Effect of hydrolysis time and acid concentration on bioethanol production of microalga Scenedesmus sp.

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Abstract. Scenedesmus sp. has tremendous potential to produce bioethanol due to its high content of carbohydrate, approximately 10-52%. This study aimed to investigate the effect of hydrolysis time and acid concentration on ethanol production fermented by Saccharomyces cerevisiae. The analysis included in this research were cell density calculation, pH level, carbohydrate content, reducing sugar content, and ethanol content tested by Gas Chromatography. Biomass of Scenedesmus sp. was hydrolyzed using 1%, 2%, and 3% sulfuric acid for 75 minutes and 105 minutes. The hydrolysate was adjusted its pH to 4-5 using NaOH 50% then it was added with nutrients. The fermentation process was carried out for 5 days and samples were collected every day. The results showed that the highest ethanol content was 1.1% and achieved on the 1st day of fermentation with 75 minutes of hydrolysis process using 3% sulfuric acid. It was followed by a 105 minutes hydrolysis process using 3% sulfuric acid which yielded a value of 1.095% on the 2nd day of fermentation. Optimization of the hydrolysis and fermentation process are needed for a further study to obtain high ethanol content.

1. Introduction

The current environmental problems, such as pollution, global warming, and even climate change, are caused by the emission of CO₂ originating from fossil fuels. This leads scientists to intensify research for the development of renewable, sustainable, and clean fuel [1]. One of the major and clean biofuels that is a good alternative as a transportation fuel is bioethanol. This biofuel has many advantages over fossil fuels, such as high octane number that prevents knocking of cylinders in engines; produces less greenhouse-effect gasses due to the higher oxygen contents; can be used directly in the current automotive industry without any modifications; and can be mixed with oil [2][3][4]. Bioethanol mainly produced from sugars of corn and sugarcane. Recently, however, microalgal carbohydrates have gained considerable interest as potential raw materials for bioethanol production.[5][6][7].

Microalgae being the most suitable candidates for bioethanol production due to their favorable characteristics, such as fast growth rate; high CO₂ fixation ability; and no need of arable land for cultivation [8][9][10][11][12]. Microalgae contain various types of carbohydrates, such as glycogen, starch, agar, and cellulose which can be easily converted to fermentable sugars for bioethanol production [13]. Scenedesmus is one of the most appropriate candidates for carbohydrate-based microalgae feedstock in bioethanol production because of its carbohydrate content, approximately 10-52% [14]. A study reported that Scenedesmus dimorphus accumulated 53.7% carbohydrate contents
and produced 80% fermentable sugars when it was hydrolyzed with sulfuric acids, indicating its feasibility for bioethanol production [15].

Carbohydrates are stored in microalgal cells and the majority are present in polysaccharide form, either as structural components or storage materials. This polysaccharide needs to be converted into monomeric sugars, such as glucose, fructose, galactose so that later can be directly converted to bioethanol via fermentation [16]. Extraction of the stored carbohydrates from microalgal cells needs pre-treatment step for lysing the cells. This is a crucial step in bioethanol production. This step can be carried out through different methods, such as enzymatic, acidic or solvent extraction [17].

Acid hydrolysis is considered as a good method which is more effective than physical and thermal methods. However, it requires high temperature treatment to degrade algal biomass and convert the polymeric sugars (e.g., starch and cellulose) to monomeric units. Strong acids, such as hydrochloric acid, sulfuric acid, and nitric acid, are commonly used. Several factors, such as acid strength, duration of treatment, and temperature considerably affect the yield of acid hydrolysis [18][19].

In this study, the potential use of *Scenedesmus* sp. as a feedstock to produce bioethanol was evaluated. The effect of hydrolysis time and acid concentration on microalgae acid pretreatment for bioethanol production were also investigated.

2. Materials and Methods

2.1. Cultivation and growth conditions of microalgae *Scenedesmus* sp.

Microalgae were cultivated in a technical medium consisting of (g/L) trisodium phosphate (0.3), ammonium sulfate (0.8), green Hyponex (1.0), and urea (1.0) under 3000 lux light intensity and continuous aeration. The culture growth was evaluated based on its cell density using Spectrophotometer UV-VIS (Hitachi U-3900H) at 680 nm. Microalgae were cultured for 12 days and harvested at late exponential phase (early stationary phase) by centrifugation (Hitachi CT6EL, Japan) at 6000 rpm for 5 minutes. Biomass was oven dried at 50 °C.

2.2. Acid hydrolysis

25 g of dry biomass was hydrolyzed using 250 ml sulfuric acid (H$_2$SO$_4$) in various concentration (1%, 2%, and 3%) then heated at 100 °C for 75 and 105 minutes. The hydrolyzed biomass was cooled at room temperature and subsequently adjusted to pH 4-5 using 50% NaOH. Hydrolysate was enriched with nutrients consisting of (g/L) NH$_4$SO$_4$ (2.0), K$_2$HPO$_4$ (1.0), KH$_2$PO$_4$ (1.0), ZnSO$_4$ (0.2), MgSO$_4$ (0.2), and yeast extract (2.0) and then homogenized [20]. The hydrolysate was sterilized at 121°C for 15 minutes and subsequently cooled at room temperature.

2.3. Carbohydrate analysis

Carbohydrate was measured by the sulfuric-phenol method [21]. The analysis was evaluated by comparing the carbohydrate content of the sample with the standard solution. 5 mg of dry biomass was added with aquadest until volume 1 ml, then added with 0.5 ml of 5% phenol and 2 ml of concentrated H$_2$SO$_4$. The solution was shaken for 1 minute until homogeneous and incubated for 30 minutes in dark condition. The absorbance was evaluated using Spectrophotometer UV-VIS at 490 nm. The making of glucose standard solution was carried out using the same procedure as samples. The standard solution was made in various concentration (0, 20, 40, 60, 80, and 100 ppm). Aquadest was used as a blank solution.

2.4. Determination of reducing sugar

Reducing sugar was analyzed according to the 3,5-dinitrosalicylic acid (DNS) method [22]. DNS reagent was made by dissolved 5 g of DNS and 8 g of NaOH in 200 ml aquadest (solution A). 15 g of sodium potassium tartrate and 4 g of sodium metabisulfite were dissolved in 200 ml aquadest (solution B). Solution A and B were mixed and added with aquadest until 500 ml. The mixture
solution was homogenized and placed on the dark bottle. The solution was incubated for 24 hours before using.

Reducing sugar was evaluated by comparing the reducing sugar of the sample with the standard solution. 5 mg of dry biomass was added with aquadest to get volume 1 ml, then added with 3 ml of DNS reagent. The solution was heated in a water bath at 100 °C for 5 minutes then it was shaken and cooled at room temperature. The absorbance was evaluated using Spectrophotometer UV-VIS at 540 nm. DNS reagent was used as a blank solution. The making of glucose standard solution was carried out using the same procedure as samples. The standard solution was made in various concentration (0, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 ppm). Aquadest was used as a blank solution.

2.5. Preparation of yeast S. cerevisiae

*S. cerevisiae* was cultured at PDA (Potato Dextrose Agar) medium. The culture was incubated at 30°C for 24 hours. A pure culture of *S. cerevisiae* was added to the liquid medium (0.7 g peptone and 0.36 g yeast extract in 120 ml aquadest) with the ratio 1:5. The culture starter was shaken for 48 hours at room temperature.

2.6. Fermentation

40 ml of sterile hydrolysate was added with 10% *S. cerevisiae* culture. The hydrolysate was shaken and fermented at room temperature for 5 days. The sample was collected every day during the fermentation process. The analysis was conducted included cell density calculation, pH level, carbohydrate content, reducing sugar content, and ethanol content.

2.7. Determination of ethanol content

The ethanol content was evaluated by Gas Chromatography (Shimadzu-17A, Japan) which equipped with Trwax column and FID (flame ionization detector). Hydrogen was used as a carrier gas (0.5 kgf/cm²) and nitrogen was used as a mobile phase (1 kgf/cm²). The temperatures of the column, injector, and detector were 160 °C, 160 °C, and 225 °C, respectively. 1 µl of sample was injected into the device.

2.8. Data analysis

The carbohydrate, reducing sugar, and ethanol contents were analyzed according to the linear regression equation:

\[ y = a + bx \]  
\[ (y: \text{sample absorbance}, a: \text{intercept}, b: \text{slope}, x: \text{concentration in ppm}) \]

The total number of cells was calculated using manual counter and the cell density was counted according to the equation:

\[ \Sigma \text{cell} = \frac{N}{9} \times 4 \times 10^6 x n \]  
\[ (\Sigma \text{cell}: \text{the total number of cells}, N: \text{number of cells in 9 square}, n: \text{dilution factor}). \]

3. Results and Discussion

3.1. The Growth of microalgae Scenedesmus sp.

The result showed that microalgae didn’t experience the lag phase (Figure1). Started from day 0 until day 10, microalgae experienced exponential phase that was indicated by the increase of growth curve significantly. This exponential growth occurred as a result of vast nutrients availability and spaces for
the cell to grow [15]. Microalgae produced a high level of carbohydrate in this phase. Therefore, the exponential phase is the right time for harvesting [23]. After the exponential phase, microalgae entered stationary phase, a condition where cell density reaches its saturated point due to most of the nutrients have been utilized and the available spaces for growth are limited. Competition of nutrients, light, and spaces for growth eventually caused the cell to enter the death phase that indicated by the decrease of the growth [15].

![Figure 1. The growth curve of microalgae Scenedesmus sp.](image)

3.2. Carbohydrate content

The result from the linear regression of standard solutions showed an equation of $y = 0.0101x + 0.0623$ with $R$ value $= 0.9830$ (Figure 2). Carbohydrate content of sample was then calculated by entering the sample absorbance value into the regression equation. This experiment was conducted in 3 replications and resulted in an average carbohydrate content of 32.06%. According to Becker [14], the carbohydrate content of *Scenedesmus* sp. is approximately 10-52%. Several strategies can be conducted in order to increase the accumulation of carbohydrate content. Controlling environmental factors, such as nutrients, light, and temperature can affect microalgae growth and biomass composition. Limitation of nutrients (such as nitrogen, sulfur, and phosphate) will force microalgae to transform protein or peptides into carbohydrates [24][25], particularly nitrogen starvation will lead microalgae to accumulate large amounts of carbohydrates and fats [26].

3.3. Reducing sugar

High level of reducing sugar will impact on high production number of molecule 3-Amino-5-nitrosalicylic acid. Therefore, the absorbance of the sample will increase as well [22]. Based on the absorbance measurement, the equation obtained from linear regression is $y = 0.0037x - 0.0879$ with $R$ value $= 0.9870$ (Figure 3).

![Figure 2. The curve of glucose standard.](image)  ![Figure 3. The curve of reducing sugar standard.](image)
Reducing sugar of sample was calculated by entering the sample absorbance value into the regression equation. This experiment was conducted in 3 replications and resulted in an average reducing sugar of 23.91%.

3.4. Acid hydrolysis
Sulfuric acid was used in this process because sulfate is a nutrient present in the yeast fermentation mediums. In addition, sulfate has a lower toxicity to yeasts when compared to chloride, and it is easily removed by overliming with calcium hydroxide [27]. The result of biomass hydrolysis shown in Figure 4.

![Figure 4. Hydrolysate of Scenedesmus sp. biomass (A: 1% H₂SO₄, B: 2% H₂SO₄, C: 3% H₂SO₄).](image)

Based on the result, different colors of hydrolysates were observed. The higher concentration of sulfuric acid, the darker the color of hydrolysate. Acid concentration of 3% gave the darkest color of hydrolysate than the other concentrations. Sulfuric acid is a dehydration agent that can absorb water content in carbohydrate-containing materials. When carbohydrates are dehydrated, then the remaining element is carbon and the color of hydrolysate will turn into black [28].

3.5. Fermentation
Fermentation is the process of converting substrates into simpler, more specific products with the help of certain microbes. This study used *S. cerevisiae*, a yeast that has a good ability to convert sugar into ethanol [14]. *S. cerevisiae* has a good ethanol tolerance (up to 20%, v/v) and capable to be growing rapidly in anaerobic conditions [29][30]. In addition, *S. cerevisiae* can thrive in a relatively high salt concentration after its acclimatization by pre-cultivation under salt-stressed conditions [20]. *S. cerevisiae* has invertase and zymase enzymes. The invertase enzyme serves to break sucrose into monosaccharides (glucose and fructose) while zymase enzyme will convert glucose into ethanol [27]. The number of cells during fermentation shown in Figure 5.

![Figure 5. The number of *S. cerevisiae* cells.](image)
The number of cells increased significantly from day 0 until day 2 (Figure 5). This is due to the fast consumed of glucose in the early stage of fermentation [31]. At day 3 until 5, the number of cells decreased, as a result of decreased amounts of reducing sugars and other nutrients that were the source of growth for \( S. \text{cerevisiae} \). In addition, there are several factors that affect fermentation, such as temperature, pH, oxygen, and substrate concentration [32].

3.6. Carbohydrate content during fermentation process
This study aimed to determine how much is the decrease in carbohydrate content that occurs during the fermentation process. The result of carbohydrate content shown in Figure 6.

![Figure 6](image)

**Figure 6.** The carbohydrate content during the fermentation process.

Based on the result, the carbohydrate content decreased during the fermentation process. The highest carbohydrate contents were 1115 ppm and 1063 ppm and achieved on 75 minutes and 105 minutes of hydrolysis time, respectively. Both treatments were carried out using 3% of sulfuric acid. An earlier study reported that acid concentration of 4% (v/v) and temperature at 125 °C for 15 min successfully produced 48% (w/w) of glucose [15]. This data indicated that the temperature positively correlated with the acid concentration in order to maximize the glucose content.

3.7. Reducing sugars during fermentation process
Measurements of reducing sugar were carried out to determine how much is the decrease in glucose content utilized by \( S. \text{cerevisiae} \) during the fermentation process. The result of reducing sugar content shown in Figure 7.

![Figure 7](image)

**Figure 7.** The content of reducing sugars during the fermentation process.
The highest contents of reducing sugar were 1245 ppm and 1324 ppm and achieved on 75 minutes and 105 minutes of hydrolysis time, respectively. Both treatments were carried out using 3% of sulfuric acid. Acid concentration affects the content of reducing sugar. The higher the concentration of acid, the higher reducing sugar [27]. During the fermentation process, the reducing sugar was decreased significantly from day 0 until day 1. This was due to the fast consumed of the reducing sugar by yeast at an early stage of the fermentation process [21]. A study by Harun and Danquah [24] reported that sugar profile displayed a declining trend with a slower consumption rate after 24 h, in line with the growth of yeast. During the fermentation process, simple sugars are consumed by yeast especially in the exponential phase and this resulted in the extracellular bioethanol production. On day 2 until day 5, the decrease in reducing sugar was not significant. This was influenced by several factors, such as temperature, pH, and sugar concentration. Therefore, S. cerevisiae couldn’t utilize the reducing sugar optimally [32].

3.8. pH level during fermentation process

The result showed that pH level decreased from initial pH (4-5) at 1st day of fermentation (Figure 8). The decreased pH level may be caused by the accumulation of side product, such as pyruvate acid, lactic acid, and acetic acid. During the fermentation process, the pH level was unstable. At some concentrations, an increase in pH level was observed. This was due to the deamination process of yeast extract that caused the increase of the medium pH. The changes in pH level were influenced by the ratio between acidic organic compounds with alkaline ammonia [33].

![Figure 8. pH level during the fermentation process.](image)

3.9. Analysis of ethanol content using gas chromatography

The result showed that the sample had ethanol content, this was indicated by the same peak between sample and standard at 1.675 min (data not shown). The measurement of ethanol content using Gas Chromatography was directly proportional to the increased growth of S. cerevisiae and the significantly decreased of carbohydrates level and reducing sugars during the first and second day of the fermentation process. The highest of ethanol content was 1.1 % and achieved on the 1st day of fermentation with 75 minutes hydrolysis process using 3% sulfuric acid. It was followed by the treatment with 105 minutes of hydrolysis process using 3% sulfuric acid which yielded a value of 1.095% on the 2nd day of fermentation (Figure 9). The higher the acid concentration used, the higher the ethanol content produced. This is due to the high acid concentration increase the reducing sugar [27]. The longer the fermentation process is conducted, the more the ethanol content will be decreased. This is due to further conversion of ethanol into other compounds, such as acetic acid and lactic acid.

The ethanol content in this study was very low when compared to the earlier study by Hamouda et al. [34]. Their study was conducted using microalgae Scenedesmus obliquus that hydrolyzed at 120 °C with 5% sulfuric acid for 20 min and resulted in the ethanol content of 20.33%. With the high content of initial carbohydrate and reducing sugar (32.06% and 23.91%, respectively), supposedly the ethanol
content produced was also high. This may indicate that the fermentation process was not running optimally. Various factors can affect the fermentation process, i.e., the formation of side products (compounds) during the hydrolysis process; thus, interfering with the fermentation process. These side compounds are such as furfural, 5-hydroxymethylfurfural (HMF), levulinic acid, acetic acid, formic acid, uronic acid, 4-hydroxybenzoic acid, vanillic acid, vaniline, phenol, cinnamaldehyde, and formaldehyde [35]. Excessive strong acids reduce sugars to furfural, thereby decreasing the final yield of monomeric sugars. In addition, furfural and its derivatives are toxic for the fermenting microorganisms, diminishing or even completely inhibiting fermentation [18][19].

Figure 9. Ethanol content from the fermentation process.

Scenedesmus has been described as microalgae with one of the most resistant cell walls. Its cell wall is composed of multilayers where the interior is cellulose covered by a hemicelluloses matrix. The exterior layer contains sporopollenin, a polyterpene which is also present in pollen grains and confers a high rigidity and resistance to chemical attack [36][37][38]. This study only used acid hydrolysis for lysing the cell. It is suggested to combine with other methods to optimize the cell lysis. Khan et al [39] explain in their study, that acid hydrolysis alone is not sufficient for lysing algal cells and production of fermentable sugars with a good yield. The most effective and efficient pretreatment method among all the tested methods was by carrying out the CaO treatment before the enzymatic and acid hydrolysis. CaO effectively lysed the algae cells and makes the further hydrolysis and saccharification process easier.

Temperature also plays a crucial role in the pretreatment process. This study used a temperature of 100°C in the acid hydrolysis process. Acid hydrolysis is effective at a high temperature. At a low temperature acid cannot break the bond between the monomers in polysaccharides. Temperature range of 100-200 °C have been reported as the effective temperature range for pretreatment of algal biomass, depending on reaction time and acid concentration to release monomeric sugars for fermentation [17][40]. However, based on the pretreatment optimization of the biomass, it revealed that 150 °C was the most suitable temperature [39]. Harun and Danquah [25] have found that pretreatment of microalgae at a concentration of 10 g/L at 160°C and 3% (v/v) of sulfuric acid for 15 min gave the maximum yield of 52 wt% (g ethanol/g microalgae). On the other hand, Miranda et al. explained pretreatment dried biomass of S. obliquus with sulfuric acid (2 N) at 120 °C for 30 min was the best way to convert the complex carbohydrates and sugars into simple sugars, prior to the fermentation process [27].

Duration of hydrolysis process must be also considered as an important factor in bioethanol production. A study investigated by Harun and Danquah showed that the use of pretreating cells with longer duration above 30 min produced a lower bioethanol production [25]. Other study reported that increasing duration of pretreatment process up to 60 min did not increase the saccharification process
of wheat straw, thus possible in reducing the bioethanol production. On the other hand, the use of a short duration pretreatment process has a positive impact on energy consumption [41].

According to normality and homogeneity tests, all data was normally and evenly distributed. Analysis of variance (ANOVA) showed that variations of acid concentration and hydrolysis time did not significantly affect the bioethanol production.

4. Conclusions

Scenedesmus sp. is actually a quite promising alternative for bioethanol production due to its high content of carbohydrate and reducing sugar. However, in this study, the resulting ethanol content was very low. The results showed that the highest ethanol content was 1.1 % and achieved on the 1st day of fermentation with 75 minutes of hydrolysis process using 3% sulfuric acid. It was followed by a treatment with 105 minutes hydrolysis process using 3% sulfuric acid which yielded a value of 1.095% on the 2nd day of fermentation. Optimization of hydrolysis and fermentation processes should be done in order to produce high ethanol content. This can be carried out by, for example, increasing the temperature or integrating the enzymatic process into the procedure.

5. References

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