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# Bioethanol production from glucose obtained from enzymatic hydrolysis of *Chlorella* microalgae

Megawati *, Zuhriyan Ash Shiddieqy Bahlawan, Astrilia Damayanti, Radenrara Dewi Artanti Putri, Bayu Triwibowo, Haniif Prasetiawan, Septian Putra Kusuma Aji, Adi Prawisnu

*Universitas Negeri Semarang, Faculty of Engineering, Department of Chemical Engineering, Gunungpati, Semarang 50299, Indonesia*

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ABSTRACT

*Chlorella* microalgae are one of the microalgae that can potentially be used as a substrate in ethanol production due to their high carbohydrate content. However, carbohydrates content in microalgae cannot be directly fermented into ethanol and need to be firstly hydrolyzed into glucose. The aim of this study was to develop a combined process of enzymatic hydrolysis and fermentation for the production of bioethanol from the *Chlorella.* The greatest glucose yield in the enzymatic hydrolysis process reached up to 80.01% under the condition of 3% (v/v) glucoamylase added, medium pH of 5.0, and hydrolysis temperature of 80 °C for five hours. The highest glucose concentration obtained from hydrolysis of *Chlorella* biomass in this study was subsequently fermented anaerobically using yeast *Saccharomyces cerevisiae*. From the fermentation process, the maximum ethanol yield reached 94.21% and the ethanol concentration was 4.80 g/L at a medium pH of 5.0 and a yeast load of 0.5 g for 30 h. Hydrolysis using enzymes has proven to be effective in the pretreatment of polysaccharides from the *Chlorella* into glucose and is suggested to be used as a substrate to produce bioethanol. Overall, *Chlorella* has great potential to be developed into sustainable renewable energy.


## 1. Introduction

With the likelihood of depletion of fossil fuels and the increasing demand for energy due to human growth in the forthcoming, the energy sector faces a challenging problem. The rising world population and economic growth have an impact on world energy needs [1]. In addition, the use of fossil energy produces a large number of carbon emissions which has a noteworthy impact on global warming and climate change [2]. One way to overcome such problems is to substitute fossil fuels with renewable energy [3]. Bioethanol with microalgae substrate has been widely researched and developed [4]. Moreover, bioethanol as alternative energy has also proven to be effective in averting global warming as its combustion can lower carbon emissions by up to 34% [5].

Bioethanol production from glucose typically employs an anaerobic fermentation process with *Saccharomyces cerevisiae* as yeast [6]. As reported in the existing literature, *S. cerevisiae* can produce up to 2.64% bioethanol with tree trunk waste as substrate [7], 7.4 g/L ethanol with a red algal substrate [8], and 40.63 g/L with carrot pulp substrate [9]. Glucose, as the primary substrate for bioethanol production, can be derived from several biomass sources. The glucose substrate for the production of bioethanol derived from sucrose or food starch is called first-generation bioethanol [10]. The first generation bioethanol has great potential to substitute for fossil fuels, but its production is hampered by the need for food and tropical forests' destruction [11]. The second-generation bioethanol utilizes non-edible substrates such as agricultural waste [12]. In the latest generation of bioethanol, i.e., the third one, glucose substrates are derived from microalgae biomass [13]. Bioethanol with microalgae biomass as a substrate has great potential and continues to be developed. Furthermore, microalgae are microorganisms that are capable of photosynthesis and have the ability to consume carbon dioxide up to 15 times that of land plants [14]. In recent years, several types of microalgae have been

* Corresponding author.
*E-mail address:* megawati@mail.unnes.ac.id ( Megawati).

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studied for bioethanol production, such as *Porphyrydium, Chlamydomonas, Scenedesmus Anabaena,* and *Spirulina* [15].

Some types of microalgae contain a moderately high carbohydrate concentration from 8% up to 64% as a food reserve and their growth [16]. *Chlorella* is one of the microalgae with carbohydrate content reaching 20.99%, thus it has great potential as a substrate for bioethanol production [17]. However, these carbohydrates are not readily processed into bioethanol products. At the first stage, it is necessary to carry out a pretreatment process of hydrolysis of polysaccharides to release monosaccharides which are then fermented into bioethanol by yeast [18]. The pretreatment process is also viewed as one of the crucial processes and contributes markedly up to 30% of the total cost production [19]. Some studies reported the hydrolysis process of microalgae into reducing sugars up to 80.00% with alkaline hydrolysis [20], 93.6% by acid hydrolysis [21], and 99% by enzymatic hydrolysis [22]. Enzymatic hydrolysis has several advantages compared to chemical hydrolyses, such as higher yields of reducing sugars without degradation, low corrosion problems, and low utility consumption [23]. In addition, enzymatic pretreatment was reported efficient for hydrolyzing cell walls in some microalgae [24]. Carbohydrate hydrolysis using enzymatic has great potential to improve efficiency in the fermentation process and can reduce costs during the production process. Thus, optimizing the hydrolysis of carbohydrates into reducing sugars is essential for more efficient and sustainable bioethanol production.

Research on the production of bioethanol with microalgae as the substrate has been extensively studied. However, studies to develop a combined process of enzymatic hydrolysis and fermentation for the production of bioethanol from the microalgae *Chlorella* are still scarcely found. In this study, alpha-amylase and glucoamylase enzymes were employed in the hydrolysis process. Reducing sugar was studied for each different operating condition in the experiment. Meanwhile, in the subsequent process, yeast *S. cerevisiae* was employed for anaerobic fermentation. At the fermentation stage, the concentration of bioethanol and residual glucose were also studied under different experimental operating conditions. It is expected that this research can contribute to finding an effective strategy for bioethanol production using microalgae *Chlorella* as the substrate and can be used as an initial stage renewable alternative energy.

## 2. Materials and method

### 2.1. Materials

The microalgae used in this study was green microalgae *Chlorella* powder purchased from Ugo Plankto Algae (Purworejo, Central Java, Indonesia). The yeast used was instant dry yeast *S. cerevisiae*, which was purchased from Lalvin 71B (France). Alpha-amylase and glucoamylase were purchased from Novozyme (Denmark) with the activity 300 KNU/g and 100 FBG/g, respectively.

### 2.2. Enzyme hydrolysis

In the enzymatic hydrolysis process, dry microalgae of *Chlorella* were diluted with distilled water until the concentration of microalgae became 60 g/L in a 250 mL volumetric flask. The solution's acidity was adjusted to a pH of 6.0 using citrate acid (Merck, Germany). The addition of alpha-amylase enzyme was performed after the temperature of the solution reached the optimum temperature of the alpha-amylase enzyme activity up to 95 °C. When the temperature of the broth decreased to 50 °C, glucoamylase enzyme was added and subsequently mixed with a mixer (DLab, China) for five hours. Enzymatic hydrolysis was carried out with an agitation rate of 400 rpm, and the glucose was analyzed in the one-hour interval.

### 2.3. Glucose analysis

The Nelson-Somogyi method was employed to analyze the glucose using hydrolysates *Chlorella* and the glucose from fermentation [25]. Glucose solutions with concentration variations of 0–600 ppm were firstly analyzed to obtain a linear correlation absorbance. 1 mL of the sample was placed in a test tube, and then 1 mL of Nelson's solution (Merck, Germany) was added. The test tube was heated at 85 °C for 20 min until a red precipitate was formed. The mixture was cooled to room temperature. Thereafter, 1 mL of arsenomolybdate (Merck®, Germany) solution and distilled water as a diluent was added and then stirred with a shaker of 400 rpm for five minutes. The solution was measured using a spectrophotometer UV–Vis (GENESYS™ 20 Thermo Fisher Scientific, Germany) with a 540 nm wavelength. The absorbance value of the sample was read with a linear graph to obtain the concentration of reducing sugar.

### 2.4. Bioethanol fermentation

Instant dry yeast of *S. cerevisiae* (0.25 – 1.00) g was inoculated into a medium solution with the composition of distilled water, 5 g/L yeast extract (Microgen, India); 5 g/L glucose (Merck, Germany); 5 g/L $(NH_4)2SO_4$ (Merck, Germany); 5 g/L $MgSO_4$ (Merck, Germany); and 5 g/L peptone (Oxoid, the USA). The medium was sterilized by using an autoclave at 121 °C for 30 min and followed by a cooling process until it reached room temperature. Inoculation was prepared in a 500 mL volume flask with 250 mL of inoculation medium then incubated at 30 °C for 24 h over the orbital shaker at an agitation rate of 100 rpm to grow the *S. cerevisiae* aerobically. The enzymatic hydrolysates of *Chlorella* with the optimum yield were used as substrates in the fermentation process. Fermentation was carried out in a 500 mL volume flask with 250 mL of distilled water enriched with nutrients (5 g/L yeast extract; 5 g/L glucose; 5g/L $(NH_4)_2SO_4$; 5 g/L $MgSO_4$; and 5 g/L peptone). The pH of the fermentation medium was adjusted to a pH of 5 using a citric buffer. 10 % (v/v) of inoculated *S. cerevisiae* was added into the flask and fermented under anaerobic conditions for 30 h. The sample was carried out every six-hour time interval to analyze the concentration of ethanol and reducing sugar.

### 2.5. Ethanol analysis

The fermented ethanol was analyzed using the Winnick-Conway method according to [26]. At the initial stage, the ethanol solutions with concentrations ranging from 0.5 to 6 g/L were analyzed, and the absorbance values were taken from each solution to acquire a linear correlation for the sample analysis. The results of *Chlorella* fermentation were filtered to separate the residual solid substrate. Dichromate acid solution was prepared by adding 4.262 g of potassium dichromate (Merck, Germany) with 100 mL of distilled water. Hereinafter, 50 mL of sulfuric acid (Merck, Germany) was added to the solution and continued dilution with distilled water until the volume became 1000 mL. The dichromate acid solution was stored at room temperature for further analysis. For ethanol analysis, 5 mL of the sample was placed on the outside side of the Conway plate with 1 mL of 20% sodium carbonate solution (Merck, Germany). Furthermore, the dichromate acid solution was diluted at 1:10 with distilled water, and 5 mL of dichromate acid was added to the center side of the Conway plate. The Conway plate was tightly protected with a cover and heated in an oven (Memmert 55, Germany) at 50 °C for two hours. The center side solution of Conway was analyzed using a UV–Vis spectrophotome-

ter (GENESYS™ 20 Thermo Fisher Scientific, Germany) with a maximum wavelength. The absorbance value of the sample was read with a linear graph to obtain the ethanol concentration.

### 2.6. Calculation of hydrolysis yield, and ethanol yield

Hydrolysis yield calculation referred to the equation from Shokkrar et al. [27], which express the hydrolysis yield as the concentration of glucose hydrolysates per total carbohydrate concentration of *Chlorella* microalgae (Equation 1).

$$\text{Hydrolysis yield}\,\% = \frac{\text{glucose concentration hydrolysates (g/L)}}{\text{concentration of carbohydrate in substrate (g/L)}} \times 100\% \quad (1)$$

To quantify ethanol yield, an equation by Im et al. [28] was used. The formula divides the ethanol concentration obtained by the glucose consumed and the theoretical ethanol from glucose (0.511) (Equation (2)).

$$\text{Ethanol yield}\,\% = \frac{\text{ethanol concentration (g/L)}}{0.511 \text{ x glucose concentration (g/L)}} x100\% \quad (2)$$

## 3. Results and discussion

### 3.1. Effect of glucoamylase enzyme concentration on glucose production

As previously mentioned, microalgae *Chlorella* has a high carbohydrate concentration of up to 20.99%. This composition makes *Chlorella* be a suitable substrate for bioethanol production. The effect of the variation of concentration of glucoamylase studied in the range of 1–3% (v/v) on carbohydrate hydrolysis of *Chlorella* is presented in Fig. 1 At the first stage, the alpha-amylase enzyme was employed with an activity of 300 KNU/g for the liquefaction process and continued with the hydrolysis process with the glucoamylase enzyme with an activity of 100 FBG/g.

From Fig. 1 it can be seen that more concentration of glucoamylase added to the substrate resulted in a rise in glucose conversion during the hydrolysis process. The addition of 3% glucoamylase enzyme led to the highest glucose yield of 80.00%, followed by the 2% and 1% glucoamylase enzyme, which yielded glucose of 78.02% and 75.44%, respectively, at the end of the hydrolysis process for five hours. The productivity rate of hydrolysis with *Chlorella* biomass changed significantly ($p > 0.05$) in the first one hour, and after that, the productivity tended to be constant in all parameters. In this finding, the variations of glucoamylase concentration did not show significant results at the end of the hydrolysis process. This may occur as the carbohydrates in *Chlorella* have been converted to glucose quickly, so it needs to be analyzed at a lower time span to determine the significance of the effect of the enzyme. This finding is also in alignment with research executed by Han et al. [29] in the hydrolysis of waste hamburgers for ethanol production and Mussatto et al. in enzymatic hydrolysis of brewer's spent grain [30]. Most of the carbohydrates in the *Chlorella* microalgae are composed of starch content [31]. In the enzymatic pre-treatment of *Chlorella* substrate, the enzyme alpha-amylase liquefied starch into oligosaccharides by hydrolyzing alpha 1,4 glycosidic bonds and followed by the glucoamylase enzyme hydrolyzing alpha 1,4 glycosidic bond of oligosaccharides into glucose [20]. In small amounts, the enzyme glucoamylase also can convert large quantities of *Chlorella* substrate. However, rising the concentration of the glucoamylase also increases the active site so that the hydrolysis process will be faster and more effective [32].

### 3.2. Effect of temperature and pH hydrolysis on glucose production

The effect of enzymatic hydrolysis temperature on *Chlorella* biomass was also studied in the temperature range of 50–90 °C. Fig. 2 depicts glucose yield at all variations of temperature increases during the hydrolysis time process. A significant increase ($p > 0.05$) in the conversion of glucose results occurred at the first one hour, and after that, it tended to be stable until the end of the hydrolysis process. This is evidenced by the decreasing productivity rate for all temperature parameters. This phenomenon may be associated with the decrease in the polysaccharide substrate in the *Chlorella* microalgae that was converted to glucose in line with the hydrolysis time. The hydrolysis temperature of 80 °C yielded the highest glucose up to 80.01% at the end of the five hours hydrolysis process and a maximum productivity rate of 6.83 g/L.h. Meanwhile, below 80 °C, glucose conversion was lower and continued to decline with decreasing process temperature. The highest productivity rate also occurred at a hydrolysis temperature of 80 °C at 6.83 g/L.h. In general, the lack of heat in the hydrolysis process leads to a decreasing productivity rate. In hydrolysis with the lowest temperature (50 °C), the productivity rate of glucose only reached 5.77 g/L.h. Meanwhile, at the maximum operating temperature of 90 °C, the glucose yield reached 79.91%, and the maximum productivity rate

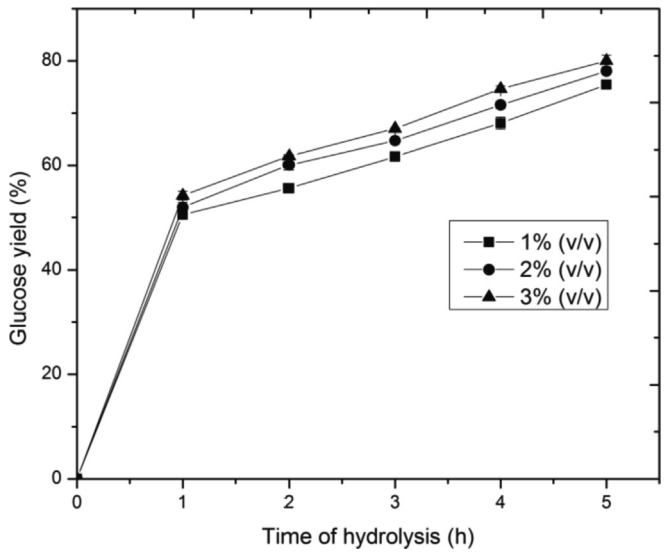


**Fig. 1.** The effect of glucoamylase concentration on glucose yield.

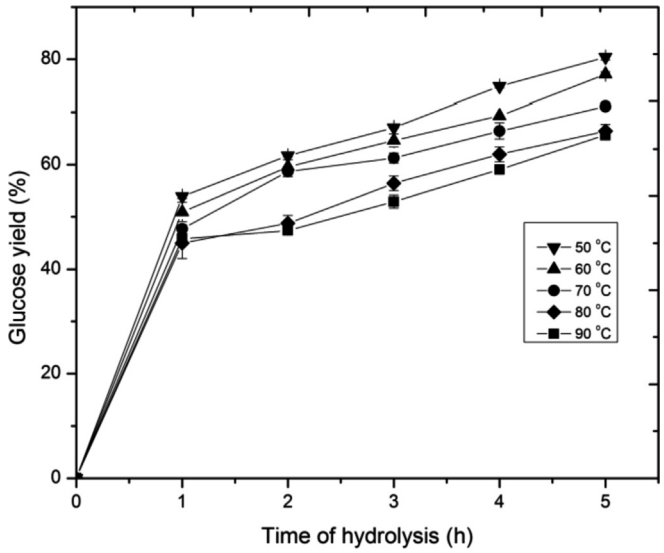


**Fig. 2.** The effect of temperature hydrolysis on glucose yield.

was 6.72. The glucoamylase enzyme can be active at a temperature of 80–90 °C, and the optimum temperature is at 80 °C [33]. The abatement in glucose conversion at high temperatures (90 °C) is due to the denaturation process and damage to some enzymes by thermal [34], so that the glucose conversion process will be decreased. This finding is also in line with the research conducted by Harun et al. [35] on the hydrolysis of *Chlorococum humicola* microalgae and Shokrkar et al. on hydrolysis of mixed microalgae culture [20].

The effect of medium pH (2.0 to 6.0) on the hydrolysis process was also investigated during the enzymatic hydrolysis process on *Chlorella* biomass. Fig. 3 indicates that at pH 5.0, the optimal glucose conversion reached 80.00%, and in more acidic conditions, the enzyme glucoamylase was ineffective for the enzymatic hydrolysis process. At the medium pH of 2.0 and 3.0, glucose conversion only reached 46.39% and 52.04%, respectively. From this finding, it is clear that the glucose conversion is not only caused by the activity of the glucoamylase enzyme, but also the acidic conditions of the medium that can hydrolyze carbohydrates. More acidic conditions of the medium can result in the denaturation of enzymes that are proteins to become less active as a biocatalyst. Other than that, extreme pH conditions also affect the enzyme to become less compact and become more hydrophobic to the substrate [36]. The active pH of the glucoamylase enzyme itself is in the range of 4.5 to 7.0. Similarly, Ruiz et al. [37] also mentioned that the optimum medium pH for glucoamylase exists at a pH of 4.5. Unfortunately, the pH of hydrolysis under alkaline conditions was not investigated in this study.

### 3.3. Effect of time fermentation

In the fermentation stage, glucose used as a substrate is the highest glucose yield from the hydrolysis process of *Chlorella* microalgae, which was 80.01% or 9.98 g/L of glucose concentration. *S. cerevisiae* was employed at this stage by an anaerobic fermentation process and incubated at a pH of 5.0 at 30 °C for 30 h to obtain optimum fermentation time. The residual glucose concentration, ethanol concentration, ethanol production, and ethanol yield at various fermentation times are presented in Fig. 4.

Fig. 4 shows that the ethanol concentration increased during the fermentation process. Meanwhile, the glucose concentration in the broth continued to decrease until the end of the fermentation process. The ethanol yield n increased significantly up to 57.31% in the first six hours of fermentation time, with the ethanol

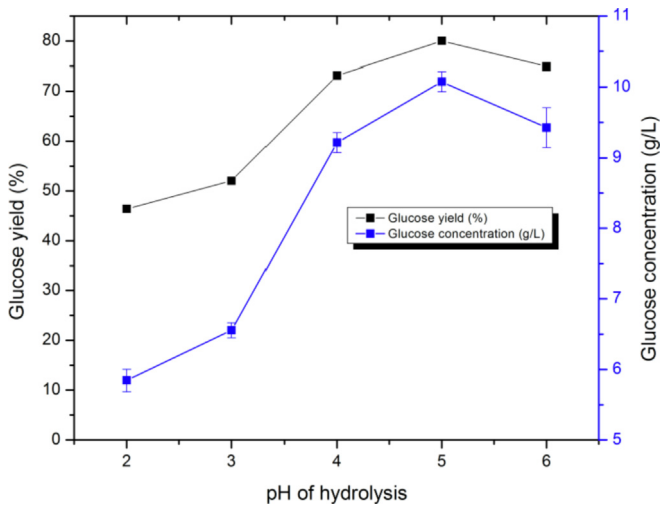


**Fig. 3.** The effect of medium pH on hydrolysis process.

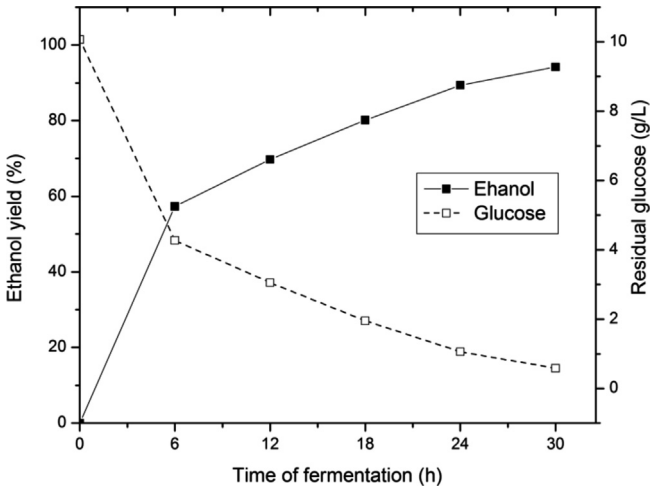


**Fig. 4.** The effect of fermentation time on ethanol yield and residual glucose.

productivity rate reaching 0.48 g/L.h. This may be linked with the rapid growth of yeast *S. cerevisiae* in the log phase. The decrease of glucose concentration from 10.07 g/L to 4.27 g/L also demonstrated that the yeast cell actively consumes glucose as the main nutrient for the growth and converts it into bioethanol. Ethanol concentration reached 4.8 g/L at the end of the fermentation process, with the ethanol yields up to 94.21%. In general, as the fermentation time increases, the productivity rate of ethanol production will decrease gradually. This is due to glucose reduction in the fermentation medium as the main nutrient [38] and causes the yeast cell to lack nutrients. The decrease in glucose during the fermentation process shows the effectiveness of *S. cerevisiae* cells in consuming glucose from hydrolysates *Chlorella* as the main substrate [39]. On the other hand, Zhang et al. [37] stated in their research that ethanol as a fermentation product from glucose is a major factor in inhibiting the growth of *S. cerevisiae* yeast so that the yeast cells will quickly enter the death phase. This finding is also in line with previous research by Kim et al. [40] in ethanol fermentation using the *Chlorella vulgaris* substrate and Lee et al. [41] using *Cholrella sp*. substrate.

### 3.4. Effect of pH fermentation

The process of fermentation and microbial growth is generally affected by the pH conditions of broth fermentation. The effect of pH on ethanol production from glucose hydrolyzed *Chlorella* was investigated with a pH range of 3.0 to 6.0. The fermentation process was studied anaerobically at 30 °C using *S. cerevisiae* yeast for 30 h. It is found in our study that the yield of bioethanol production is affected by the pH of the broth. The result of ethanol yield and glucose concentration is presented in Fig. 5.

The maximum production of ethanol was at the pH of 5.0, which yielded 94.21% ethanol. At a pH of 3.0, which is more acidic, yeast *S. cerevisiae* produced the lowest ethanol yields (87.65%). This result may be linked to the critical role of enzymes in yeast *S. cerevisiae,* whereas the enzyme can work effectively at the optimum pH for their cell growth and metabolism. This study is also in accordance with the research conducted by Tan et al. [42] in which an acidic medium will encourage acetic acid as a side product so that it will interfere with the fermentation process of ethanol production. In addition, at lower pH conditions in the fermentation broth, $H^+$ ions in the media can cause changes in the cell wall membrane, thus affecting the essential nutrients in the cell and making the enzymes work inefficiently [43]. When the enzyme does not work optimally, the yeast *S. cerevisiae* cannot grow effectively,

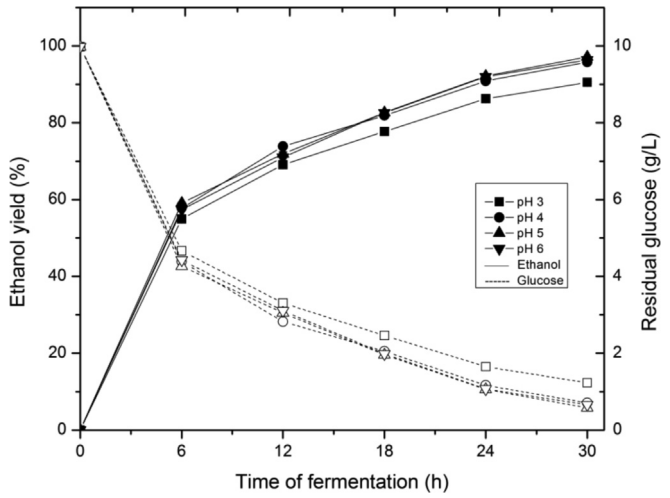


**Fig. 5.** The effect of pH fermentation on ethanol yield and residual glucose.

thereby inhibiting the production of glucose into ethanol. Banosa et al. [44] reported in their study that the yeast *S. cerevisiae* can grow at a tolerance of pH of 4.0 to 6.0. This is in accordance with our finding that there was an increase in ethanol concentration from a pH of 3.0 to 6.0. Unfortunately, the medium pH under alkaline conditions was not studied in the present study.

### 3.5. Effect of yeast load

The effect of dry yeast of *S. cerevisiae* cell load in the fermentation process was studied at an operating temperature of 30 °C, a medium pH of 5.0, and a fermentation time of 30 h to determine the optimum conditions (Fig. 6). Our investigation showed that adding 0.25 g dry yeast load mass in the fermentation process results in very sluggish glucose consumption by yeast cells. As the yeast mass was increased to broth up to 1.00 g, the glucose consumption by the yeast will be more rapid. Yeast load mass of 0.75 g and 1.00 g glucose consumption was active in the first six hours of fermentation and tended to be constant after six hours. The 0.50 g dry yeast mass added showed the greatest glucose consumption at the end of the fermentation process (30 h). More dry yeast load mass added can affect the number of active yeast cells

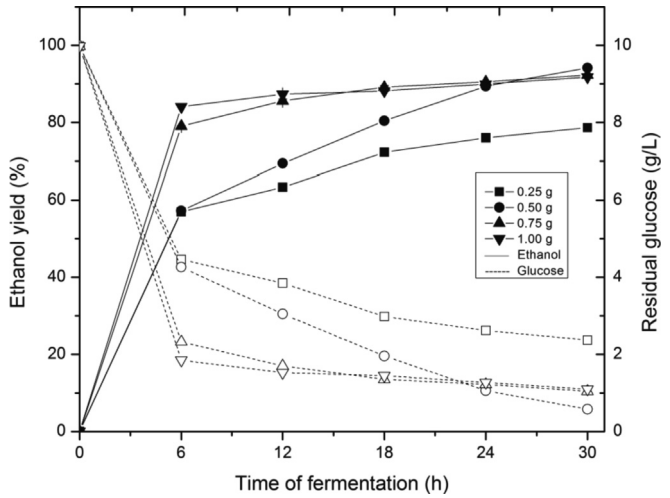


**Fig. 6.** The effect of yeast load on ethanol yield and residual glucose.

in the broth so that the consumption of substrate by yeast cells can be faster [45].

Although the addition of 1.00 g of yeast consumed glucose as the main substrate resulted in the fastest glucose consumption by yeast cells, the ethanol yield obtained at the end of the fermentation only reached 91.78%. Meanwhile, the addition of 0.50 g of yeast reached the optimum ethanol production yield up to 94.21%. In other words, adding a larger amount of dry yeast to the broth did not result in a high ethanol yield. This can be associated with the inefficient glucose conversion to ethanol products due to the presence of yeast. Yeast cells can grow extremely rapidly and abundantly in the fermentation medium [46]. The rapid depletion of glucose in the medium causes cells to lack nutrients for their metabolism and growth so that this condition may shorten the log phase of the yeast cell, and the death phase comes more quickly. Nevertheless, the rapid increase in the concentration of *S. cerevisiae* cells could lead to a shorter fermentation time and direct glucose consumption to produce ethanol [47]. This finding is in accordance with the research conducted by Kumoro et al. [48] on ethanol fermentation with oil palm empty fruit bunches (OPEFB) as a substrate.

## 4. Conclusions

In this study, hydrolysis with alpha-amylase and glucoamylase enzymes on *Chlorella* microalgae substrate under varying conditions of glucoamylase concentrations, the temperature of the process, and medium pH was investigated. Our results showed that the maximum glucose conversion reached 80.01% or 10.07 ± 0.02 g/L under the condition of 3% (v/v) glucoamylase added, medium pH of 5.0, and a temperature of 80 °C for five hours. Meanwhile, the highest hydrolysis productivity rate was 6.83 g/L.h, and occurred in the first one hour. The heat in the hydrolysis process affects the glucose yield obtained. The glucoamylase enzyme is active at temperatures above 80 °C, and when the heat is reduced, the hydrolysis process does not run effectively. With the addition of 50 °C heat, the obtained glucose yield only reached 65.63%, with a productivity rate of 2.89 g/L.h. Hydrolysis using enzymes has proven to be effective in the pretreatment of polysaccharides from the microalgae *Chlorella* into glucose which was used as a substrate in the fermentation process. However, this investigation found that there was an insignificant effect on the concentration of glucoamylase, and further research is needed for cost efficiency in the pretreatment process. After the pretreatment process, glucose hydrolysates of *Chlorella* microalgae were converted into ethanol by employing yeast *S. cerevisiae*. In the fermentation stage, the effect of pH of broth and yeast load was also studied with a fermentation time of 30 h. The maximum ethanol yield reached 94.21% or 4.80 g/L at a pH of 5.0 and a yeast load of 0.5 g for 30 h. At a lower pH of the medium, *S. cerevisiae* cannot convert glucose into ethanol optimally. At a lower pH of the medium, the concentration of hydrogen ions in the fermentation medium can change the charge of the cell membrane of *S. cerevisiae* so that glucose as the main substrate will be inhibited from entering the cell nucleus and affect the conversion process of glucose into ethanol optimally. In addition, the addition of yeast load to the fermentation medium also accelerates the fermentation process due to more cells converting glucose into ethanol. However, the ratio between yeast load and the substrate should be considered as more yeast load will require more substrate for cells' nutrients.

## CRediT authorship contribution statement

**Megawati:** Conceptualization, Methodology, Funding acquisition, Supervision. **Zuhriyan Ash Shiddieqy Bahlawan:** Formal

analysis, Writing- Original draft preparation, Writing – review & editing. **Astrilia Damayanti:** Data curation. **Radenrara Dewi Artanti Putri:** Investigation. **Bayu Triwibowo:** Software, Validation. **Haniif Prasetiawan:** Project administration. **Septian Putra Kusuma Aji:** Resources. **Adi Prawisnu:** Visualization.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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