner layer consists of hemicellulose, cellulose, and glycoprotein [13].

Macroalgae also grow at a fast rate and produce huge biomass [21]. Macroalgae contain several types of carbohydrates, such as, cellulose, laminar, carrageenan, mannan, mannitol, alginate, and ulvan, which are absent in lignocellulosic biomass. Macroalgae are classified into three groups based on pigments: *Rhodophyta* (red algae), *Phaeophyta* (brown algae), and *Chlorophyta* (green algae) [13]. The common macroalgae are known as seaweeds and are normally seen attached to rocks. Besides, several species of macroalgae, such as *Ulva lactuca, Undaria pinnaitifida, Sargassum ilicifolium, Eucheuma cottonii, or Gelidium amansii*, feature potential as bioethanol raw material given their dry-matter carbohydrate content (26–66%) [22].

Carbohydrates consist of 70% (dry weight) part of the *Chlorococcum* species distorted in the chloroplast. Therefore, it requires an excessive temperature and pressure to help the microalgae cells break during the supercritical fluid extraction from fat. This process results in the release of polysaccharides beginning from cell walls [16]. Rabelo *et al*. [23] reported that hydrolysis is commonly used because it can release carbohydrates from the cell wall and convert them into fermentable sugars for bioethanol production. Hydrolysis includes several kinds, such as acid hydrolysis, alkaline hydrolysis, or enzymatic hydrolysis. The enzymatic hydrolysis is commonly used for bioethanol production from algae [24]. A previous study reported that maximum glucose release reached 68.2% (w/w) after hydrolysis of *Chloroccum humicola* with 20 mg cellulase at 40ºC for 72 h. Enzymatic hydrolysis shows the potential for applications in microalgal biomass. However, the enzymatic hydrolysis presents several disadvantages, such as long processing time and high cost, indicating the need for more treatments for the production of an optimal product [25].

For these reasons, this study performed acid hydrolysis with H_2SO_4. The advantages of this method include low cost, non-requirement of the acid recovery process, and zero acid loss during the hydrolysis process [26]. This work investigated the effect of acid concentration on hydrolysis acid using raw materials, such as *Spirulina platensis, Chlorella sp.,* and *Ulva lactuca*. Then, *Ulva lactuca* was used to investigate the effect of hydrolysis time and temperature on the bioalgal content. The highest total sugar concentration under the hydrolysis temperature and time of *Ulva lactuca* was used for bioethanol production using fermentation followed by separate hydrolysis and fermentation (SHF) method.

2. METHODOLOGY

2.1 Raw Materials

The algal raw materials used for this study included *Chlorella* sp., *Spirulina platensis*, and *Ulva lactuca*. The dry-powder of algal biomass was obtained from Semarang and Jogjakarta, Indonesia. Pure commercial H_2SO_4 (p.a) (Merck 98 wt %) was used for the hydrolysis process. The other materials used in this research included yeast extract (Merck), maltose (Merck), peptone (Merck), and glucose (Merck). Bacteriological agar (Oxoid) was used to cultivate the yeast *Saccharomyces cerevisiae.*

2.2 Experimental Methods

2.2.1 Acid hydrolysis

Each alga (*Spirulina platensis*, *Chlorella* sp., and *Ulva lactuca*) at 10% (w/v) was mixed with different concentrations of H_2SO_4 (0.5; 1; 1.5; and 2 N) to reach a 100 ml solution. The suspension was hydrolyzed in a water bath at 80°C for 60 min with an agitation rate of 200 rpm. The highest total sugar content of the three types of microalgae and macroalgae with variations in H_2SO_4 concentration was chosen for further study on the effect of temperature and hydrolysis time. The algae with maximum total sugar were hydrolyzed at varying temperatures (40, 60, 80, and 100°C) and times (60, 80, 100, and 120 min). The hydrolysate was cooled to room temperature and was adjusted to pH 6.5–7 using $CaCO_3$ solution. Then, the hydrolysate was used for the fermentation process.

2.2.2 Fermentation

The pure culture of *Saccharomyces cerevisiae* was incubated on an agar solid medium containing: 3 g/L yeast extract, 3 g/L maltose, 5 g/L peptone, 15 g/L agar, and 10 g/L glucose. The medium was autoclaved at 121°C and 1 atm. Then, the yeast was incubated at 30°C for 24 h. The solid phase was used for preculture.

The preculture medium for *Saccharomyces cerevisiae* (100 ml) contained 10 g/L yeast, 26 g/L peptone, 20 g/L glucose, and distilled water and was sterilized in an autoclave at 121°C and 1 atm. The yeast was incubated at 200 rpm at 30°C for 24 h. The preculture was centrifuged for 2 min at 600 x g. The solid phase was used for the main culture.

The main culture of 10 g/L yeast was cultivated from preculture. A 500 ml medium consisted of 26 g/L peptone, 20 g/L glucose, and distilled water and autoclaved at 121°C and 1 atm. The medium was incubated at 200 rpm for 24 h at 30°C. The main culture was centrifuged for 2 min at 600 x g. The liquid phase was discarded, and the solid phase was repeatedly neutralized with 1% H_3PO_4 (v/v) until the residual sugars were removed.

The hydrolysate obtained using 2 N H_2SO_4 at 80°C for 60 min hydrolysis of *Ulva lactuca* 10% (w/v) was used for fermentation. The hydrolysate was added with 1

M citrate solution until pH 4.5. Then, the mixture was sterilized with autoclave at 121°C for 15 min at 1 atm.

For fermentation of *Ulva lactuca* hydrolysates, the main culture of *Saccharomyces cerevisiae* was added to 100 ml hydrolysate containing peptone (5 g/L) and yeast extract (3 g/L) to obtain a 10% concentration (w/v). The incubation process was conducted at 24, 48, and 72 h. Then, the bioethanol content was separated using distillation at 79°C. The distillate was used for the analysis of bioethanol content by Gas Chromatography (GC). The slurry from fermentation was used for the analysis of total sugar content and glucose concentration by High-Performance Liquid Chromatography (HPLC).

2.2.3 Analytical procedures

2.2.3.1 Microalgae composition

Proximate analysis was used to determine the water, ash, and carbohydrate contents following the method from the work of Lynch *et al.* [27]. Water content was measured by drying at 105°C until constant weight. The protein content was calculated using the Kjeldahl method with a factor of N = 6.25 [28]. Furthermore, the lipid content was deduced using the soxhlet method [27].

The lignin, cellulose, and hemicellulose contents were measured with the method of Chesson-Datta [29]. A mixture containing 1 g dried algal (*Spirulina platensis* or *Chlorella* sp. or *Ulva lactuca)* and 150 ml distilled water was heated at 100°C for 1 h. 1 g dried sample is written as the symbol (a). The residues were filtered and washed by hot water (300 ml). The residues were oven-dried until constant weight that a value of (b) was obtained. 150 ml H_2SO_4 (1 N) was mixed with dried residues and heated at 100°C for 1 h. Then, the mixture was filtered and washed by distilled water (300 ml), and the residues were dried until constant weight that was written as a symbol (c). The dried residues were mixed with 10 ml of 72% H_2SO_4 at room temperature for 4 h. Then, 150 ml H_2SO_4 (1 N) was added and refluxed at 100°C for 1 h. The residues were filtered and washed by distilled water (400 ml) until neutral and dried in an oven until constant weight that written as a symbol (d) The residue was heated until it became ash and weighed. The percentage of hemicellulose, cellulose, and lignin was calculated as follows:

$$\% \text{ hemicellulose} = (c-b)/a \times 100\% \quad (1)$$

$$\% \text{ cellulose} = (d-c)/a \times 100\% \quad (2)$$

$$\% \text{ lignin} = (e-d)/a \times 100\% \quad (3)$$

Where;

(a) = 1 g dried sample

(b) = The dried residue after reflux with hot water

(c) = The dried residue after reflux with sulfuric acid H_2SO_4 (1 N)

(d) = The dried residue after reflux with sulfuric acid (72% H_2SO_4)

2.2.3.2 Scanning Electron Microscopy (SEM)

The surface of the microstructure of *Chlorella sp., Spirulina platensis,* and *Ulva lactuca* before H_2SO_4 hydrolysis was analyzed with a scanning electron microscope (SEM; JEOL type JSM-6510LA, JEOL Ltd., Japan). Firstly, the samples were fractured in liquid nitrogen. Further, the sample was mounted on an aluminum disk with double surface tape, and the sample holder was placed and evacuated in a sputter-coater with gold. The images of sample surface morphology were obtained at a specific magnification at 500x, 1000x, and 5000x.

2.2.3.3 Determination of total sugar content

All samples obtained from hydrolysis with varying acid concentrations, temperatures, and the sample from the fermentation process are separated from the slurry to obtain a supernatant. The supernatant was centrifuged for 3 min at 5000 x g. Then, the liquid phase was neutralized with $CaCO_3$ solution until pH 6.5–7, after that. The solid phase was removed by centrifugation at 4000 x g for 15 min. The liquid phase was used to determine the total sugar content. Total sugar content was analyzed using the Somogyi modification method. Total sugar was measured by diluting 25 mL sample with distilled water until 100 ml volume. A total of 20 ml sample solution added 4 ml HCl was then heated to boil and neutralized with 4 N NaOH. The solution was neutralized with 45% NaOH and diluted to a volume of 100 mL. Testing was performed by taking a 1 ml sample by adding 10 ml Somogyi-Nelson reagents [30] and hydrolyzation for 30 min at a temperature of 95°C. Then, the sample was cooled to room temperature and titrated.

2.2.3.4 Determination of glucose content

The slurry from the fermentation process was centrifuged for 3 min at 5000 x g. Then, the liquid phase was neutralized with $CaCO_3$ solution until pH 6.5–7. The solid phase was removed by centrifugation at 4000 x g for 15 min. The liquid phase was filtered using a 0.2 µm membrane filter for analysis. The identification and quantification of glucose during the fermentation process were carried out by HPLC (Shimadzu Corporation, Japan) supplemented with Shimadzu LC (LC-20AD) and Shimadzu refractive index detector. The column used WAS RP-18 C18 Hibar (250 mm x 4.6 mm, 5 µm). The sample was filtered through a syringe filter and injected into HPLC at the column temperature of 50°C. A mixture of acetonitrile and water (85:15 v/v) was used as the mobile phase with a flow rate of 1 ml/min. The injection volume was 20 µL. Glucose concentrations were calculated using a calibration curve obtained from the standard solution of this compound.

2.2.3.5 Determination of ethanol content

The distillate was used to determine the bioethanol concentration by GC (model Agilent 6820, Agilent Technologies, United States). The flame ion detector and a polar capillary column HP-5 (P/N: 1909 LJ-413; length: 30 m, diameter: 320 µm) were used. The detector and oven temperatures were maintained at 250°C and 90°C, respectively. Helium gas was used as the carrier gas. A total of 1 µL sample was injected into the GC syringe for the calculation of bioethanol content.

3. RESULTS AND DISCUSSION

3.1. Characterization of Algae

3.1.1 Proximate analysis

Table 1 presents the proximate analysis of dried algae types *Spirulina platensis, Chlorella sp.,* and *Ulva lactuca*. Proximate analysis can provide information about the percentage of lipids, proteins, carbohydrates and ash and water contents of the algal biomass. Based on Table 1, *Spirulina platensis* contained 53.85%, 32.11%, 1.89% DW carbohydrate, proteins, and lipids, respectively. Previous studies showed that *Spirulina platensis* contained carbohydrates, proteins, and lipids with 8–14%, 46–63%, and 4–9% dry matter content, respectively [30]. These findings indicate that the carbohydrate content *of Spirulina platensis* in this study was higher than that of previous studies. The difference in carbohydrates, proteins, unsaturated fatty acids, and pigments results from the quantity and quality of nutrients and light during cultivation, which affects the formation of *Spirulina platensis* carbohydrates [31].

Table 1 shows the proximate analysis results of *Chlorella*, which contained 55.39% carbohydrates, 14.32% proteins, and 11.37% lipids. A previous study on *Chlorella vulgaris* reported yields of 12–17% carbohydrates, 51–58% proteins, and 14–22% lipids [30]. Thus, the *Chlorella sp.* from this study contained the highest carbohydrate content compared with *Chlorella vulgaris* and *Chlorella pyrenoidosa* from previous studies.

The *Ulva lactuca* from this study consisted of 74.82% carbohydrates, 14.74% proteins, and 2% lipids. According to Ortiz *et al.* [32], the carbohydrate, protein, and lipid contents of *Ulva lactuca* totaled 61.5%, 27.2%, and 0.3%, respectively. Thus, the carbohydrate and lipid content of *Ulva lactuca* in this study was higher than that of previous studies. Moreover, the protein content of *Ulva lactuca* from this study reached 14.74%. Thus, the protein content of this study is notably lower than that of a previous study [32].

The comparison of *Ulva lactuca, Spirulina platensis and Chlorella sp*. shows that the highest carbohydrate content of 74.82% was obtained from *Ulva lactuca*, agreeing with the result of previous research reporting that several types of *Ulva* species such as *Ulvaria oxysperma* contain rich carbohydrate content. The differences in carbohydrate content are due to the accumulation of carbohydrates, especially in cell walls, in response to environmental conditions, indicating the high photosynthetic process in the algal habitat.

Table 1. The proximate analysis of dried algae type *Spirulina platensis*, *Chlorella* sp., and *Ulva lactuca*.

No	Components	Component (%)					Reference
		Carbohydrates	Proteins	Lipids	Ash	Water	
1	*Spirulina platensis*	53.85	32.11	1.89	7.69	4.45	This study
2	*Spirulina platensis*	13.6	-	-	-	-	Um and Kim 2009
3	*Chlorella sp.*	55.39	14.32	11.37	7.89	11.0	This study
4	*Chlorella vulgaris*	12-17	51-58	14-22	-	-	Um and Kim 2009
5	*Chlorella pyrenoidosa*	26	57	2	-	-	Um and Kim 2009
6	*Ulva lactuca*	74.82	14.74	2	2.97	5.44	This study
7	*Ulva lactuca* (flour)	61.5	27.2	0.3	-	-	Oritiz *et al.* 2006
8	*Ulvaria oxysperma*	46-72	-	-	-	-	Harun *et al.* 2009

3.1.2 Hemicellulose, cellulose, and lignin content

Table 2 shows the hemicellulose, cellulose, and lignin contents of several algae, including *Spirulina platensis, Chlorella sp., and Ulva lactuca*. Carbohydrates such as starch accumulate in the algal plastids or become a cell wall, which contains cellulose, hemicellulose, glycoprotein, pectin, alginate, and agar [13]. As shown in Table 2, *Spirulina platensis* contained 37.82% cellulose, 47.47% hemicellulose, and 13.28% lignin. Also, *Chlorella sp.* comprised 30.3% cellulose, 67.37% hemicellulose, and 0.77% lignin. *Ulva lactuca* from this study contained 45.07% cellulose, 18.24% hemicellulose, and 7.02% lignin. In general, from this work, the highest cellulose content was achieved by *Ulva lactuca*, whereas the lowest cellulose content was obtained from *Chlorella sp. A* previous study reported that *Ulva lactuca* contains 9% cellulose, 20.6% hemicellulose, and 1.7% lignin [33]. Sui *et al.* [34] reported that *Chlorella* cells main contain 8.6% polysaccharides.

The comparison showed that the cellulose content of *Ulva lactuca* from this study is higher than that of previous research. However, the hemicellulose obtained from this study is lower than *Spirulina platensis* and *Chlorella sp*. The difference in cellulose content of each type of algae possibly occurred depending on the chemical composition of algae, which varies with species, habitat, maturity, and environmental condition [34]. Cellulose and hemicellulose are bound to rigid cell walls making it challenging to release starch as a source of carbon for the fermentation process and to convert cellulose and hemicellulose into simple sugars [35].

Table 2. Hemicellulose, cellulose, and lignin contents of algal materials.

Algae	Component			Reference
	Hemicellulose (%)	Cellulose (%)	Lignin (%)	
Spirulina platensis	47.47	37.82	13.28	This study
Chlorella sp.	67.37	30.30	0.77	This study
Ulva lactuca	18.24	45.07	7.02	This study
Ulva lactuca	20.60	9.13	1.56	Yaich *et al.* (2011)

From this work, the highest lignin content was achieved by *Spirulina platensis*, whereas the lowest lignin content was obtained from *Chlorella sp*. The lignin content of *Ulva lactuca* in this study was higher than that of previous research.

The lignin obtained in this study, as also observed in a previous work, was tightly bound to cellulose and hemicellulose. This component is a long chain or branch that forms in the cell wall [36]. The presence of lignin in macroalgae also was reported by Ramachandra and Hebbale [37] who observed that macroalgae contained high concentration of structural polysaccharides and low lignin contents, therefore requiring mild and low-cost processes for the extraction of sugars.

3.1.3 Morphology evaluation

Figure 1 shows the surface structure of (a) *Chlorella sp.,* (b) *Spirulina platensis*, and (c) *Ulva lactuca* raw materials using SEM analysis at magnification of 500–5000 x. *Spirulina platensis* and *Chlorella sp*. featured smooth, irregularly shaped, and stiff surface structure, whereas *Ulva lactuca* exhibited a denser and unhollowed surface than *Chlorella sp*. and *Spirulina platensis*. Similar to a previous study that analyzed the surface structure of sea algae (*Monostroma nitidum*) with SEM, the SEM images showed the framework of the green alga before hydrothermal pretreatment, allowing for observation [38].

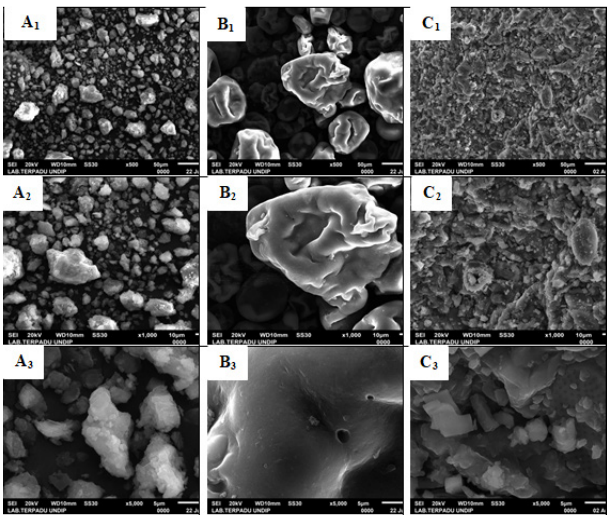

Fig. 1. SEM micrographs of (A) *Chlorella* sp., (B) *Spirulina platensis*, and (C) *Ulva lactuca* raw materials at 500 (A_1–C_1), 1000 (A_2–C_2), and 5000x (A_3–C_3) magnification.

3.2 Analysis of Algae

3.2.1 Effect of sulfuric acid concentration on acid hydrolysis

The hydrolysis process breaks the rigid algal cell wall by breaking the intermolecular connection between hemicellulose and other polymer components, thus enhance the accessibility of yeast in the fermentation process. High cellulose and hemicellulose contents are used to produce sugar monomers in the form of glucose [36]. Several factors, such as acid concentration, hydrolysis time, and temperature affect the acid hydrolysis of algal biomass into fermentable sugars.

Figure 2 shows the effect of H_2SO_4 concentration on the total sugar of *Spirulina platensis, Chlorella sp., and Ulva lactuca*. Water hydrolysis was used as a control and acid hydrolysis with different H_2SO_4 concentrations (0.5–2 N) at 80°C for 60 min were used for evaluation. The water hydrolysis of *Spirulina platensis, Chlorella sp., and Ulva lactuca* produced total sugars of 0.87–3.04% (v/v), whereas the acid hydrolysis yielded higher total sugar content of 1.97–13.11% (v/v). The highest total sugar content (10.44%; v/v) was obtained from *Spirulina platensis* biomass at 2 N H_2SO_4 at 80°C for 60 min. The acid hydrolysis of *Spirulina platensis* using 0.25–2.5 N HNO_3 was also investigated by a previous study [19]. The maximum reducing sugar yield (98%; v/v) was obtained at 2.5 N HNO_3 for 90 min at high acid concentrations. This result was due to the positive effect of increased acid concentration on the hydrolysis rates at the temperatures of 80–100°C [19]. Previously, acid pretreatment of microalgae that had different carbohydrates contents had been frequently studied. It showed that mostly carbohydrates contents was stored in the cell wall, therefore acid pretreatment were used for cell wall disruption. The high concentration of sulfuric acid was necessary to release the entrapped carbohydrates for use as a carbon source during the fermentation process [39].

Figure 2 shows the effect of H_2SO_4 concentration on the *Chlorella sp.* biomass. The highest total sugar content (4.37%; v/v) was obtained at 2 N H_2SO_4 for 90 min, whereas the lowest value of 1.54% (v/v) was observed when using 0.5 N H_2SO_4. The result was in agreement with previous studies of acid hydrolysis of *Chlorella sp*. that reported the increasing HCl concentration from 0.5 to 2% led to produce an increase of total sugar from 6.77 to 43.78%. The results show that the acid catalyst was required to convert many feedstocks in *Chlorella sp*. to fermentable sugars [40].

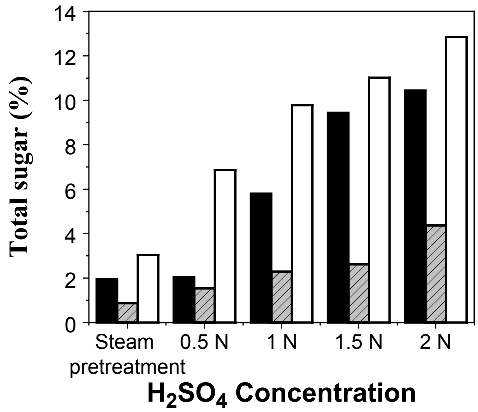

Fig. 2. Effect of H_2SO_4 concentration on the acid hydrolysis of *Spirulina platensis* (■), *Chlorella* Sp. (▨) and *Ulva lactuca* (□) (80°C, 60 min).

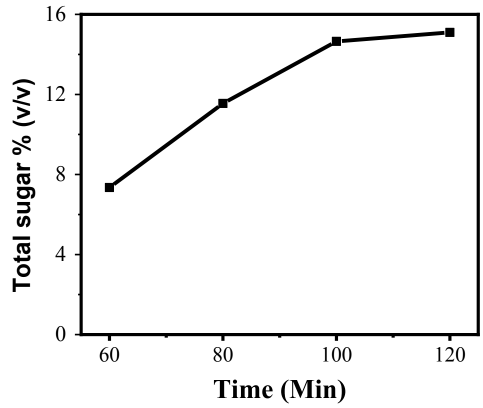

Fig. 3. Acid hydrolysis of *Ulva lactuca* at different times using 2 N H_2SO_4 at 80°C.

Figure 2 also shows the effect of H_2SO_4 concentration on the *Ulva lactuca* biomass. The highest total sugar concentration of *Ulva lactuca* (12.85%; v/v) was obtained at 2 N H_2SO_4 at 80°C for 60 min. A previous study investigated the acid hydrolysis of *Ulva lactuca* [41]. The highest reducing sugar concentration (0.07 g.g-1) of *Ulva lactuca* was obtained using 1 N H_2SO_4. Acid pretreatment by H_2SO_4 (2 N), at 120°C for 30 min was examined using the dried biomass of microalga *Scenedesmus obliquus*. The results revealed that the pretreatment with sulfuric acid was crucial for the conversion of complex carbohydrates and sugars into simple sugars [18]. Previous studied on the pretreatment using acids such as H_3PO_4 on the trees (*Samanea saman*) to produce bioethanol had also been carried out. Total sugar after pretreatment of 9.4% was obtained from the H_3PO_4 treatment. The results also showed that acid pretreatment could increase the total of sugar released [42]. However, the acid concentration is a major operational parameter that could affect the saccharification of microalgal biomass. When the concentration of acid was raised from 4 to 7% H_2SO_4 concentration, a small increase in reducing sugars was observed, but the content diminished when a 10% H_2SO_4 was applied. The decrease in reducing sugar might be attributed to the degradation of monosaccharides into sugar degradation products (such as furfural, hydroxymethylfurfural (HMF), propionic acid, acetic acid, formic acid, and lactic acid) [43]. Moreover, the use of high concentrations of acid (more than 3%) led to the corrosion of the experimental equipment. Pretreatment with dilute acid at low acid concentrations was carried out to avoid the use of high amounts of neutralizing agents in acid hydrolysis [44]. From Figure 2, the comparison between *Spirulina platensis,Chlorella sp.,* and *Ulva lactuca* shows that the total sugar of *Ulva lactuca* at 2N H_2SO_4 of 12.8% (v/v) was the highest than that of *Spirulina platensis* and *Chlorella sp*. after hydrolysis. Therefore, *Ulva lactuca* was further used as a raw material to investigate the effect of time and temperature on the hydrolysis process and fermentation.

3.2.2 Effect of hydrolysis time

Figure 3 shows the relationship between different hydrolysis times (60–120 min) and the total sugars obtained after acid hydrolysis of *Ulva lactuca* biomass. The hydrolysis process was conducted using 2 N H_2SO_4 at 80°C. The increased total sugar concentration between 7.35–15.10% (v/v) was obtained with increasing hydrolysis time. The highest total sugar concentration (15.10%; v/v) was reached at 120 min. The results obtained in this study are similar to those obtained by Nguyen *et al.* [44], who reported that prolonging the hydrolysis time of *Chlamydomonas reinhardtii* increased the release of sugar, until the saturation level was influenced by acid dosage and temperature conditions. Trivedi *et al.* [45] evaluated the effect of time hydrolysis of *Ulva fasciata Delile* as raw material by using cellulase. The reducing sugar yields of 72.73–168.15 $mg.g^{-1}$ were obtained at an increasing incubation time of 6–36 h. However, when hydrolysis time increased to 42 h, reducing sugar yields gradually decreased to 151 $mg.g^{-1}$. The decreased reducing sugar confirmed that the prolonged residence time for hydrolysis causes sugars to degrade to form inhibitor agents, such as HMF or furfural. Hydrolysis time can affect the total sugar, the longer the hydrolysis time can increase the total sugar value. However, this investigation only focuses on the effects of time and the best of time condition does not use to investigate the effect of temperature.

3.2.3 Effect of hydrolysis temperature

Figure 4 shows the effect of different temperatures (40–100°C) on the total sugars after acid hydrolysis of *Ulva lactuca* biomass. The hydrolysis was carried out using 2 N H_2SO_4 at 60 min. The results show that the highest total sugar concentration (23.04%; v/v) was obtained at a temperature of 100°C. This result corresponded with that of previous studies. Microalgal saccharification using dilute acid hydrolysis at different temperatures (23–90°C) was also investigated by Chng *et al.* [46]. Their results showed that the sugar yield significantly increased when the temperature was 80–90°C. By contrast, the lowest sugar yield was obtained at lower temperatures of 23–30°C and 45–55°C. Hydrothermal acid pretreatment of *Chlamydomonas reinhardtii* for ethanol production was studied previously [44]. The results showed that the maximum yields of glucose increased with the increase in acid concentration and temperature. The maximum release of glucose was 28.5 g/l, which corresponded to 58% (w/w) of dry cell weight, at 110°C after 30 min of residence time with 3% sulfuric acid. Temperature affects the sugar released during hydrolysis because it can extend residence time so and the more sugar is released.

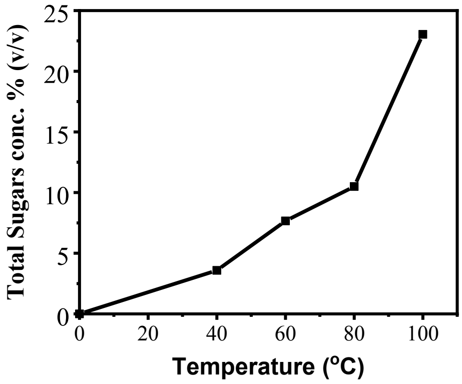

Fig. 4. Acid hydrolysis of *Ulva lactuca* using 2 N H_2SO_4 at different temperatures for 60 min.

3.3 SHF Process

Figure 5 shows the concentration of bioethanol and total residual sugars from the fermentation process of *Ulva lactuca* hydrolysate using 2 N H_2SO_4 at 80°C for 60 min. The condition was chosen according to the main conditions when searching for the highest total sugar value in the microalgae variation (*Spirulina platensis, Chlorella sp*., and *Ulva lactuca*). Figure 5 shows that the bioethanol concentration sharply increased, and the highest bioethanol concentration of 1.45% (v/v) was obtained at 72 h. By contrast, the total sugar concentration decreased until all sugars have been consumed within 48 h of fermentation. A similar finding on the fermentation process of carbohydrate-rich *Scenedesmus dimorphus* was also reported by Chng *et al.* [46]. Glucose was consumed at a fast rate, but the amount of bioethanol produced was comparatively low. *Saccharomyces cerevisiae* metabolism that converts sugar to ethanol occurred slower due to the use of an acetate buffer in enzyme hydrolysis, which affects the acidity of fermentation. The acid hydrolyzed by microalga *Chlorella vulgaris* FSP-E was investigated for ethanol production via the SHF process using optimal acidic hydrolysis conditions (1% sulfuric acid, 121°C, and 20 min hydrolysis time) by Ho *et al* [15]. The results revealed that the maximum ethanol concentration of 11.66 g/L was obtained within 12 h. The feasibility of dilute acid hydrolysis is recommended due to its lower cost and cost-effectiveness compared with enzymatic hydrolysis, leading to a fivefold shorter SHF operation time (from 60 h to 12 h). The acid hydrolysis of carbohydrate in iles-iles starch as raw material for bioethanol was reported by Kusmiyati *et al.* [47]. The results of the study produced higher ethanol (8.63%) at similar time fermentation (72 hours) than that from *Ulva lactuca.*

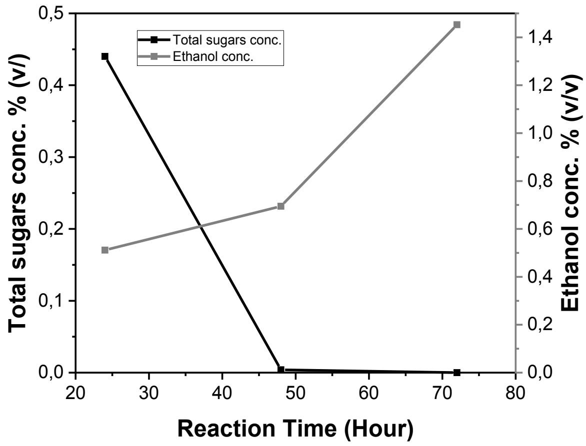

Fig. 5. Fermentation of *Ulva lactuca* biomass hydrolysate for ethanol production by the SHF process.

Table 3 shows the effect of retention time on the fermentation of *Ulva lactuca* for 24–72 h time on residual glucose. The lowest residual glucose concentration of fermentation (329.04 mg/L) was obtained at the retention time of 72 h. When the retention time of fermentation was increased from 24−72 h, the residual glucose concentration decreased significantly from 1648.38 to 329.04 mg/L. Ho *et al.*[15] reported that the residual glucose concentration from simultaneous saccharification and fermentation of *Chlorella vulgaris* FSP-E decreased from 0.5 g/L to nearly 0 g/L when the fermentation time was increased from 12 to 36 h. Furthermore, the residual glucose concentration from the SHF of *Chlorella vulgaris* FSP-E has slowly decreased from 23.6−0 g/L when the fermentation time was increased from 6 h to 24 h. The decreased residual glucose concentration with increased time was also confirmed by Chng *et al.* [46].

Table 3. Concentration of glucose during fermentation.

Time fermentation (h)	Glucose (mg/L)
24	1648.38
48	1414.02
72	329.04

4. CONCLUSION

The acid hydrolysis of microalgae *Spirulina platensis* and *Chlorella sp*. and macroalga *Ulva lactuca* has been studied following the SHF methods for bioethanol production. Low acid concentrations for the hydrolysis process show potential for overcoming obstacles, which include the carbohydrate starch granules that are bound within the rigid cell walls of algae. Results showed the highest total sugar concentration of acid hydrolysis (12.85%; v/v) when hydrolysis was performed on *Ulva lactuca* biomass using 2 N H_2SO_4 at 80°C for 60 min. Given this result, *Ulva lactuca* was used to investigate the effect of time and temperature on acid hydrolysis. The maximum total sugars of 23.04% (v/v) from *Ulva* *lactuca* was obtained at hydrolysis conditions of 2 N H_2SO_4 at 80°C for 60 min. Then, the fermentation process using Saccharomyces cerevisiae for *Ulva lactuca* hydrolysate resulted in the highest bioethanol concentration of 1.45% (v/v) at 72 h fermentation. The fermentation time showed a significant effect on the production of bioethanol from *Ulva lactuca*. A prolonged fermentation process gives the yeast chance to grow and convert monomer sugars into bioethanol. However, fermentation with a long duration will result in a toxic effect on yeast growth given the high ethanol accumulation.

ACKNOWLEDGEMENT

The authors gratefully thank for research grant received from the Ministry of the Higher Education, Research and Technology of Indonesia (KemenristekDikti) based on contract number; 7/E/KPT/2019; 228/SP2H/LT/DRPM/2019; 026/L6/AK/SPH.1; 060/A38.04/UDN-09/V/2019.

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