

Evaluation on Microalgae Biomass for Bioethanol Production

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Abstract. The depletion of energy resources has triggered worldwide concern for alternative sources, especially renewable energy. Microalgae biomass offers the most promising feedstock for renewable energy because of their impressive efficient growing characteristics and valuable composition. Simple cell structure of the microalgae would simplify the pretreatment technology thus increase the cost-effectiveness of biofuel production. *Scenedesmus dimorphus* is a carbohydrate-rich microalgae that has potential as biomass for bioethanol. The cultivation of *Scenedesmus dimorphus* under aeration of carbon dioxide enriched air resulted 1.47 g/L of dry biomass with composition of 12% w/w total lipid, 53.7% w/w carbohydrate and 17.4% protein. Prior to ethanolic fermentation with *Saccharomyces cerevisiae*, various pre-treatment methods were investigated to release and degrade the complex carbohydrate in cell biomass thus obtaining the maximal amount of digestible sugar for ethanolic yeast. In this study, sulfuric acid was used as hydrolysis agent while amyloglucosidase as enzymatic agent. Dried biomass via hydrothermal acidic hydrolysis yielded sugar which is about 89% of total carbohydrate at reaction temperature of 125 °C and acid concentration of 4% v/v. While combination of organosolv treatment (mixture of methanol and chloroform) with enzymatic hydrolysis yielded comparable amount of sugar with 0.568 g glucose/g treated-biomass. In this study, the significant information in pre-treatment process ensures the sustainability of the biofuel produced.

1. Introduction

Bioethanol as the choices as alternatives fuels is always limited and actively debated due to fuel vs food issue. Ease of pretreatment on biomass and compatibility of treated biomass to subsequent ethanolic microbe is the core area of research, to increase the overall bioethanol production efficiency. As such, microalgae with simple cell structure become the biomass in spot for fermentation, with stipulation that treated biomass is preferred for ethanolic microbe.

Microalgae as a renewable and promising resource for biofuels production are an attention of researcher all over the world [1]. It is urgently needed as clean energy source due to heavy carbon dioxide emission that leads to severe pollution and climate change problems. Microalgae with its inherent properties, grows extremely rapidly and commonly double their biomass within 24 hours. The biomass mainly composed with lipid, carbohydrate and protein. With this, sustainable production of biofuels needs a bundle of research on the production technologies from algae strain to biofuels product (biodiesel, bioethanol, biogas). The production chain involves selection of algae strain, algae



cultivation for desired requirement (higher lipid and carbohydrate productivity), harvesting, compounds extraction and biofuels production reaction [2].

Scenedesmus dimorphus is a potential microalgae species for production of bioethanol due to its capability in accumulating carbohydrate content as stored carbon in the cell. However, the cell need to be disrupted and the complex biopolymers need to be converted to simple sugars before fermentation, through pretreatment process such as hydrolysis. This is because current industrial ethanologenic yeast only consume simple sugar such as glucose and galactose. Microalgae have the beneficial for the fermentation process with its simple cell structure and some of it is unicellular species. Most of the researches examine pretreatments to degrade the carbohydrate into simple sugar that ready for fermentation by microorganism [3,4,5]. The pretreatment in study are physical, chemical and physicochemical pretreatment. Acid hydrolysis and enzymatic pretreatment are two most common and efficient steps in discuss. The quality of the hydrolysate from the treatments process have the significant effect to subsequent fermentation technology.

In this study, the potential use of *S. dimorphus* as a feedstock to produce bioethanol was evaluated. In order to provide more precise perspective of *S. dimorphus* as a promising commercial feedstock, microalgae cultivation growth and starch content were study. Few pre-treatment methods was conducted to investigate the quality of hydrolysate for yeast fermentation. Attention was placed on the fermentable sugar, i.e., glucose, sucrose and galactose, to produce bioethanol.

2. Procedure

2.1. Microalgae cultivation

Scenedesmus dimorphus (UTEX 1237, University of Texas) were cultured in a 2-L bioreactor and continuously aerated with 2 L/min of air and CO₂-enriched air. Illumination was provided continuously with 2 fluorescent lamps located at one side of the reactor. The medium consisted of 20 mL of 25 g/L NaNO₃, 7.5 g/L MgSO₄·7H₂O, 2.5 g/L NaCl, 7.5 g/L K₂HPO₄, 17.5 g/L KH₂PO₄, 11.4 g/L H₃BO₃; 2 mL of 8.82 g/L ZnSO₄·7H₂O, 1.18 g/L MnCl₂·2H₂O, 1.193 g/L Na₂MoO₄·2H₂O, 1 g/L CuSO₄, 0.401 g/L CoCl₂·6H₂O; 2 mL of 50 g/L EDTA-Na₂ and 4.48 g/L FeSO₄·7H₂O; 2 mL of 0.2 g/L Thiamine Hydrochloride B1 and 0.01 g/L Cyanocobalamin B12. The initial inoculum size was 79.2 x 10⁶ cells. Growth was assessed daily by measuring the optical density with a UV vis spectrophotometer at a wavelength of 600 nm.

Dry weight of biomass was obtained by centrifuging and drying the collected culture solution at 60 °C oven until constant weight was achieved. The dry weight of the algae biomass was determined gravimetrically and growth was expressed in terms of dry weight per liter culture.

The pH of culture was measured daily with pH meter. Starch content was measured from late exponential phase till early stationary phase of growth by targeting higher biomass productivity at that cultivation stage. All experiments were conducted in triplicate and data reported in average value.

2.2. Biomass harvesting by natural sedimentation

Natural sedimentation was employed in order to harvest the cultured microalgae. The culture aeration was stopped and subjected to gravity sedimentation. The top liquid phase of cultures was decanted and cells that deposit at bottom phase were harvested by centrifugation at 5500 rpm for 5 min. The cells were washed twice with distilled water after centrifugation. The cell supernatant was dried at 60 °C for 24 hr and keep in desiccator. The recovery was calculated based on the OD difference at bottom point.

Recovery efficiency (%),

$$\eta = \frac{OD_i - OD_f}{OD_i} \quad (1)$$

Where OD_i is the initial optical density of medium before sedimentation at 5 cm from bottom of culture bottle and OD_f is the final optical density of medium after sedimentation at 5 cm from bottom of culture bottle.

2.3. Biomass characterization

2.3.1. Total carbohydrate quantification. The total carbohydrate content was determined using two-step acid hydrolysis method. The method is a modified method from National Renewable Energy Laboratory [6]. First step of analysis is sulfuric acid at 72% (w/w) was added into glass tubes that filled with 3 g of dried biomass. The solution was vortex to thoroughly mix the acid and biomass. The tubes were kept in a $30\text{ }^{\circ}\text{C} \pm 3\text{ }^{\circ}\text{C}$ water bath for one hour. Subsequent step of hydrolysis was carried at concentration of 4% (w/w) for one hour at temperature of $121\text{ }^{\circ}\text{C}$ using autoclave. The hydrolysate was neutralized to pH between 5-7 using calcium carbonate. Neutralized samples are filtered with $0.22\text{ }\mu\text{m}$ nylon filter for HPLC analysis. Monomer sugar concentrations (mg/mL) were calculated from HPLC calibration and total carbohydrate was determined by summing the monomer sugars.

2.3.2. Total starch quantification. The starch content was determined using a modified method based on the National renewable Energy Laboratory using amyloglucosidase from *Aspergillus niger* (aqueous solution, $\geq 300\text{ U/mL}$, Sigma-Aldrich, Malaysia) as a hydrolysis enzyme [7]. One hundred milligrams of dried biomass was added to a boiling tube. Simultaneously, 5 mL of distilled water and 1 mL of 2N NaOH were added, and the solution was stirred for 20 min in a preheated water bath set at $90\text{ }^{\circ}\text{C}$. The solution was neutralized with 1 mL of 2N HCl, and the pH of the solution was maintained by adding 2 mL of acetate buffer (50 mM, pH 4.5). The reaction was started by adding 1 mL of amyloglucosidase (60 units of activity per millilitre), and the solution was stirred for 60 min at $40\text{ }^{\circ}\text{C}$. The resultant samples were identified with high performance liquid chromatography (HPLC).

2.3.3. Total lipid quantification. The total lipids were obtained by extracting the dry biomass with a mixture of chloroform and methanol (ratio of 1:2), which is a modification of the method proposed by Bligh and Dyer [8]. One gram of dried biomass was stirred in the solvent mixture at room temperature for 24 h. The solution was filtered with filter paper, and washed again with solvent mixture. The washing and filtration steps were repeated three times. Lastly, the solvent was evaporated by gentle heating until a constant weight was reached; this residual mass was the total lipid content.

2.3.4. Total protein quantification. Protein content was measured with the AOAC 988.05 method. Biomass was go through digestion and distillation process. The total protein was calculated based on the products of 6.25 and percentage of nitrogen content.

2.4. Pretreatment

2.4.1. Solvent treatment. The dried biomass of 2 g was added to a solvent mixture of methanol and chloroform (ratio 2:1). The solution was stirred (750 rpm) for 24 hours at room temperature. After the extraction process, the solution was filtered with filter paper. The extracted biomass was oven dried at $60\text{ }^{\circ}\text{C}$ until a constant weight was achieved and stored in desiccators.

2.4.2. Hydrothermal acid hydrolysis. Acid hydrolysis was conducted using concentrated sulphuric acid (97%). One hundred milligrams of biomass was treated with 1.0 to 5.0% (v/v) of H_2SO_4 with constant working volume of 10 mL. The mixture was autoclaved for 15 minutes at $110\text{ }^{\circ}\text{C}$, $120\text{ }^{\circ}\text{C}$ and $125\text{ }^{\circ}\text{C}$ each.

2.4.3. Enzymatic hydrolysis with pre-heated biomass. Effect of heating to enzymatic hydrolysis was carried out by directly added enzyme into biomass solution without stirring solution at $90\text{ }^{\circ}\text{C}$.

Hydrolysis conditions were maintained at pH 4.5, enzyme concentration at 60 units/mL and temperature at 55 °C.

Enzymatic hydrolysis was carried out using the amyloglucosidase enzyme (One unit of the enzyme can convert 1 mg of starch to glucose in 3 minutes at a pH and temperature of 4.5 and 55 °C, respectively). A biomass of 100 mg was added to the boiling tube, followed by 7 mL of sodium acetate buffer solution. The solution was stirred for 15 minutes in a preheated water bath at 90 °C. One millilitre of enzyme (60 units/mL) was added and stirred for 60 minutes at various reaction temperatures (30°C, 35°C, 40°C, 55°C, and 60°C) and pH values (4.0, 4.5, 5.0, 5.5, and 6.0).

2.5. Analysis of sugar content with high performance liquid chromatography (HPLC)

The system (Agilent series 1200) consisted of an evaporative light scattering detector (ELSD) and a Hi-Plex Ca column. The nebulizer and evaporator temperature were set at 50 °C and 80 °C, respectively. The analysis was run at a flow rate of 0.6 mL/min using deionized water as the mobile phase. The sugar content was calculated based on a glucose calibration curve.

3. Results and Discussion

3.1. *Scenedesmus dimorphus* growth monitoring

Figure 1 demonstrates the changes of dry weight and pH during cultivation period. The curves showed typical growth pattern with exponential phase, stationary phase and death phase. The dry weight with CO₂ enrichment aeration was apparently higher than aeration with air only throughout the cultivation period. The addition of CO₂ shorten the exponential phase and extend the stationary phase with higher biomass density. The pH of the culture aerated with air continues to increase to approximately 10 at day 10th and drop gradually to 7-8, as revealed in figure 1. While the addition of CO₂ maintained the pH value fluctuated at the range of 5-6.5, which is favourable for microalgae culture [9].

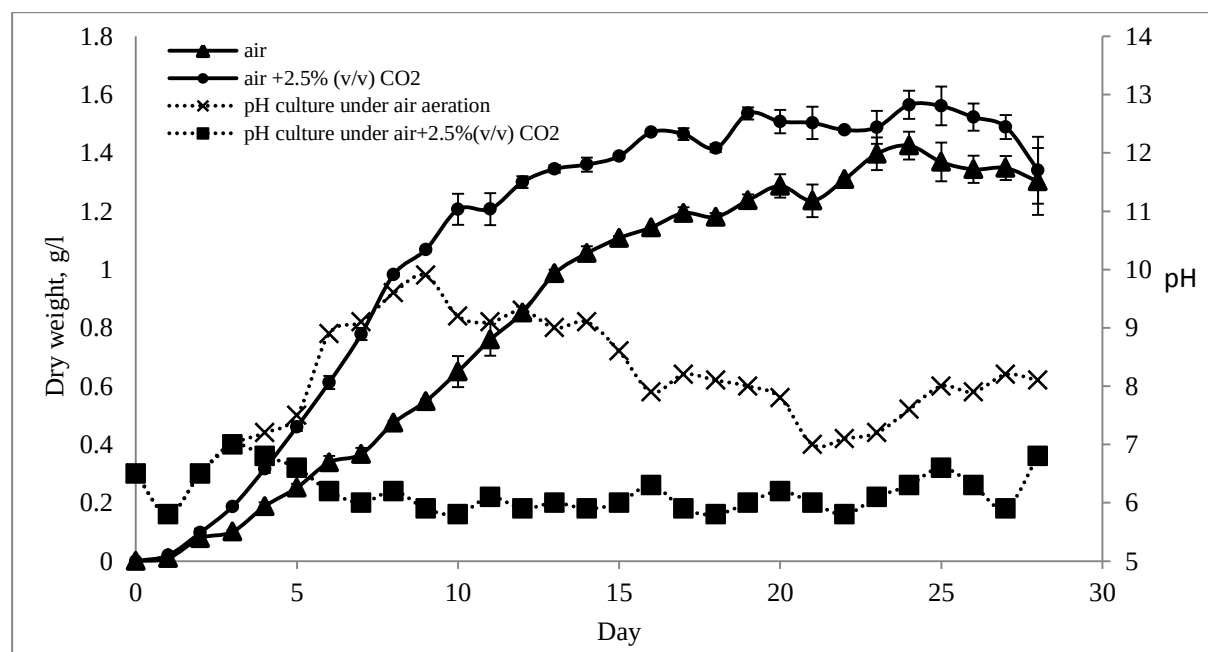


Figure 1. Dry weight and pH monitoring during cultivation time.

The starch content monitoring during cultivation period was presented in figure 2. The measurement was not calculated from day 1 to day 7 because the amount of collected biomass was too

little for starch content analysis. Starch content for both aeration increase steadily from day 8 to day 16, but decrease gradually starting day 16. The starch content of 2.5% (v/v) CO₂-enriched air accumulated higher starch content compared to air aeration. The optimum value was found at day 16 with starch content of 57.36% (w/w) and 47.43% (w/w) under aeration of CO₂ enrichment air and air, respectively.

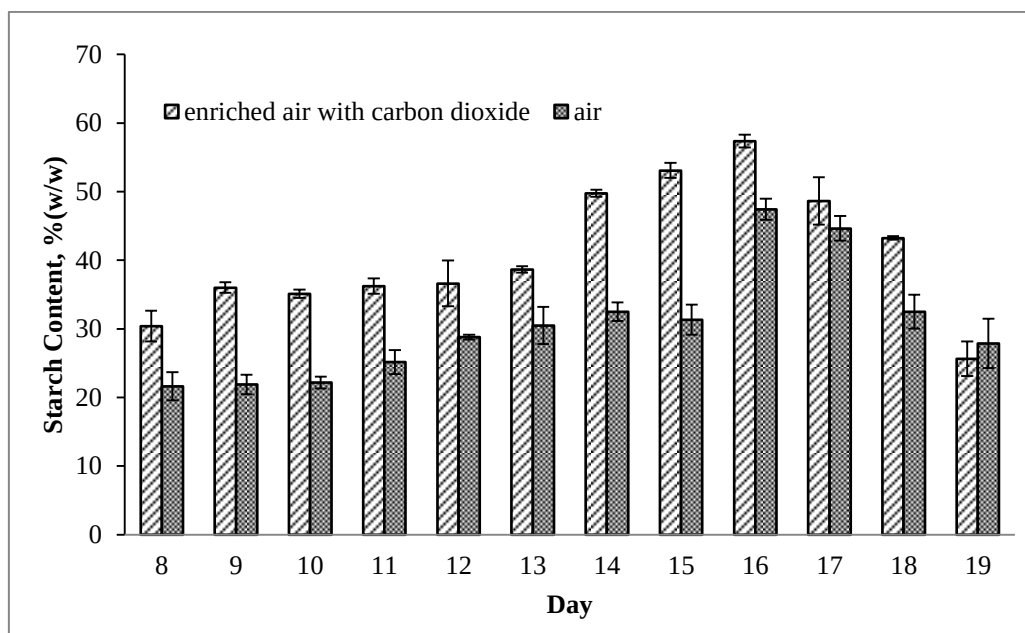


Figure 2. Starch content of biomass during cultivation time

Based on the growth monitoring presented, exponential growth occurred at initial phase when there are vast nutrient and room for cell to grow. However, growth entered stationary phase when cell density reached its saturated point where most of the nutrients had been utilized and the available spaces are limited for growth. The competition for nutrients, light and spaces for growth eventually caused the cell into death phase. The addition of carbon dioxide significantly increased the starch content and maintained a maximum growth rate. There is no additional organic or inorganic carbon sources included in the cultivation medium, thus carbon dioxide is the sole carbon source for microalgae to synthesize carbohydrates and accumulate starch in the microalgae cell [10]. As such, gaseous CO₂ first dissolves in water to form carbon dioxide (CO₂), carbonic acid (HCO₃⁻) and bicarbonate ion (CO₃²⁻) [11]. The dissociated components become the nutrient for microalgae growth. At the same time, presence of carbonic acid balanced the increasing pH that coupled with cell growth thus maintained the pH of the culture near to neutral [12,13]. This explained the minimal changes of pH for CO₂ enrichment aeration and substantial changes of pH profile for air aeration. Therefore, the CO₂ content in air is too low to support high cell growth rate. Since *S. dimorphus* have highest starch content and biomass density at day 16, the biomass was harvested for subsequent analysis and reaction.

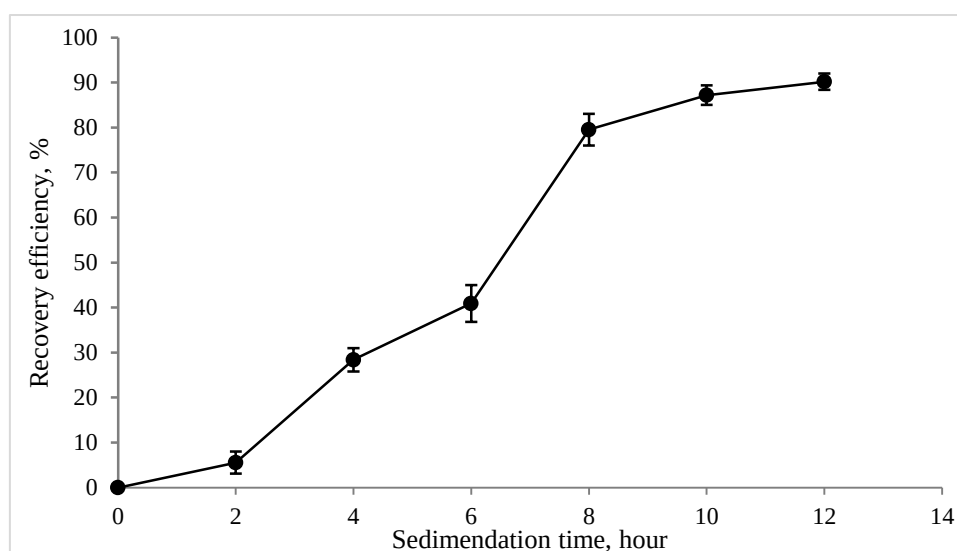
S. dimorphus biomass was characterized and the composition was presented in table 1. The biomass contains about 53.7 % w/w of total carbohydrate, 12 % w/w of total lipid and 17.4 % w/w of protein. The total carbohydrate consisted about 44.5% (w/w) of starch, while the remainder consisted of structural polysaccharides, especially from the cell wall. Based on the HPLC chromatogram after two-step acid hydrolysis, the sugar profile indicated the presence of dextrin, sucrose, glucose and galactose. According to reported studies [14,15], green algae like *S. dimorphus* have unicellular cell wall built from cellulose and store carbon as starch. The degradation of the cellulose cell wall could yield simple sugar which is mannose, galactose and glucose [16].

Table 1. Carbohydrate content of *S. dimorphus* compared to other bioethanol feedstock.

Bioethanol feedstock	Carbohydrate content (% w/w)
Corn	59.5 [17]
Chlorella vulgaris	51.0 [18]
<i>S.dimorphus</i> (this study)	53.7

3.2. Biomass harvesting with natural sedimentation

S. dimorphus was harvested with natural sedimentation where aeration was ceased on day 16. The day of harvest was decided based on the starch content and dry weight of biomass as discussed earlier. Figure 3 illustrate the performance of natural sedimentation. The recovery efficiency of sedimentation was presented in figure 3 based on the optical density difference. The OD value was drop from 2.169 to 0.160 within 12 hours. At the first 4 hours of precipitation, the recovery efficiency increased from 0% to 28%. Substantial recovery is observed between 4-8 hours of sedimentation from 28% to 79.5%, but minimal increase was found afterwards from 79.5% to 90%. Microalgae cell culture potential to sediment due to gravity and formation of biological floc if the culture in stagnant situation particularly at stationary phase. In addition, the density of carbohydrates (1500 kg/m^3) is higher than those of proteins (1300 kg/m^3) and lipids (860 kg/m^3) [19]. The accumulation of carbohydrates inside the cell biomass was maximized on day 16, which further enhanced the density difference of the medium with the cell [20]. Furthermore, *S. dimorphus* cells tend to clump together in a high cell density environment. Consequently, this phenomenon increases the efficiency of sedimentation when substantial cell density is accumulated at the bottom of bottle. This explained the sedimentation pattern of *S. dimorphus* cell.

**Figure 3.** Recovery efficiency of microalgae with natural sedimentation.

3.3. Effects of hydrothermal acid hydrolysis at various conditions

Acid hydrolysis is a common method and sulphuric acid is the best catalyst among others after decades of study on it. Total sugar content will be presented instead of glucose since acid is capable to break most of the complex biopolymer in biomass into simple sugar. Figure 4 shows the effect of temperature and acid concentration in sulphuric acid hydrolysis. Total sugar content was increased with increasing temperature for acid concentration range of 1%-5%(v/v). Meanwhile increasing acid

concentration increased the total sugar content significantly. Both parameters have interaction effects in degrading the polysaccharides to simple sugar. At higher temperature of 125 °C, considerable increase in total sugar content is observed for lower acid concentration from 1% to 2%. The yield was optimum at 4% of acid concentration and indicated declining trend at higher concentration when temperature is high enough for degradation. Therefore, the hydrolysis yield was maximized at 125 °C, which successfully produced 48% (w/w) of glucose in 15 min at an acid concentration of 4% (v/v). The data indicated that the temperature positively correlated with acid concentration in order to maximize the glucose content. Hydrolysis yield can be as high as 89.4% at optimum condition and as low as 2.4% at lowest temperature and acid concentration.

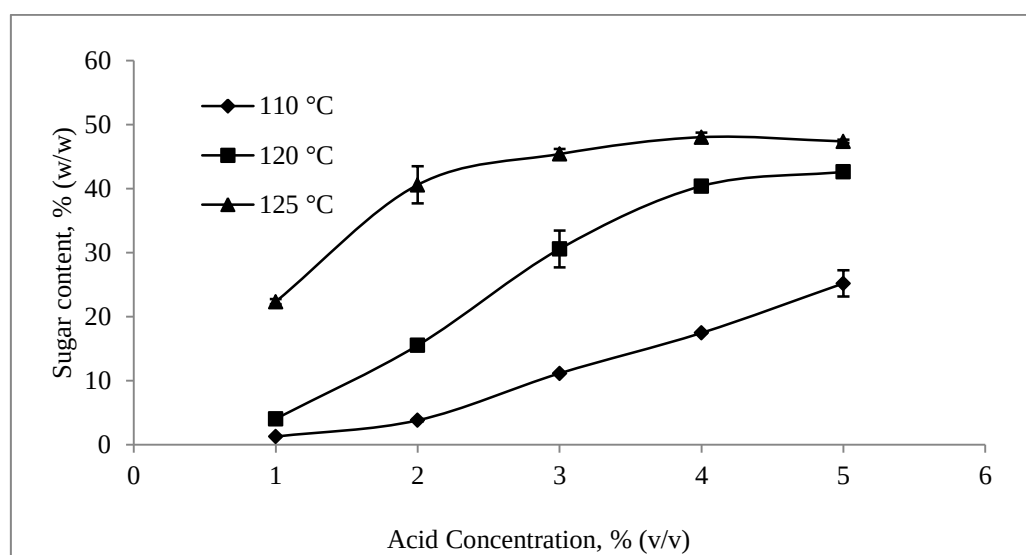


Figure 4. Effect of acid hydrolysis condition to sugar content.

Few studies had revealed that the performance of acid hydrolysis depends strongly to the cell wall rigidity of species [21,22]. For instance, Park et al. [23] concluded that the treatment of *Chlorella vulgaris* biomass with hydrochloric acid obtained the highest sugar conversion yield(92.5%); while Choi et al. [24] reported that 69.5 % of sugar yield was obtained by the treatment of *Golenkinia sp.* biomass with 2.0% (w/w) of sulphuric acid at 150 °C for 15 minutes.

Based on the HPLC chromatogram, the main sugar presence in hydrolysed products were glucose, sucrose and galactose. The hydrolysed products contains different percentage of glucose, sucrose and galactose at different hydrolysis conditions. At optimum condition, most of the sugar content attributed by glucose. The carbohydrate profile at optimum condition for sucrose, glucose and galactose are 0.053 g/g biomass, 0.363 g/g biomass, and 0.062 g/g biomass, respectively. The content of sucrose and galactose were reduced accordingly because most of them was degraded to glucose at higher temperatures and acid concentration. Further increased the temperature and acid concentration could degrade the sugar to furfural products thus reduced the yield of hydrolysis [25]. This explained the declining pattern of sugar content at higher operating temperature and acid concentration. Therefore, the acid concentration should be reduced if higher temperature was applied for optimum yield.

The usage of sulphuric acid in this treatment potential to generate side products and neutralization is required to increase the pH for yeast fermentation, mostly pH value of 4-6. The neutralization process resulting formation of waste, mostly insoluble salts that potential to create environmental issue.

3.4. Effects of enzymatic hydrolysis at various conditions

The enzymatic hydrolysis coupled with hydrothermal effect was investigated here. Table 2 presented the effect of heating to enzymatic hydrolysis. Hydrolysis without pre-heating the biomass reduced 13.9%(w/w) of glucose content. Therefore, effect of enzymatic operating conditions were evaluated by pre-heated the biomass at 90 °C in distilled water for 15 minutes. Figure 5 illustrates the effect of enzyme under various working environment. Enzymatic hydrolysis in this study was strictly targeted to liberate starch and transform it into glucose using amyloglucosidase enzyme. Thus, glucose is the only sugar found in hydrolysed products.

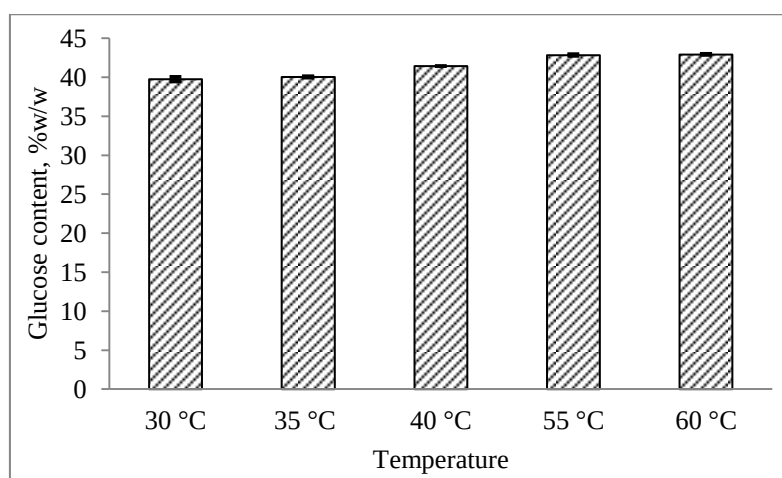
Table 2. Effect of pre-heating to enzymatic hydrolysis.

Treatment	Glucose content (% w/w)	Conversion yield (%)
Heating at 100 °C for 15 minutes	41.84±0.36	94.21±0.8
No heating	27.91±1.13	62.85±2.56

Figure 5(a), (b) and (c) show the effect of temperature, pH and enzyme loading to enzymatic function, respectively. In one hour time of reaction, considerable increase in glucose content from 39.7% to 42.8% is exhibited when higher temperature was applied from 30 °C to 55 °C. While temperature higher than 55 °C do not significantly improved the efficiency of enzyme. Thus, optimum temperature for enzymatic degradation was found at 55 °C with conversion yield of 96.1% based on the starch content of biomass. Therefore, effect of pH was investigated at reaction temperature of 55 °C owing to slight differences of glucose content(0.1%) between 55 °C and 60 °C.

Figure 5(b) demonstrates that the glucose content decreases with increasing pH of the medium. The glucose content gradually reduced to 34.9% when pH increased to 6. The highest glucose content of 42.6% (w/w) was obtained at pH 4. There is only 0.5% different in glucose content between pH 4 and 4.5. It is reasonable to conclude that the enzyme is active in acidic environment.

Performance of amyloglucosidase under various concentration was illustrated in figure 5(c). Enzyme activity performed well with 92.1% of glucose yield under concentration of 75 units/mL in one hour of hydrolysis period. Concentration above or below the value yielded glucose content that below 40% (w/w). This is because lower concentration did not sufficient to convert all the starch to glucose within the reaction time. Meanwhile, higher concentration of enzyme could reduce the enzyme activity.



(a)

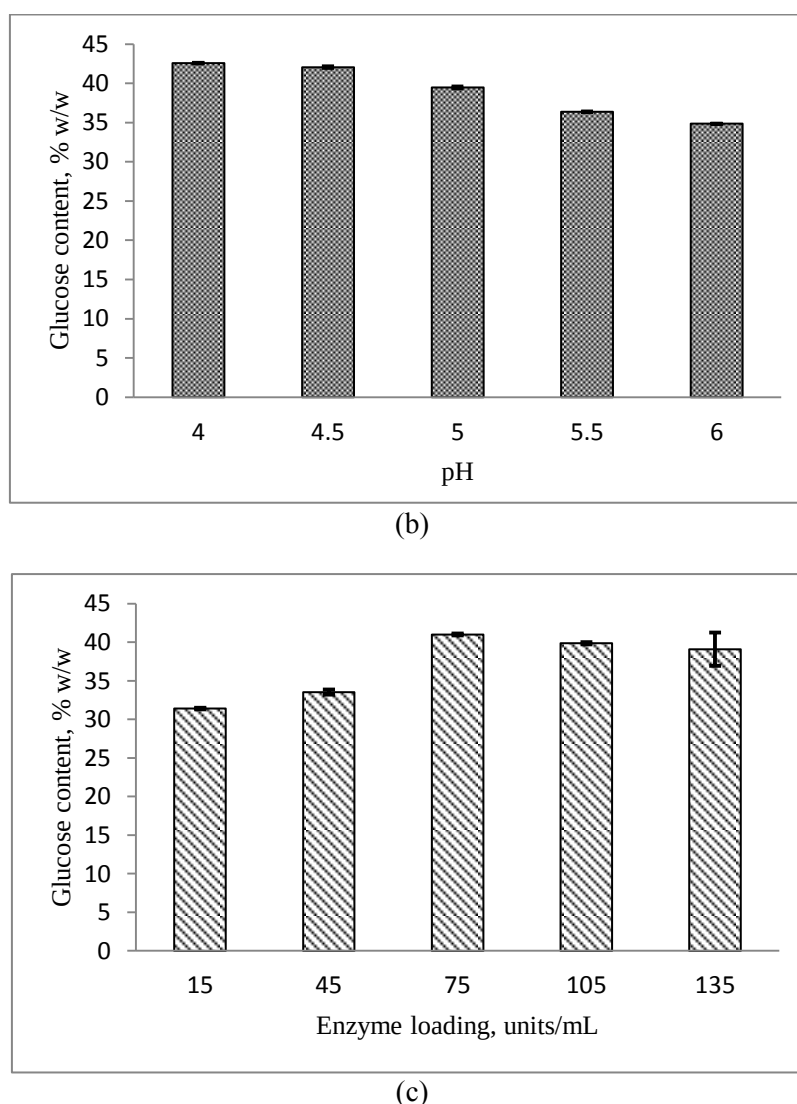


Figure 5. Enzymatic hydrolysis of *S. dimorphus* biomass at (a) various temperature, (b) various pH, and (c) enzyme loading, error bars indicate standard deviation.

As a result, it is suggested that saccharification temperature of 55 °C and a working pH of 4.5 that require less energy is reasonable for optimum performance of hydrolysis. This operating conditions agreed well with supplier suggested value and others research finding which using same type of enzyme. The performance of this enzyme over a wide range of reaction conditions is advantageous for fermentation, especially in an SSF configuration, where yeast only perform well under 30-40 °C [5]. The results clearly showed that enzymatic hydrolysis performed well under mild conditions during the glucose production step, which can be directly utilized for the subsequent fermentation process. Moreover, current production technology of enzyme have make cost of enzyme reduce significantly, thus reduce the concern on overall biofuel production cost.

3.5. Combination of solvent treatment with enzymatic hydrolysis

Single treatment with solvent only do not produce large amount of fermentable sugar when the treated biomass dissolve in distilled water. HPLC chromatogram depicted that the solution only contains 1-5% w/w of sucrose, glucose and galactose. Most of the carbohydrate remains as soluble complex

biopolymer such as starch and dextrin. Therefore, enzymatic hydrolysis at optimum conditions was experimented on the solvent-treated biomass without pre-heating process. The process resulted about 0.568 g glucose/g treated-biomass. The yielded hydrolysate is a promising input for subsequent fermentation because there are no side products detected in the solution, which could harm the ethanologenic yeast.

3.6. Economic feasibility of microalgal bioethanol production

Bioethanol production has been limited by extensive economic and energy restrictions, which are considered to be linearly proportional. Economic assessment considers upstream and downstream processes to define the minimum required cost before commercialization [26]. The National Energy Laboratory (NREL) has projected the cost of bioethanol production based on a production rate of 1,339 dry U.S. ton/day of microalgae, specifically *Scenedesmus sp.* and *Chlorella sp.* [27]. Based on the analysis, the feedstock contribute the largest portion to fuel price which need significant effort to reduce it. This analysis predicted minimum bioethanol prices of \$4.35/GGE [27]. Similar report have addressed the same issue that microalgae-based bioethanol production is only feasible with substantial reduction in feedstock price and improvement in conversion process [3].

4. Conclusions

Scenedesmus dimorphus is promising as an alternative source for bioethanol. The microalgae are offering outstanding feedstock for bioethanol with 1.47 g/L biomass and 53.7% of carbohydrate content. The high yield of sugar content which is more than 80 % is obtainable with simple pretreatment process, thus fermentable via counterpart fermentation. This study become the essential information for further detail study in the production technology to establish a sustainable and cost effective bioethanol. Moreover, the side products of fermentation, CO₂ could be the carbon source for microalgae cultivation.

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