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The Effect of Acidic pH on Growth Kinetics, Biomass Productivity, and Primary Metabolite Contents of *Euglena* sp.

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Abstract

Euglena is a microalga with the potential to be an environmentally friendly renewable energy resource. The pH value is a crucial factor in micro-algal cultivation. Changes in pH affect the growth and development of microalgae, including the production of biomass and primary metabolites, such as proteins, carbohydrates, and lipids. In this study, *Euglena* sp. was grown on Cramer-Myers medium and subjected to various acidic conditions. This study aimed to determine the effect of pH on the growth kinetics, biomass, carbohydrate, lipid, and protein contents of *Euglena* sp. The *Euglena* sp. culture was optimized at various pH values of 2.5, 3.5, and 4.5. The results were analyzed by one-way analysis of variance at a 95% confidence level, followed by Duncan's multiple range test. As results, *Euglena* sp. had the best growth rate, the greatest biomass, and the highest carbohydrate, protein, and lipid contents at pH 3.5 compared to the other pH conditions. The average biomass in the pH 3.5 treatment was 1.600 ± 0.229 g/L, and the carbohydrate, protein, and lipid contents were 5.983 ± 0.056 g/L, 0.196 ± 0.023 µg/mL, and 0.300 ± 0.020 g/L, respectively.

Keywords: acidic pH, biomass, Euglena sp., growth kinetics

Introduction

Euglena sp. is a promising micro-algal species because it can be used to make biofuels, such as bioethanol and biodiesel [1]. A large amount of biomass is produced during the cultivation of *Euglena* sp., which can be converted into a renewable energy source. Attempts have been made to discover renewable energy sources by utilizing inexpensive but environmentally friendly and promising subjects. *Euglena* sp. has been cultivated and processed into various products due to its high productivity [2]. *Euglena* sp. has the potential to be used as bioenergy or biofuel, such as biodiesel and bioethanol. Additionally, *Euglena* sp. can be processed into functional food sources or food mixtures to improve nutrition, feed, cosmetics, and medicines [3].

High biomass productivity and metabolite content are required to realize these goals. These can be accomplished by optimizing the cultivation of *Euglena* sp. Metabolic processes and the growth of microalgae are strongly affected by environmental factors, such as pH. This study was carried out to culture *Euglena* sp. at several acidic pH values. *Euglena* sp. lives and grows optimally in the pH range of 3–7.4 [3]. Cells grown at pH 5 and 6 have high respiration rates, whereas those grown at pH 7 have the lowest respiration rates [4].

Olaveson *et al*. [5] reported that *Euglena mutabilis* grows rapidly at pH 5.5, with a maximum biomass growth rate and productivity at pH 3–4. Suzuki [6] reported that euglenoids have a high tolerance for acidic environments, as *Euglena* sp. can grow and survive at a pH of 3.5. Contaminants cannot live in the culture at such a low pH, providing *Euglena* with the conditions for survival. Low pH values were chosen for the present study due to a preliminary study using various pH ranges that revealed the best results in the acidic pH range. In addition, the *Euglena* sp. strain used in this study was collected from peat swamps in Dieng, Central Java. This area had acidic peat water at the time of sampling. The degree of acidity plays a role in the absorption of nutrients by cells, as it affects the solubility and availability of mineral ions. Furthermore, pH variations can affect micro-algal growth and development because pH affects metabolism, enzyme activities, the balance of inorganic carbon, and

cell physiology [7]. Previous studies did not provide information related to the effect of an acidic pH on the carbohydrate, lipid, and protein contents of *Euglena* sp. In addition, the use of *Euglena* sp. as a micro-algal model is still uncommon, particularly in Indonesia. As a result, this study was conducted to determine the effect of variations in pH on the growth rate, biomass productivity, and primary metabolite contents of *Euglena* sp.

Materials and Methods

Cultivation of *Euglena* sp. A *Euglena* sp. culture was added to a 500 mL culture vial containing 300 mL of Cramer-Myers (CM) medium [8]. The culture medium was adjusted to pH values of 2.5, 3.5, and 4.5. Our preliminary study using various pH values revealed that the acidic pH range produced the best results.

***Euglena* sp. growth rate.** Cell density is an indicator of the growth rate of micro-algal cells. Optical density (OD) was calculated by measuring absorbance at a wavelength of 680 nm using a spectrophotometer [6]. The chlorophyll content of the *Euglena* sp. cells in the sample was previously determined using a 680 nm wavelength [2]. Thus, this OD measurement was taken every day for the entire 18-day observation period.

Growth kinetics modeling of *Euglena* sp. *Euglena* sp. growth kinetics modeling was carried out using the logistic and Gompertz models. The logistics model predicts the number of stable populations by using the maximum growth rate per day as a parameter. The following formula was used to calculate the logistic model (Equations 1 and 2), where X represents cell density, X_0 represents initial cell density, Xmax represents maximum cell density, and μmax represents the maximum specific growth rate [9].

$$\frac{dx}{dt} = \mu_{max}\left(1 - \frac{x}{\mu_{max}}\right)x \quad (1)$$

Followed by:

$$x = \left(\frac{Xo.\exp(\mu_{max}.t)}{1-[(\frac{Xo}{X_{max}})(1-\exp(\mu_{max}.t)]}\right. \quad (2)$$

The Gompertz model was also used to represent the cell population at the exponential phase. However, the parameters used in this model are more complex, including maximum cell production (r_m) and lag time (t_L). The model was carried out using Equations 3 and 4, where SSR is the sum of squares residual and SST is the total sum of squares [9].

$$x = Xo + \left[X_{max}.\exp[-\exp((\frac{r_m.\exp(1)}{x_{\max}})(t_l - t) + 1\right] \quad (3)$$

Followed by:

$$R^2 = (1 - \frac{SSR}{SST}) \quad (4)$$

Biomass measurement of *Euglena* sp. Biomass was measured once every 3 days during the 18-day cultivation period by weighing the dry biomass. The sample was taken in 2 mL increments and centrifuged for 10 min at 3,300 rpm. The supernatant was removed. The pellets were heated to constant weight in an oven at 30–50 °C and then reweighed on an analytical balance. The following formula was used to calculate dry weight [10] (Equation 5). Biomass productivity was calculated using Equation 6.

$$\text{DW (g/L)} = \frac{\text{total weight} - \text{weight after drying}}{volume\ of\ samples} \quad (5)$$

Biomass productivity was calculated through the following formula (Equation 6):

$$\text{Productivity } (\frac{g}{L}/day) = \frac{\Delta x}{t} \quad (6)$$

Where Δx is the difference in biomass on day t_1 and day t_0 and t is the time interval (days).

Calculation of the growth rate and percentage change in *Euglena* sp. A digital spectrophotometer was used to take the daily OD measurements at 680 nm to calculate cell growth [11]. Additionally, the specific growth rate (μ) and doubling time (T_d) of *Euglena* sp. were calculated using Equations 7 and 8, where OD_t is the change in optical density over time (days), OD_0 is the optical density at time t (days).

(8), N_t is the density at time t, N_0 is the initial density, t_t is the observation time, and t_0 is the start time.

$$\mu = \frac{ln\ OD_t - ln\ OD_0}{\Delta t} \quad (7)$$

$$T_d = \frac{ln\ 121}{\mu} \quad (8)$$

Carbohydrate content analysis. The Phenol Sulfuric Acid method [12] was used to determine carbohydrate content. The supernatant from the centrifuged sample was treated with 0.5 ml of 5% phenol and 1 mL of concentrated sulfuric acid (H_2SO_4) for 10 min at 4 °C. Then, the absorbance was measured at 490 nm with the spectrophotometer. The total carbohydrate levels in standard glucose solutions were calculated using a standard linear regression equation at concentrations of 500, 1,000, 1,500, 2,000, 2,500, 3,500, 4,000, 6,000, and 8,000 μg/mL.

Protein content analysis. The Bradford method was used to determine the *Euglena* sp. protein content. The culture was centrifuged and then an SDS solution was added to the supernatant. Following a 5-min incubation at 95 °C, a 5-min incubation was performed at 4 °C. The incubated samples were treated with Bradford's solution [13]. Absorbance was read at 595 nm using an ELISA

reader. Protein content was calculated from standard bovine serum albumin solutions of 20, 50, and 100 µg/mL using standard linear regression.

Lipid content analysis. The total lipid content of *Euglena* sp. was determined using the Bligh and Dyer method [14]. A 1:2 ratio of chloroform and methanol was used to extract the lipids, followed by a 1:1 ratio of chloroform and DI water [14]. The solution was centrifuged until three layers formed, and the bottom layer was removed and incubated in an oven at 30 °C for 24 hours.

$$\text{Total Lipids (g/L)} = \frac{Final\ sample\ weight - Initial\ sample\ weight}{5\ mL} \quad (9)$$

Data analysis. The results were analyzed by one-way analysis of variance followed by Duncan's multiple range test using IBM SPSS Statistics 23 software (SPSS Inc., Chicago, IL, USA). A p-value < 0.05 was considered significant.

Results and Discussion

The growth rate of *Euglena* sp. *Euglena* sp. experienced differences in growth among the pH treatments by day 10 of culture, indicating that different pH media can affect cell growth and development of euglenoids. Different pH media affect the metabolism and growth of micro-algal culture, as well as the balance of organic carbon, nutrient availability, and cell physiology [15]. The death phase was faster in the pH 2.5 treatment than in the other treatments because of disturbances in nutrient absorption. *Euglena* cells can survive at pH 1.3–2.6 but do not undergo maximum growth [16]. Natasya [17] reported that the pH of the media determines the solubility and availability of mineral ions, which affects the absorption of nutrients by the cells. The low cell density at pH 2.5 was caused by impaired nutrient absorption due to the acidic culture media. Cell walls degrade under a low pH, as these pH conditions activate enzymes that break the bonds between wall-forming polysaccharides, resulting in the loosening of the cell wall structure [18]. The cell density and growth of *Euglena* sp. were determined at a wavelength of 680 nm [19].

According to Figure 1, *Euglena* sp. grew best at pH 3.5. *Euglena* sp. grew slowly at pH 2.5, and the rate began to decline on day 9. *Euglena* grew well at a pH of 4.5, but the resulting OD value was lower than that of cultures with a pH of 3.5. *Euglena* sp. can survive in acidic environments, as previous research has shown that *Euglena* sp. can grow and survive at pH 3.5 [6]. Euglenoids have been reported to have a high tolerance for acidic environments [20]. The pH 3.5 treatment had the highest cell growth rate because of the long log phase. *Euglena* sp. cells are in a constant state of division in the log phase and survived longer in the pH 3.5 culture because it corresponded to acidic medium conditions when isolated. The region in which *Euglena* sp. was originally isolated from (their natural habitat) has low pH (acidic conditions). So, the author suggest that the survival of the *Euglena* sp. was due to that it doesn't need to change its characteristic to adapt with the surroundings, because that was the environment it suitable with. Olaveson and Stokes [5] studied *E. mutabilis* population growth in the pH range of 1.5–9.0 and discovered intense growth at pH 5.5, with maximum growth at pH 3–4.

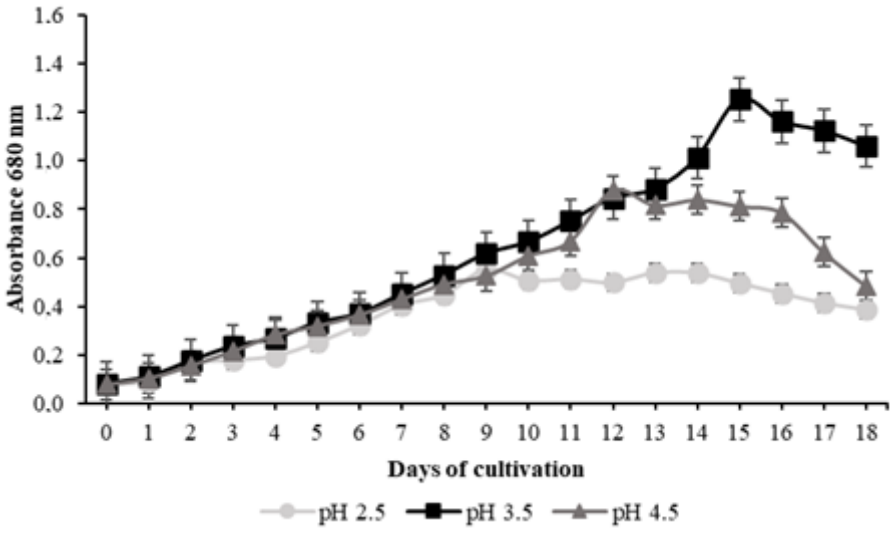

Figure 1. Growth Rate of *Euglena* sp. Cultivated for 18 Days in CM Media at pH Values of 2.5, 3.5, and 4.5

The pH 2.5 cultures had the lowest cell density compared to the other pH treatments. This follows the research of Hargreaves and Whitton [16] who reported that *Euglena* cells can survive at pH 1.3–2.6 but do not experience maximum growth. It also agrees with the treatment conditions in the pH 2.5 medium, in which the cells experienced early death and had the lowest average density. The pH 2.5 medium was toxic to *Euglena* sp., thereby inhibiting cell growth. Drastic changes in the pH of the medium changed cell enzyme activities. Kawaroe *et al*. [20] reported that pH affects the processes of growth, metabolism, and absorption. The highest cell growth rate was observed in the pH 3.5 treatment with an average absorbance value of (1.041 ± 0.159), followed by pH 4.5 and pH 2.5 with average respective absorbance values of (0.733 ± 0.051) and (0.418 ± 0.007). The average absorbance values were significantly different among the different pH treatments at the 95% confidence level.

Table 1 shows the time needed for *Euglena* to double in number, and the specific growth rates at the different pH levels. The specific growth rate at pH 3.5 was 0.104 ± 0.006 per day, resulting in a maximum OD value of 1.041 on day 15 and a doubling time of 1.637 ± 0.120 days. The specific growth rate at pH 2.5 was 0.123 ± 0.003 per day, with a maximum OD of 0.418 on day 9, and a doubling time of 0.693 ± 0.068 days. The specific growth rate at pH 4.5 was 0.061 ± 0.007 per day, with a maximum OD value of 0.733 on day 12, and a doubling time of 1.009 ± 0.088 days.

The level of acidity affects the solubility and availability of mineral ions, which affect nutrient absorption. In addition, variations in pH affect enzyme activities and metabolic processes, including the balance of inorganic carbon and cell physiology as well as the growth of microalgae. A pH change generally affects the biomass, lipid, primary metabolite, and pigment contents [7]. Euglenoids have a high tolerance for acidic environments [20]. According to our findings, *Euglena* sp. had the highest growth rate at pH 3.5. These findings are consistent with previous research that *Euglena* growth is highest at pH 3–7.4 [3]. The *Euglena* sp. in this study died rapidly at pH 2.5. Acidic pH conditions disrupt cell biochemical processes that affect cell growth [22]. This was suspected to be the cause of the low cell density in the pH 2.5 treatment. The pH 2.5 treatment was toxic and disturbed metabolism of *Euglena.* Hargreaves and Whitton [16] stated that pH *Euglena* cells can survive but cannot grow or multiply. The lower pH results in the degradation of cell walls. Low pH conditions activate enzymes that break the bonds between the polysaccharides that comprise the cell walls, causing the cell walls to become loose [23].

Table 1. ***Euglena*** **sp. Growth Parameters Obtained at Different pH values**

Value of pH	Growth rate (μ/day)	Doubling time (Td)
2.5	0.123±0.003[a]	0.693±0.068[a]
3.5	0.181±0.011[c]	1.637±0.120[c]
4.5	0.156±0.006[b]	1.009±0.088[b]

Note. The numbers in the columns are averages ± SDs. Numbers followed by a different letter in a column are significantly different according to Duncan's multiple range test ($p < 0.05$)

The *Euglena* sp. growth curve is presented in Figure 1. Cells undergo adjustment to the new environmental conditions and do not actively divide during the lag phase [24]. Once the cell survives, it undergoes the log phase to actively multiply, and cell density increases [21]. The results showed differences in the growth rates among the pH treatments. The pH 2.5 treatment had the lowest growth rate compared to the pH 3.5 and 4.5 treatments. The pH 3.5 treatment had the highest average cell growth rate because that treatment had the longest log phase.

Suitable kinetics models are needed to understand the dynamics of the growth of micro-algal biomass. These models are used to predict the performance and optimize the photo-bioreactor operating conditions [25]. The logistic and Gompertz nonlinear models were chosen for this study., as these models have been typically used for organisms with rapid population growth [26]. The logistic and Gompertz models are simple models of microbial growth because they are not limited by substrate type or consumption.

The biomass growth rates of *Euglena* sp. at pH 3.5 and 4.5 were fitted to the Gompertz and logistic models (Figure 2), as these treatments had higher OD values than those of the pH 2.5 treatment.

Based on the logistic model, the maximum specific growth rate (μmax) at pH 3.5 was 0.292/day with an R^2 of 0.97. Based on the logistics model, the μmax at pH 4.5 was higher than that at pH 3.5, which was 0.322/day with an R^2 of 0.82. According to the Gompertz model, the maximum cell production rate (rm) of *Euglena* sp. at pH 3.5 was 0.036 cells/mL. The maximum cell production rate (rm) at pH 4.5 was 0.055 cells/mL. The pause times (tl) of *Euglena* sp. at pH 3.5 and 4.5 were −0.533/day and 0.532/day, respectively. Furthermore, the R^2 values at pH 3.5 and 4.5 were 0.99 and 0.81.

Cell density of *Euglena* sp. Table 2 shows **t**he highest cell density was detected at pH 3.5 ($73 \times 10^5 \pm 1.000$ cells/mL) on day 15. The pH 4.5 and 2.5 treatments followed with cell densities of ($65 \times 10^5 \pm 0.578$ cells/mL) on day 12 and ($11 \times 10^5 \pm 3.605$ cells/mL) on day 9. Figure 3 shows the pH 3.5 treatment had the

highest average cell growth rate because that treatment had the longest log phase. The treatment can affect rapid cell growth with high cell density. The pH 2.5 treatment had the lowest cell density compared to the other pH treatments. *Euglena* cells can survive at pH 1.3–2.6 but do not experience maximum growth [16]. This agrees with the conditions of the pH 2.5 treatment, which experienced early death and had the lowest average cell density. The average cell density was significantly different among the treatments at a confidence level of 95%.

***Euglena* sp. Biomass.** Biomass or dry weight was measured once every 3 days during the 18-day culture. An important factor for obtaining high biomass productivity of microalgae is the nutrient content and the culture conditions. The use of certain nutrients in the presence of control of environmental conditions can change production costs and affect the growth or composition of biomass [27, 28]. Variations of nutrients (such as media components) and modifications of environmental factors (such as pH, light intensity, temperature, etc.) will affect the number of micro-algal biomass (dry weight, wet weight of biomass produced) and also the biomass compositions (such as secondary metabolites (Flavonoid, carotenoid, etc.) and primary metabolites (lipid, protein, carbohydrate, etc.) contained in the biomass. The biomass produced during cultivation at the three pH values is presented in Figure 4.

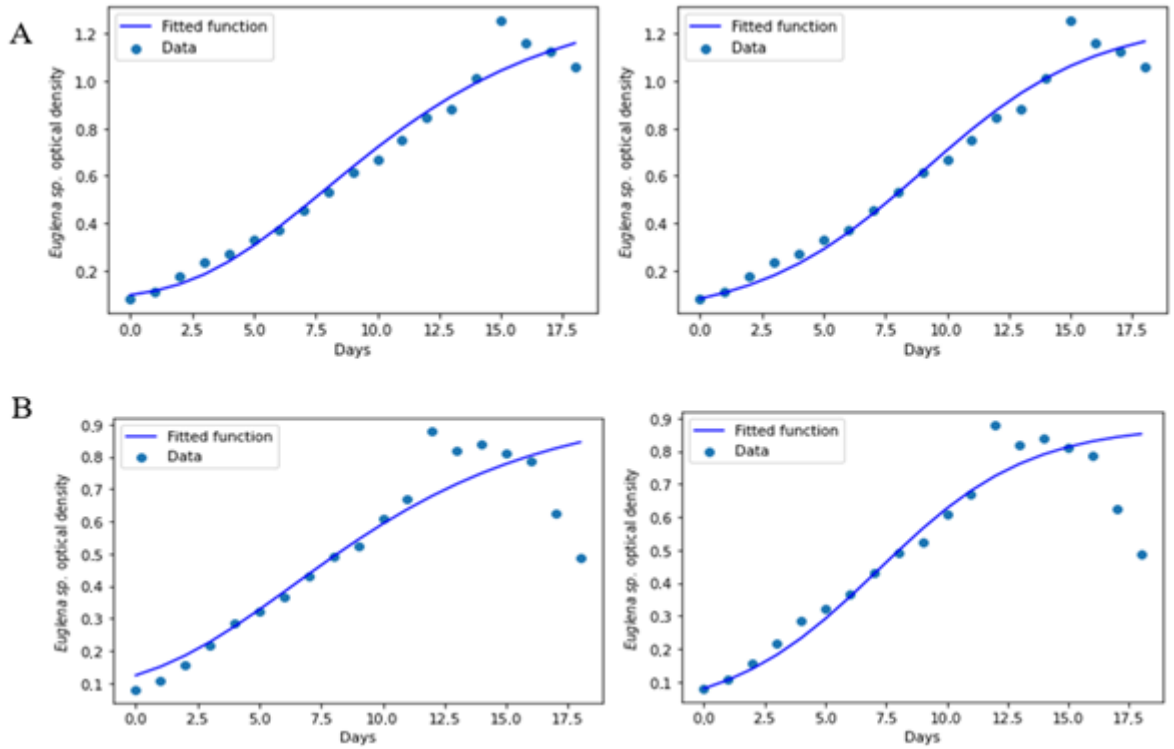

Figure 2. A. Gompertz (Left) and Logistic (Right) Growth Model at pH 3.5. B Gompertz (Left) and Logistic (Right) Growth Model at pH 4.5.

Table 2. *Euglena* sp. Cell Density at Different pH Values

pH	Cell density ± SD* (cell/mL)
pH 2.5	11 x10^5±3.605^a
pH 3.5	73 x10^5±1.000^c
pH 4.5	65 x10^5±0.578^b

Note. The numbers in the columns are averages ± SDs. Numbers followed by a different letter in a column are significantly different according to Duncan's multiple range test ($p < 0.05$)

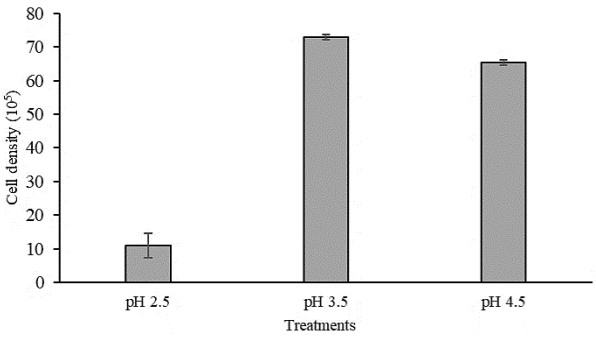

Figure 3. Cell Density of *Euglena* sp. Cultivated in CM at pH Values of 2.5, 3.5, and 4.5 for 18 Days

Figure 4 shows the biomass growth pattern of *Euglena* sp. at pH values of 2.5, 3.5, and 4.5 measured on days 0, 3, 6, 9, 15, and 18. In the pH 2.5 treatment, there was an increase in biomass until day 9 and then there was a decrease from days 12 to 18. The pH 3.5 treatment increased the rate of biomass accumulation until day 15. This correlated with the results of cell growth where cell growth was in the log phase on day 15 and began to decrease on days 16 to 18. Furthermore, there was an increase in biomass at pH 4.5 until day 12, followed by a decrease on days 15 and 18. Table 3 shows that pH 3.5 treatment had the highest biomass with an average biomass of (1.600 ± 0.229 g/L), followed by the pH 4.5 treatment with an average biomass of (1.033 ± 0.333 g/L). The pH 2.5 treatment had the lowest average biomass of (0.467 ± 0.104 g/L). Biomass productivity is the rate of *Euglena* sp. biomass production per day (g/L/day). Biomass productivity was (0.107 ± 0.015 g/L) at pH 3.5, (0.069 ± 0.015 g/L) at pH 4.5, and (0.031 ± 0.007 g/L) at pH 2.5. In addition, the biomass of *Euglena* sp. cultivated at pH 3.5 was higher than that of the other treatments. This is because the log phase lasted longer than the other treatments. The pH of the medium also affected cell density. The pH 3.5 treatment had cultures with higher cell density than those of the other pH treatments [28]. In addition, *Euglena* sp. can survive for longer periods at pH 3.5 treatment because it corresponded to pH condition of the environment (peat water) when *Euglena* sp. was isolated. The region in which *Euglena* sp. was originally isolated (taken) from (their natural habitat) has low pH (acidic conditions). So, the author suggest that the survival of the Euglena sp. was due to that it doesn't need to change its characteristic to adapt with the surroundings, because that was the environment it is suitable with (same conditions as its original wild environment). *Euglena* sp. can survive for longer periods because it corresponds to acidic medium conditions when isolated [29]. The average absorbance values of the treatments were significantly different at the 95% confidence level.

Carbohydrate, lipid, and protein contents of *Euglena* sp. Table 4 shows that the highest carbohydrate content of *Euglena* sp. was found in the pH 3.5 treatment (5.983 ± 0.056 g/L), which had carbohydrate productivity of 0.399 ± 0.003 g/L/day. Carbohydrate productivity is the rate of carbohydrate production by *Euglena* sp. per day (g/L/day). Carbohydrates produced by microalgae can be used as a carbon source during fermentation to produce bioethanol. Carbohydrates are converted to glucose, which is fermented to produce bioethanol [30]. Carbohydrate content was positively correlated with biomass and cell density, suggesting that carbohydrates are abundant because they are the primary metabolites produced by cells. The highest lipid content of *Euglena* sp. was observed in the pH 3.5 treatment (0.300 ± 0.020 g/L), and lipid productivity was 0.020 ± 0.001 g/L/day. Lipid productivity is the rate of *Euglena* sp. lipid production per day (g/L/day). Lipids comprise a certain percentage of the biomass produced, which is in line with why lipids were correlated with biomass. When biomass is high, total lipids are also at high levels [31].

The highest protein content (0.196 ± 0.023 µg/mL) in *Euglena* sp. was detected in the pH 3.5 treatment and protein productivity was 0.013 ± 0.001 µg/mL/day. Protein productivity is the rate of *Euglena* sp. protein production per day (µg/mL/day). According to Wang *et al.* [32] and Hayasi *et al.* [33], pH affects the protein content of *Euglena* sp. during the stationary phase. The stationary phase lasted the longest in the pH 3.5 treatment, so more protein was formed. However, protein

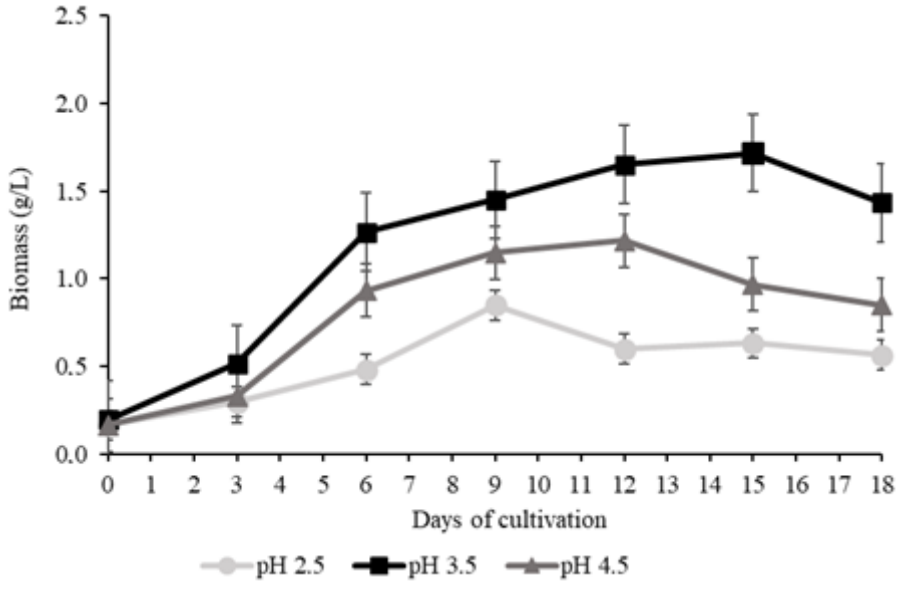

Figure 4. A. Biomass of *Euglena* sp. Cultivated on CM Media at pH Values of 2.5, 3.5, and 4.5 for 18 Days

Table 3. *Euglena* sp. Biomass and Biomass Productivity at Different pH Values

pH	Biomass±SD* (g/L)	Biomass Productivity±SD* (g/L/day)
pH 2.5	0.467±0.104[a]	0.031±0.007[a]
pH 3.5	1.600±0.229[c]	0.107±0.015[c]
pH 4.5	1.033±0.333[b]	0.069±0.022[b]

Note. The numbers in the columns are averages ± SDs. Numbers followed by a different letter in a column are significantly different according to Duncan's multiple range test ($p < 0.05$)

Table 4. *Euglena* sp. Carbohydrate, Lipid, and Protein Contents, as Well as Their Productivities at Different pH Values

pH	Carbohydrate± SD* (g/L)	Carbohydrate Productivity± SD* (g/L/day)	Lipid± SD* (g/L)	Lipid Productivity ± SD* (g/L/day)	Protein ± SD* (µg/mL)	Protein Productivity± SD* (µg/mL/day)
2.5	3.601±0.211[a]	0.240±0.014[a]	0.067± 0.046[a]	0.005±0.003[a]	0.031± 0.024[a]	0.002±0.002[a]
3.5	5.983±0.056[c]	0.399±0.003[c]	0.300± 0.020[b]	0.017±0.001[b]	0.196± 0.023[c]	0.013±0.001[c]
4.5	4.933±0.122[b]	0.329±0.009[b]	0.260 ± 0.125[b]	0.020±0.008[b]	0.139± 0.023[b]	0.009±0.002[b]

Note. The numbers in the columns are averages ± SDs. Numbers followed by a different letter in a column are significantly different according to Duncan's multiple range test ($p < 0.05$)

production was quite low. This was due to the effect of the acidic pH on the denaturation of enzymes. Denaturation of enzymes is a cause of low protein yield. According to Scopes [34], enzymes lose activity due to denaturation, inactivation of the active site, and proteolysis. Extreme pH, temperature, and denaturant compounds contribute to denaturation. *Euglena gracilis* contains 39–61% protein and has been used as a dietary supplement and food source [35]. *Euglena* sp. metabolite content is positively correlated with biomass, as metabolite content is higher when biomass is present in large quantities. The carbohydrate, protein, and lipid contents and their productivities were significantly higher in the pH 3.5 treatment than those in the other treatments.

The pH is one of several factors that affect biomass production by microalgae. Microalgae store chemical energy in the form of carbohydrates, lipids, and proteins to adapt to ambient conditions. The phenol sulfuric acid method was used to assess the carbohydrate content in this study [12]. This method uses 5 % phenol and concentrated sulfuric acid (H_2SO_4). Sulfuric acid breaks down polysaccharides, oligosaccharides, and disaccharides into mono-saccharides. Phenols are used to detect simple sugars contained in the sample. Pentose and hexose compounds are dehydrated and converted to furfural and hydroxymethyl furfural, respectively. These dehydrated compounds react with phenols resulting in a yellow-gold color change. The highest carbohydrate content occurred in the pH 3.5 treatment and the lowest was in the pH 2.5 treatment. The average carbohydrate content was positively correlated with biomass and cell density.

Protein content was determined using the Bradford method with the Bradford solution. SDS was also added, which lyses the cells. SDS is an anionic detergent that coats proteins and provides a negative electric charge to all proteins in proportion to their molecular weight. The Bradford test involves Cromassive Brilliant Blue dye, which binds to the proteins in an acidic solution to give them a bluish color [27]. Protein is the highest constituent component in *Euglena* sp. cells. The highest amount of protein is realized when the cells have reached the peak growth phase, and protein content is positively correlated with cell density. According to Wang *et al*. [32] and Hayashi *et al*. [33], the protein content of *Euglena* sp. is most affected by pH during the stationary phase. The pH 3.5 treatment had the highest protein content because the stationary phase lasted the longest, allowing more protein to form.

The total lipid content of *Euglena* sp. was determined using the Bligh and Dyer method [14]. The Bligh and Dyer method extracts lipids using a 1:2 ratio of chloroform: methanol, followed by 1:1 chloroform: water [14]. The pH 3.5 treatment had the highest lipid content because of the long-lasting log phase with a high cell density so the biomass was also high as well. Biomass is correlated with total lipid content [36].

The biomass of *Euglena* sp. contained lipids suitable for biodiesel production, and the main components were palmitic, linolenic, and linoleic fatty acids [37]. Cultivating microalgae to produce biomass reduces CO_2. Microalgae have a rapid growth rate and life cycle with high productivity per hectare. Furthermore, the bio-

refinery process produces many products based on lipids, carbohydrates, and proteins [38].

Conclusions

Our analysis of the effects of pH on *Euglena* sp. revealed that variations in pH significantly affected the growth rate, biomass productivity, and primary metabolite content of *Euglena* sp. The pH 3.5 treatment had the highest cell growth rate and biomass productivity, as well as carbohydrate, lipid, and protein contents compared to the other treatments. Therefore, further studies are needed to optimize biomass productivity and primary metabolite contents during mass-scale cultivation to produce a large amount of biomass needed for feedstock during bio-refinery activities.

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References

[1] Gissibl, A., Sun, A., Care, A., Nevalainen, H., Sunna, A. 2019. Bioproducts from *Euglena gracilis*: Synthesis and Applications. Front Bioeng. Biotechnol. 7: 108–114, https://doi.org/10.3389/fbioe.2019.00108.

[2] Harun, R., Singh, M., Forde, G.M., Danquah, M.K. 2010. Bioprocess Engineering of Microalgae to Produce a Variety of Consumer Products. Renew. Sust. Energy Rev. 14(3): 1037–1047, https://doi.org/10.1016/j.rser.2009.11.004.

[3] Jones, C.R., Cook, J.R. 1978. Culture pH, CO_2 Tension, and Cell Division in *Euglena gracilis* Z. J. Cell. Physiol. 96(2): 253–259, https://doi.org/10.1002/jcp.1040960214.

[4] Danilov, R.A., Ekelund, N.G.A. 2001. Effects of pH on the Growth Rate, Motility and Photosynthesis in *Euglena gracilis*. Folia Microbiol. 46: 549–554, https://doi.org/10.1007/BF02818001.

[5] Olaveson, M.M. Stokes, P.M. 1989. Responses of the acidophilic alga *Euglena mutabilis* (Euglenophyceae) to carbon enrichment at pH 3. J. Phycol. 25(3): 529–539, https://doi.org/10.1111/j.1529-8817.1989.tb00259.x.

[6] Suzuki, K. 2017. Large-Scale Cultivation of *Euglena*. In Schwartzbach, S.D., Shigeoka S. (eds.), Euglena: Biochemistry, Cell and Molecular Biology, 1st ed. Springer Cham. New York. pp. 285–293.

[7] Almutairi, A.W., Toulibah, H.E. 2017. Effect of Salinity and pH on Fatty Acid Profile of The Green Algae *Tetraselmis suecica*. J. Pet. Environ. Biotechnol. 8(3): 1–6, https://doi.org/10.4172/2157-7463.1000333.

[8] Cramer, M., Myers, J. 1952. Growth and photosynthetic characteristics of *Euglena gracilis*. Arch. Mikrobiol. 17: 384–402, https://doi.org/10.1007/BF00410835.

[9] Phukoetphim, N., Salakkam, A., Laopaiboon, P., Laopaiboon, L. 2017. Kinetic Models for Batch Ethanol Production from Sweet Sorghum Juice Under Normal and High Gravity Fermentations: Logistic and Modified Gompertz Models. J. Biotechnol. 243: 69–75, https://doi.org/10.1016/j.jbiotec.2016.12.012.

[10] Lee, Y.K., Shen, H. 2004. Basic Culturing Techniques. In Richmond, A. (eds.), Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Blackwell Publishing Ltd. Oxford.

[11] Humphrey, I., Chendo, M.A.C., Njah, A.N., Nwankwo D.I. 2021. Optimization of microalgae growth for biofuel production using a new empirical dynamic model. Biofuels. 12(10): 1209–1224, https://doi.org/10.1080/17597269.2019.1608012.

[12] Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F. 1956. Colorimetric Method for Determination of Sugars and Related Substances. Anal. Chem. 28(3): 350–56, https://doi.org/10.1021/ac60111a017.

[13] Bradford, M.M. 1976. A Rapid and Sensitive Method for the Quantitation Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem. 254(1–2): 248–254, https://doi.org/10.1016/0003-2697(76)90527-3.

[14] Bligh, E.G., Dyer, W.J. 1959. A rapid Method for Total Lipid Extraction and Purification. J. Biochem. Physiol. 37(8): 911–917, https://doi.org/10.1139/o59-099.

[15] Wijanarko, A., Dianursanti, Gozan, M., Andika, S.M.K., Widiastuti, P., Hermansyah, H., *et al*. 2006. Enhacement of Carbondioxide Fixation by Alteration of Illumination during *Chlorella vulgaris*-Buitenzorg's Growth. Biotechnol. Bioproc. Eng. 11: 484–488, https://doi.org/10.1007/BF02932071.

[16] Hargreaves, J.W., Whitton, B.A. 1976. Effect of pH on Tolerance of *Hormidium rivulare* to Zinc and Copper. Oecologia. 26: 235–243, https://doi.org/10.1007/BF00345292.

[17] Natasya, G.Y. 2009. Pengaruh Sedimen Berminyak Terhadap Pertumbuhan Mikroalga *Isochrysis* sp. [Thesis]. Institut Pertanian Bogor, Bogor.

[18] Jelizanur, Padil, Muria, S.R. 2019. Kultivasi Mikroalga Menggunakan Media AF6 pada Berbagai pH. Jurnal Online Mahasiswa Bidang Teknik dan Sains. 6(2): 1–5.

[19] Suzuki, K., Mitra. S., Iwata, O., Ishikawa, T., Kato, S., Yamada, K. 2015. Selection and characterization of *Euglena anabaena* var. *minoras* a new candidate *Euglena* species for industrial application. Biosci. Biotechnol. Biochem. 79(10): 1730–1736, https://doi.org/10.1080/09168451.2015.1045828.

[20] Olaizola, M. 2003. Commercial development of microalgal biotechnology: from the test tube to the marketplace. Biomol. Eng. 20(4–6): 459–466, https://doi.org/10.1016/s1389-0344(03)00076-5.
[21] Kawaroe, M., Prartono, T., Sunuddin, A., Wulan, D., Augustine, D. 2010. Mikroalga Potensi dan Pemanfaatannya untuk Produksi Bio Bahan Bakar. Institut Pertanian Bogor Press. Bogor.
[22] Lane, A.E., Burris, J.E. 1981. Effects of Environmental pH on the Internal pH of Chlorella pyrenoidosa, Scenedesmus quadricauda, and Euglena mutabilis. Plant Physiol. 68(2): 439–442, https://doi.org/10.1104/pp.68.2.439..
[23] Andersen, R.A. 2005. Algae Culturing Technique, 1st ed. Elsevier Academic Press. United Kingdom.
[24] Galvao R.M., Santana T.S., Fontes C.H.O., Sales E.A. 2013. Modeling of Biomass Production of *Haematococcus pluvialis*. Appl Math 4: 50–56, https://doi.org/10.4236/am.2013.48A008.
[25] Lam, M.K., Yusoff, M.I., Uemura, Y., Lim, J.W., Khoo, C.G., Lee, K.T., *et al*. 2017. Cultivation of *Chlorella vulgaris* using nutrients source from domestic wastewater for biodiesel production: growth condition and kinetic studies. Renew. Energy 103: 197–207, https://doi.org/10.1016/j.renene.2016.11.032.
[26] Soni, R.A., Sudhakar, K., Rana, R.S. 2017. *Spirulina*-From growth to nutritional product: A review. Trends Food Sci. Technol. 69(Part A): 157–171, https://doi.org/10.1016/j.tifs.2017.09.010.
[27] Morales-Sánchez, D., Martinez-Rodriguez, O.A., Martinez, A. 2017. Heterotrophic cultivation of microalgae: production of metabolites of commercial interest. J. Chem. Technol. Biotechnol. 92(5): 925–936, https://doi.org/10.1002/jctb.5115.
[28] Bhattacharya, I. 2022. Microalgae: An Exquisite Oil Producer. In Zepka, L.Q., Jacob-Lopes, E., Deprá, M. Progress in Microalgae Research-A Path for Shaping Sustainable Futures. Intechopen. Rijeka.
[29] Anam, K. 2010. Pengukuran Kadar Protein dengan Metode Bradford. Institut Pertanian Bogor. Bogor.
[30] Sobari, R., Susanto, A.B., Susilaningsih, D., Rahman, D.Y. 2013. Kandungan Lipid Beberapa Jenis Sianobakteria Laut Sebagai Bahan Sumber Penghasil Biodiesel. J. Mar. Res. 2(1): 112–119.
[31] Wang, Y., Seppänen-Laakso, T., Rischer, H., Wiebe, M.G. 2018. *Euglena gracilis* growth and cell composition under different temperature, light and trophic conditions. PLoS ONE. 13(4): e0195329, https://doi.org/10.1371/journal.pone.0195329.
[32] Hayashi, M., Toda, K., Ishiko, H., Komatsu, R., Kitaoka, S. 1994. Effects of Shifting pH in the Stationary Phase of Growth on the Chemical Composition of *Euglena gracilis*. Biosci. Biotechnol. Biochem. 58(11): 1964–1967, https://doi.org/10.1271/bbb.58.1964.
[33] Scope, R.K. 1994. Proteins Purification, 2nd ed. Spinger-verleg. New York. pp. 246–252.
[34] Becker, E.W. 2007. Micro-algae as source of protein. Biotechnol. Adv. 25(2): 207–210, https://doi.org/10.1016/j.biotechadv.2006.11.002.
[35] Mahapatra, D.M., Chanakya, H.N., Ramachandra, T.V. 2013. *Euglena* sp. as a suitable source of lipids for potential use as biofuel and sustainable wastewater treatment. J. Appl. Phycology. 25: 855–865, https://doi.org/10.1007/s10811-013-9979-5.
[36] Khan, U.I., Othman, M.H.D., Hashim, H., Matsuura, T., Ismail, A.F., Rezaei-DashtArzhandi, M., *et al*. 2017. Biogas as a Renewable Energy Fuel. A Review of Biogas Upgrading, Utilization and Storage. Energy Conversion Manage. 150: 277–294, https://doi.org/10.1016/j.enconman.2017.08.035.
[37] Mondal, M., Goswami, S., Ghosh, A., Oinam, G., Tiwari, O.N., Das, P., *et al*. 2017. Production of biodiesel from microalgae through biological carbon capture: a review. 3 Biotech. 7(99): 1–21, https://doi.org/10.1007/s13205-017-0727-4.