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Research Article

Growth and Metabolite Enhancement of Acidophile *Euglena* sp. Isolated from Indonesia under Different Photoperiod Cycles

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Abstract

Euglena sp. is a unicellular, flagellated microalga considered one of the most promising microalgal feedstock species for biofuels. Reducing the level of liquid waste pollutants can be done biologically by using microalgal organisms. Its metabolites, including lipids, proteins, carbohydrates, and pigments, are appropriate for producing biorefinery products such as biodiesel and jet fuels. They can be isolated from extreme environments, such as highly acidic and ammonia-rich environments, that are not conducive to their proliferation. This study sought to determine the effect of the photoperiod or (light: dark) cycle (24 L:0 D, 12 L:12 D, 14 L:10 D, and 16 L:8 D) on the growth, biomass, metabolite content consisting of lipids, carbohydrates, and proteins, and the rate of CO_2 uptake by *Euglena* sp. As stated previously, the study was conducted by cultivating *Euglena* sp. on a laboratory scale with four photoperiod regimens. The results indicated that optimal growth, biomass content, and metabolite content were obtained with a 24 D:0 L lighting cycle. The control treatment (24 L: 0 D) had the highest biomass productivity (0.032 $g.L^{-1}.day^{-1} \pm 0.004$), lipid content (0.387 $g.L^{-1} \pm 0.031$), protein content (0.542 $mg.Ml^{-1} \pm 0.007$), carbohydrate content (0.409 $x10^4$ $g.L^{-1}$), chlorophyll a (6.237 $g.L^{-1} \pm 0.184$), chlorophyll b (2.838 $g.L^{-1} \pm 0.253$), and total carotenoid (1.566 $g.L^{-1} \pm 0.105$). Full light illumination (24 L:0 D) was significantly producing carotenoid content, including phaeophytin a, phaeophytin b, violaxanthin, 9'-cis-neoxanthin, dino xanthin, and fucoxanthin.

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1. Introduction

Climate change is a global issue that receives significant attention worldwide. This phenomenon is caused by an increase in carbon dioxide (CO_2)-based greenhouse gases (Mondal *et al.,* 2017). Carbon dioxide emissions from power plants, transportation, industrial facilities, and cement production can reach 5 gigatons per year. In addition, fossil fuel combustion and human activities contribute to the increase in CO_2 gas emissions in the atmosphere (Jajesniak *et al*., 2021). In tandem with the rising demand for energy, the annual increase in CO_2 emissions is escalating rapidly. Carbon dioxide emissions increased from 3 tons to 8,230 tons between the 18th and 21st centuries, according to the Carbon Dioxide Information Analysis Center (Iglina *et al*., 2022). Biological materials, specifically microalgae, can assimilate CO_2 efficiently. Microalgae are inherently capable of absorbing CO_2 via the process of photosynthesis. In addition, microalgae can reduce the level of liquid waste pollutants (Dewinta et al., 2020). *Euglena* is a promising variety of microalgae that has the capacity to absorb CO_2 and can be utilized as a renewable energy source (Seckbach and Libby, 1970).

Currently, there is unexploited biodiversity in Indonesia, particularly microorganisms that play an essential function for humans (Erfianti *et al*., 2021; Irawan *et al*., 2023), including microalgae. Microalgae are micro-cell factories that can produce useful compounds such as protein, lipids, carbohydrates, and antioxidants (Zulkarnain *et al*., 2021). *Euglena* sp. is a green microalga in an acidic environment (pH 2.5-3.5); therefore, *Euglena* sp. is called an acidophile microalgae. Due to its ability to produce biofuel-synthesizable lipids, *Euglena* sp. has recently gained widespread industrial application (Nur *et al*., 2023). Under extreme conditions, *Euglena* sp. was effectively isolated as a member of the genus *Euglena.* Dieng Peatland has a pH range of 2.0 to 3.5, which is optimal for the proliferation of *Euglena*. Increasing the production of lipids and fatty acids in *Euglena* sp. through cultivation engineering, metabolic engineering, and genetic engineering are three methods to increase biofuel production (Erfianti *et al*., 2023). He *et al*. (2021) also reported that *E. gracilis* is a potential microalga that contains many high-value products, such as vitamins, amino acids, pigments, unsaturated fatty acids, and carbohydrate paramylon, which are metabolites whose contents vary in response to different extracellular environments. Kato and Nam (2021) demonstrated that the number of cells increased rhythmically when *E. gracilis* was grown under 8 h/8 h light/dark cycles, but the time of maximal growth rate was independent of the external light/dark cycle.

To enhance the proliferation of microalgae, environmental factors such as temperature, pH, salinity, inorganic carbon, and light are crucial (Krzemińska *et al*., 2014). Light becomes a crucial factor capable of regulating the physiology of microalgae (Khoeyi *et al*., 2012). The quality and quantity of this light can determine the availability of energy to be used during photosynthesis, particularly in dark and light conditions, and light intensity. One of the most important factors in favor of microalgae cultivation, particularly *Euglena*, is light and photoperiods. Changes in the amount of light in microalgae are able to induce changes in the microalgae's composition or biochemical processes. It is believed that increasing the frequency of light-dark cycles increases the efficiency and productivity of photosynthesis (Grobbelaar, 2009). Microalgae, such as *Chlamydomonas reinhardtii* (Merchant *et al*., 2012; Scaife *et al*., 2015), *Chlorella sorokiniana* (Gao *et al*., 2022), *Dunaliella tertiolectra* (Minhas *et al*., 2023), *Nannochloropsis oculata* (Minhas *et al*., 2023), and *Chlorella vulgaris* (Levasseur *et al*., 2018), have been cultivated through the modification of the photoperiod cycle to increase their growth, biomass, and bioactive compounds. Amelia *et al.* (2023) reported that *Dunaliella* sp. was successfully cultivated by mixing with *Azospirillum* sp. under light stress to enhance astaxanthin content.

In a previous report by Mangal *et al.* (2022), they used *E. gracilis* with a 16:8 light/dark photoperiod at a light intensity of 96 µmol photons m^{-2} s^{-1}, and a pH of 3.5 in an environmental chamber. This treatment was to increase the viability of *E. gracilis*. Hagiwara *et al.* (2002) reported that the phenomenon of photoperiodic regulation of the cell division cycle was observed in the photoautotrophic organism *E. gracilis*. When algae that were exposed to 24-hour light-dark cycles (with 14 hours of light) were moved to a continuous period of darkness at the eighth hour of the last light-dark cycle, the progression of the cell cycle was halted at either the G1, S, or G2 phase. In addition, reports related to the effect of photoperiod on the improvement of primary and secondary metabolites of indigenous *Euglena* from Indonesia are still limited.

E. gracilis is regarded as a promising candidate for the development of a photosynthetic biorefinery (O'Neill *et al*., 2015), and companies such as Euglena Co. Ltd. (Suzuki, 2017) and Algaeon Ltd. are currently

producing *E. gracilis* biomass and purified β-1,3-glucan for the food and feed industries (Wang *et al.*, 2018). Yoshioka *et al.* (2020) reported that *E. gracilis* is a unicellular eukaryotic microalga found in aquatic environments. It is able to adapt its morphology in response to environmental stressors such as temperature, radiation, and ion concentration changes. *E. gracilis* cells excrete succinate and amino acids under conditions of darkness and anaerobic conditions. Richter *et al.* (2014) also demonstrated that *E. gracilis* can tolerate various light profiles. Due to their ability to live in dark or light conditions, *Euglena* sp. is suggested as a potential candidate to produce high lipids and other metabolites such as protein, carbohydrates, and pigments with low energy consumption for cultivation. This study was the first to isolate and characterize the local acidophile microalgae *Euglena* sp. isolated from Dieng Plateau, Central Java, Indonesia, and optimize the strain using dark or light illumination to enhance the growth, biomass, and metabolite contents of microalgae.

Indigenous *Euglena* sp. isolated from Dieng has unique characteristics. It can be grown in acidic conditions, which are around 2.5-3.5. Considering this potential, *Euglena* also has the ability to break down many organic carbon sources even in the presence of light (Ogbonna *et al.*, 1998), thus, it has been studied under both autotrophic and heterotrophic conditions. Photoautotrophic cultivations of *E. gracilis* can yield greater than 3 g L^{-1} of biomass (Grimm *et al.*, 2015). Under favorable growth conditions, the specific growth rate of *E. gracilis* in autotrophic cultures is generally 1.1 d^{-1} (Kitaya *et al.*, 2005). Through the regulation of photoperiod, it can influence metabolite biosynthesis in microalgae, similar to wavelength and light intensity. It is believed that the presence of a moderately dark period facilitates the accumulation of lipids (Chandra *et al.*, 2021). This study was designed to evaluate the ability of the indigenous strain *Euglena* sp. from Indonesia grown under different photoperiod regimes to enhance their metabolite production, including lipids, proteins, carbohydrates, and pigments (chlorophyll a, b, and carotenoid).

2. Materials and Methods

Microalgae *Euglena* sp. was isolated from Dieng Plateau, Wonosobo, Central Java, Indonesia. The CM medium (Cramer and Myers) was used to cultivate microalgae on a lab scale. The composition of the CM medium includes $(NH4)_2SO_4$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $Fe_2(SO_4)_3 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $CoSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $Na_2MoO_4 \cdot 2H_2O$, Vitamin B1, and Vitamin B12. The medium was sterilized using an autoclave at 121ºC for 15 minutes.

2.1 Microalgae Cultivation

The culture of *Euglena* sp. was added to a 500-mL culture vial that included 300 mL of CM (Cramer-Myers medium) medium with four photoperiod cycles, or light and dark (24 L:0 D, 12 L:12D, 14 L:10 D, and 16 L:8 D). In addition, several environmental factors need to be considered, such as light, aeration, and temperature. The light intensity given was 180 µmol/m^2/second for 24 hours.

2.2. Measurement of the Growth

Growth curve measurements of *Euglena* sp. were done by measuring optical density (OD) every day using a Genesys UV-Vis spectrophotometer at 680 nm and 860 nm. The density of the cells was calculated using a spectrophotometer with a wavelength of 680 nm and optical density based on absorbance. One milliliter of material was obtained, deposited in a cuvette, and quantified using a spectrophotometer. Distilled water is employed as an inert solution. The doubling time (td) was calculated based on the following formula:

$$T_d = \ln 2t / \ln (N_t/N_o) \quad \text{....Eq 1}$$

Note:

t = time interval
Nt = number of cells acquired during the final phase of the exponential phase
N0 = number of cells acquired during the beginning of the exponential phase

Furthermore, the specific growth rate (µ) was calculated using the following formula by Lee and Shen (2004).

$$\mu = 0.693/t_d \quad \text{.....Eq 2}$$

2.3 Growth Kinetic Modeling

For predicting photobioreactor performance and optimizing operating conditions, kinetic modeling can be utilized. Appropriate kinetic modeling is useful for understanding the dynamics of microalgal biomass growth (Galvão *et al.*, 2013). For rapid population growth in an organism, the Logistic and Gompertz nonlinear models are commonly employed (Lam *et al.*, 2017). The Logistic and Gompertz model were the

most straightforward model for microbial proliferation because it was not constrained by substrate type or consumption. Therefore, Logistic and Gompertz were chosen for modeling the growth kinetics of *Euglena* sp. The logistic model predicts the number of stable populations based on the maximal daily growth rate. This formula was used to calculate the logistic model. max is the maximal specific growth rate (Phukoetphim *et al*., 2017; Hanief *et al*., 2020).

Logistic Model:

$$\frac{dX}{dt} = \mu_{max}\left(1 - \frac{X}{X_{max}}\right)X \quad \text{Eq 3}$$

$$X = \frac{X_0 exp\ (\mu_{max}\ t)}{1-\left[\frac{X}{X_{max}}(1-exp\ (\mu_{max}\ t)\right]} \quad \text{Eq 4}$$

Additionally, the Gompertz model is used to calculate the cell population during the exponential phase. However, this model uses more complex parameters, such as maximal cell production (*rm*) and lag time (*tL*).

$$X = Xo + \left[X_{max}.exp\left[-exp\left(\left(\frac{r_m.exp(1)}{X_{max}}\right)(t_L - t) + 1\right)\right]\right] \quad \text{Eq 5}$$

SSR is the sum square residual, and SST is the sum square total (Phukoetphim *et al*., 2017; Hanief *et al*., 2020). This formula was used to determine the model (Phukoetphim *et al*., 2017; Hanief *et al*., 2020).

$$R^2 = \left(1 - \frac{SSR}{SST}\right) \quad \text{Eq 6}$$

2.4 Biomass Measurement

The biomass of *Euglena* sp. was determined using filter paper. Previously, filter paper was measured using an analytical balance. Then, 5 mL of culture samples were withdrawn and inserted into the tube. The samples are then centrifuged at 4000 revolutions per minute for 15 minutes. To produce the natant, the supernatant was discarded. The samples were then desiccated in an oven at 40°C until the weight of each tube was constant. The biomass was measured three times, and the average of the results was calculated. This dry biomass was calculated every day. The following formula was used to calculate biomass (Richmond, 2004):

$$Biomass\ \left(\frac{g}{L}\right) = \frac{(Filter\ Paper\ Final\ Weight) - (Filter\ paper\ Initial\ Weight)}{(Sample\ Volume)} \quad \text{Eq 7}$$

Biomass productivity was calculated through the following formula:

$$Productivity\ \left(\frac{g}{L}/day\right) = \frac{\Delta x}{t} \quad \text{Eq 8}$$

Notes:

Δx = Difference in biomass on day t1 and day t0

t = Time interval (day)

2.5 Measurement of Lipid Content

The lipid content was measured using a modified version of the Bligh and Dyer method (1959). Each 15 mL sample was collected and placed in a conical vial. The samples were centrifuged for 15 minutes at 4°C and 4000 rpm. The supernatants were then extracted, leaving particles behind. As much as 2 mL of methanol reagents and 1 mL of chloroform were added to the particles. They were then vortexed for one minute. After adding 1 mL of chloroform and 1 mL of distilled water, the mixture was vortexed for one minute. After 15 minutes of centrifugation, three strata were formed. The clear solution (upper layer) was removed, and the yellow liquid (lower layer) was poured into a petri dish. The Petri dishes were heated for one day in the oven. The lipid content is then calculated using the following formula:

$$Lipid\ content\ \left(\frac{mg}{ml}\right) = \frac{weight\ of\ extracted\ total\ lipid}{weight\ of\ alga\ cells - culture\ volume} \quad \text{Eq 9}$$

2.6 Protein Content Analysis

Protein content was analyzed using the method according to Bradford (1976), with some modifications according to Chia *et al.* (2019). The standard protein used is Bovine Serum Albumin (BSA) protein. The concentrations used include 1, 2, 3, 4, 5, 6, 7, and 8 ppm. The tool used is the ELISA Reader BioTek at a wavelength of λ595 nm. A total of 2 mL of sample was taken, put into a microtube, and centrifuged at 3000 rpm for 10 minutes. Centrifugation was carried out to separate the pellet and supernatant phases. The pellet is then taken, and 1 mL of a 10% SDS solution is added. The sample was then heated at 95°C for five minutes.

Then the samples were incubated at 4ºC for five minutes. Samples were taken as much as 8 µL and put into the microplate. Afterward, 200 µL of Bradford's solution was added to the sample, and readings were carried out using the BioTek ELISA Reader at a wavelength of λ595 nm.

2.7 Carbohydrate Measurement

The phenol-sulfate method was used to measure carbohydrate content (Dubois *et al*., 1956), with some modifications according to Nurafifah *et al.* (2023). A total of 10 minutes were spent centrifuging samples at 3000 rpm for 10 minutes. The supernatant was separated from the cells. The pellets were mixed with 0.5 ml of a 5% phenol solution. After homogenization and incubation for 10 minutes, the samples were analyzed. The granules were then mixed with 1 mL of H_2SO_4 and homogenized. Twenty minutes were spent incubating the samples. At 490 nm, a spectrophotometer measured the concentration of the samples in a cuvette containing the samples.

2.8 Chlorophyll and Total Carotenoid Measurement

Carotene and chlorophyll a and b concentrations were determined by collecting 5 mL of the samples. The samples were centrifuged, and the supernatant was separated. The remaining pellets were then homogenized using a vortex. Afterward, 2 mL of methanol was added to the remaining particles. After incubating the sample for 24 hours in a refrigerator, absorbance measurements were conducted using UV-vis spectrophotometry at wavelengths of 480 nm, 652 nm, and 665 nm. The absorbance results were calculated using the following equation:

$$\text{Chlo-a}((\mu g)/L)=11.85\times(\lambda 664)-1.54\times(\lambda 647)-0.08\times(\lambda 630) \quad \text{Eq 10}$$

$$\text{Chlo-b}((\mu g)/(L))=21.03\times(\lambda 647)-5.43\times(\lambda 664)-2.66\times(\lambda 630) \quad \text{Eq 11}$$

Note
Chlo-a : Chlorophyll – a content (µg/L)
Chlo-b : Chlorophyll – b content (µg/L)
λ664 : Absorbance value at a wavelength of 664 nm
λ647 : Absorbance value at a wavelength of 647 nm
λ630 : Absorbance value at a wavelength of 630 nm

$$\text{Carotene}((\mu g)/L)=4\times(\lambda 480nm) \quad \text{Eq 12}$$

Note
Carotene: Carotene Content (µg/L)
λ480 : Absorbance value at a wavelength of 480 nm

2.9 Measurement of Carotenoid Content

A total of 5 mL samples were taken for the extraction of carotenoid content. Samples were centrifuged at 2000 rpm using a centrifuge. Samples were previously extracted using ethanol and methanol. Afterwards, samples were incubated overnight in dark conditions. Carotenoid derivative pigments were analyzed using a spectrophotometer (400–700 nm), according to Thrane *et al.* (2015).

2.10 Statistical Analysis

The growth rate, biomass, carbohydrate, lipid, and protein content of *Euglena* sp. was analyzed using ANOVA, and mean differences were analyzed using the Duncan Multiple Range Test (DNMRT) using IBM's Statistical Product and Service Solutions (SPSS) software (Version 26, IBM Corporation, USA). The significance of the data was determined by a p-value of 0.05 at a significant value is 95%. Data of carotenoid derivative pigments were analyzed using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/).

3. Results and Discussion

3.1 Cell Growth Characteristic

Euglena sp. reached the exponential phase on the 2nd day of cultivation. On the 10th day, the cell entered the stationary phase, in which the cell density remained constant. Notably, the accumulation of metabolites within the cells causes the biomass concentration to continue to increase. The control treatment, which was full light for 24 hours, showed the best growth curve, followed by the P3 treatment (18 L:6 D) (Figure 1). On the other hand, the P1 (12 L:12 D) and P2 (14 L:10 D) treatments resulted in the lowest optical density. Overall, the optical density trend steadily increased from day 0 to day 10 in all treatments. From day 10 to day 11, it has entered the stationary phase.

The number of cells was measured using a hemocytometer. The highest cell number was in the 24-hour illumination (57 x 10^4 cells/mL), followed by P3 treatment (48 x10^4 cells/mL), P2 treatment (43 x10^4 cells/mL), and P1 treatment had the lowest cell density (31 x10^4 cells/mL) (Figure 2). The cells of *Euglena* sp. had a spherical shape, were green, and contained an eye spot, which had an orange or red color as a photoreceptor. *Euglena* sp. has a length of around 9.91–13.62 µm (Figure 3).

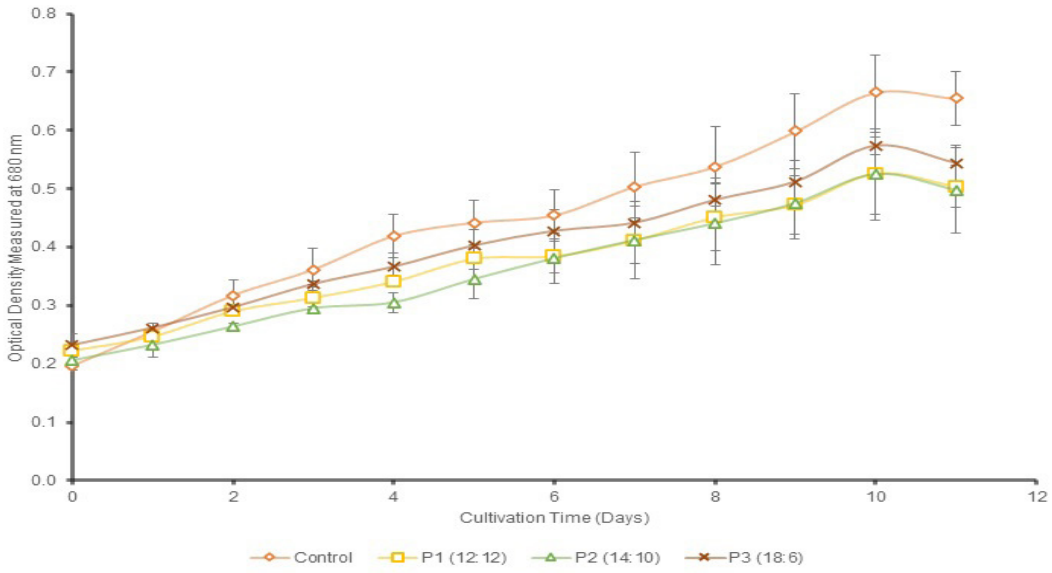

Figure 1. Growth of *Euglena* sp. on various photoperiod cycle

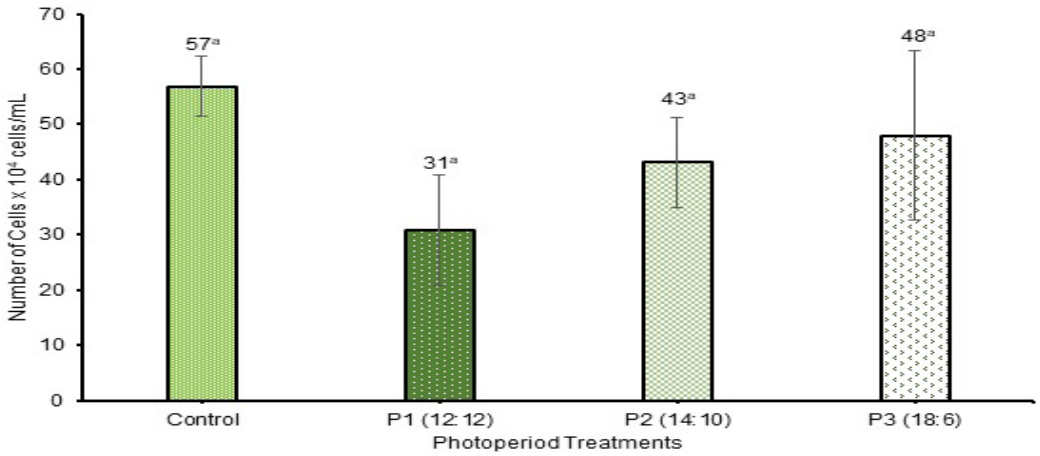

Figure 2. Number of cells of *Euglena* sp. on various photoperiod cycle.

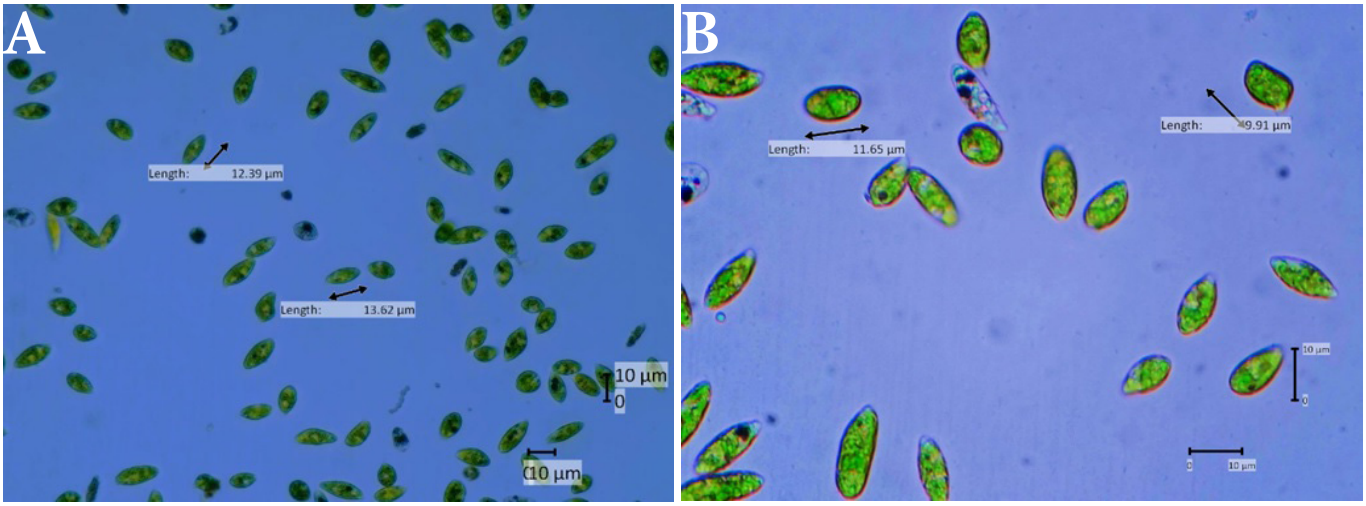

Figure 3. *Euglena* sp. cells under microscopic observation; (a) under 20x magnification; (b) under 40x magnification

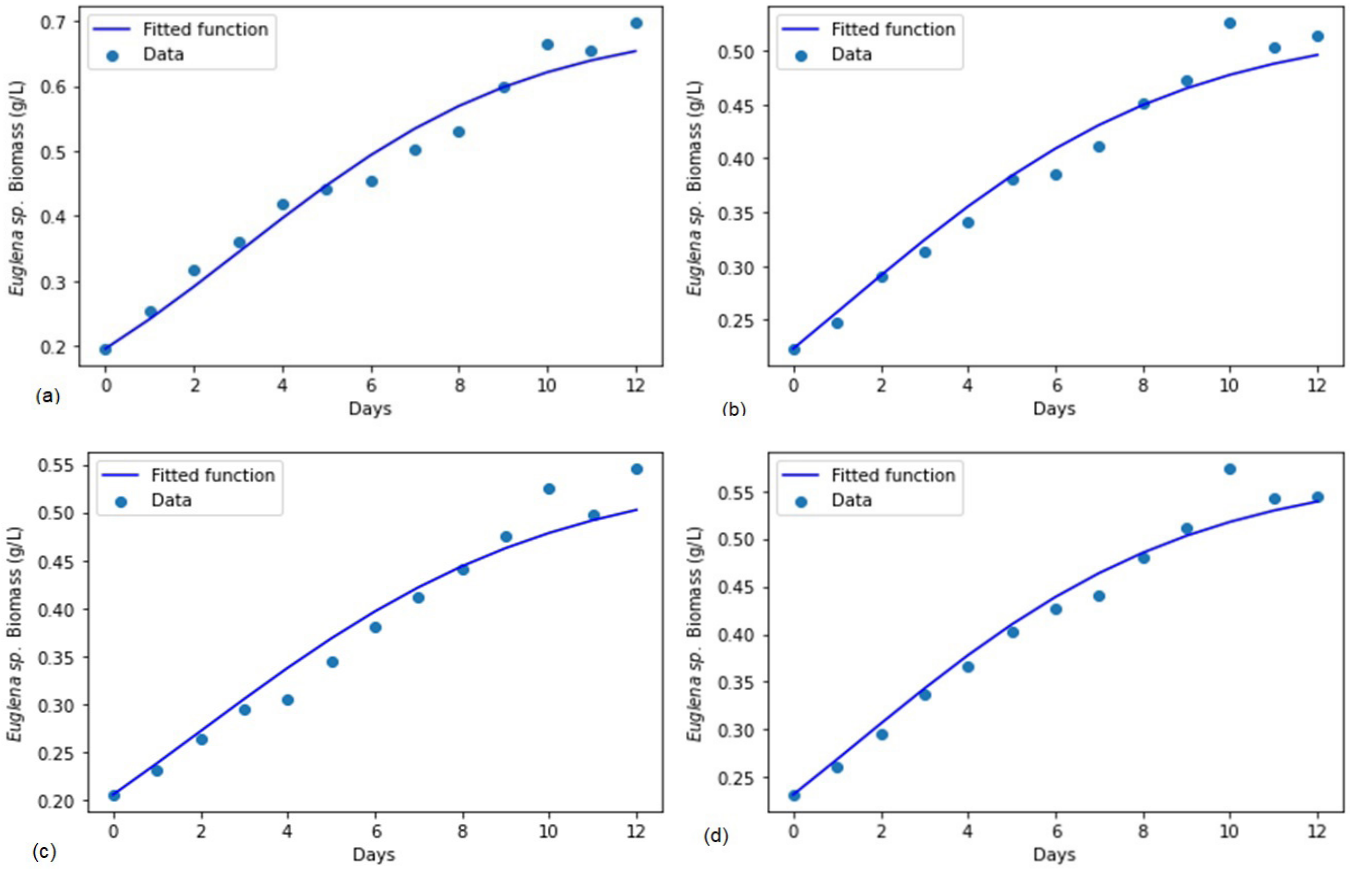

Figure 4. Fitting of Logistic Model of *Euglena* sp. (a) control treatment (24 L: 0 D); (b) P1 treatment (12 L: 12 D); (c) P2 treatment (14 L: 10 D); (d) P3 treatment (16 L: 8 D).

Cell growth of *Euglena* sp. was observed based on optical density (OD). OD was calculated using a spectrophotometer at a wavelength of 680 nm. According to Suzuki *et al.* (2015), the growth rate of the *Euglena* genus was evaluated based on the optical density at a wavelength of 680 nm (OD_{680}) using a spectrophotometer. In this study, microalgae grown under various photoperiod cycles increased until day 10 and then decreased. 24 hours of continuous illumination showed the highest OD value compared to other cycles. On the other hand, P2 treatment presented the lowest growth of *Euglena* sp. The number of cells showed that the control treatment also produced the highest cell number (57 x 10^4 cells/mL) compared to other treatments. Similar to this result, Vélez-Landa *et al.* (2021) reported that under 24 hours : 0-hour (light : dark) cycles, produced higher biomass in *Verrucodesmus verrocusos*. Kishore *et al.* (2018) demonstrated that light intensity and photoperiod are vital parameters for the cultivation of microalgae. Borowitzka (2018) also reported that microalgae use light as an important source of energy for synthesizing the cell protoplasm, and light intensity clearly affects microalgae growth. According to morphological observation, our results indicated that *Euglena* sp. cells had a spherical shape, with the length of the cell around 9.91–13.62 µm. These results are in agreement with previous findings by Al-Ashra *et al.* (2014) demonstrating that the cells of *Euglena gracilis* are cylindrical or spindle-shaped, slightly attenuated and rounded anteriorly, narrowing posteriorly, and terminating in a short, non-hyaline, obtuse apex.

3.2 Growth Kinetic Modeling

Based on logistic modeling, the maximum specific growth rate (µmax) of 24:0, 14:10, 12:12, and 16:8 was 0.304 day^{-1}, 0.246 day^{-1}, 0.259 day^{-1} and 0.261 day^{-1}, respectively (Figure 4). The R^2 errors were 0.968, 0.958, 0.965, and 0.970 for 24 L:0 D, 14 L:10 D, 12 L:12 D, and 16 L:8 D photoperiod treatments of *Euglena* sp., respectively. The results showed the logistic fitting model for photoperiod treatment of *Euglena* sp. experimental growth data (Table 1). In addition, the values of the coefficient of determination R^2 established the goodness of fit of the logistic model in the study. In the growth kinetic modeling, X is cell density, X_0 is the initial cell density, X_{max} is maximum cell density, and µmax is the maximum specific growth rate (Phukoetphim *et al.*, 2017; Hanief *et al.*, 2020).

Our result indicated that the growth rate data from the treatment 24L:0D and 16L:9D are suitable data for the logistic model.

Table 1. Growth rate parameter of logistic model of *Euglena* sp.

Parameter	24L: 0D	12L: 12D	14L: 10D	16 L: 8D
μ_{max}	0.304	0.259	0.246	0.261
R^2	0.968	0.965	0.958	0.970

Growth kinetic modeling of *Euglena* sp. under photoperiod treatments to analyze the biomass growth of microalgae by kinetic modeling needs to be developed for predicting the performance and optimization of photobioreactor operating conditions (Galvão *et al.* 2013). Logistic and Gompertz models of the two non-linear models were suitable for rapid population growth of organisms, such as microalgae (Lam *et al.*, 2017). Since it is not limited by substrate type or consumption, the Logistic and Gompertz models are the simplest and can be used for general microalgal growth rates. In this study, the logistic model was used to predict the monoculture of *Euglena* sp. The logistic model is used to predict the number of stable populations using the maximum growth rate per day as its parameter.

3.3 The Effect of the Photoperiod Cycle on the Biomass of Euglena sp.

Biomass was measured to evaluate the dry weight of *Euglena* sp. after cultivating under photoperiod cycles. Based on the result above, the highest biomass productivity was reached by 24 hours of full light (0.032 ± 0.004), followed by P3 (16 L:8 D) reaching 0.021 ± 0.004, P2 (14 L:10 D) reaching 0.022 ± 0.005, and P1 (12 L:12 D) reaching 0.021 ± 0.004 (Table 2). In addition, the doubling time (Td) of the P2 treatment (14 L:10 D) was higher than the other photoperiod treatments (Td = 8.667 ± 1.101). For specific growth rate (SGR), control treatment had the highest SGR value (0.105 ± 0.010), followed by P3 treatment (16 L:8 D), P1 treatment (12 L:12 D), and P2 treatment (14 L:10 D). According to the ANOVA test, biomass productivity in the control treatment was significantly different from other treatments ($p < 0.05$). While the SGR values in the control treatment and P3 treatment (16 L:8 D) were in the same group, however, for doubling time, control treatment, P1, and P3 were in the same group.

The result of this study showed that the biomass content of 24 hours (continuous light) was the highest, followed by 16 L:8 D. In addition, the treatment of 12 L:12 D and 14 L:10 D was almost similar for biomass production (Table 2). Such an effect was previously demonstrated by Krzemińska *et al.* (2014), who found that the maximum biomass productivity of microalgae (*Botryococcus braunii* and *Scenedesmus obliquus*) was 0.155 and 0.150 g L^{-1} day^{-1} under continuous light conditions. The photoperiod is also an important factor in increasing algal biomass. There are several reports on the influence of the photoperiod on the productivity and growth rate of *C. vulgaris* (Khoeyi *et al.*, 2012), *Dunaliella* spp. (Janssen, 2002), the biomass concentration of *B. raunii* (Ruangsomboon, 2012), and the biomass growth of *S. obliquus* (Mata *et al.*, 2012).

Table 2. Effect of photoperiod cycle on biomass productivity, specific growth rate (μ) and doubling time (T_d) of *Euglena* sp.

Photoperiod Treatments	Biomass Productivity	SGR (μ)	Doubling Time (T_d)
Control (24 L:0 D)	0.032 ± 0.004^{a}	0.105 ± 0.010^{a}	6.600 ± 0.624^{a}
P1 (12 L:12 D)	0.021 ± 0.004^{b}	0.085 ± 0.009^{b}	8.200 ± 1.044^{ab}
P2 (14 L:10 D)	0.022 ± 0.005^{b}	0.080 ± 0.010^{b}	8.667 ± 1.101^{b}
P3 (16 L:8 D)	0.023 ± 0.002^{b}	0.090 ± 0.010^{ab}	7.700 ± 0.964^{ab}

3.4 Effect of Different Photoperiod Cycles on Lipid, Protein, and Carbohydrate Content

In this study, the effect of photoperiod on *Euglena* sp. was tested to determine the optimum light and dark cycles for producing the highest lipid content. Cells grown under 24 hours of full light showed the highest amount of lipid content, 0.287 $g.L^{-1}$ (Figure 5). The result showed that lipid content under various photoperiod cycles ranged from 0.387 $g.L^{-1}$ to 0.287 $g.L^{-1}$ (Figure 5). The content of lipids was significantly ($p < 0.05$) different in the control group, P1 treatment (12 L:12 D), and P3 treatment (16 L:8 D) compared with P2 treatment (14 L:10 D).

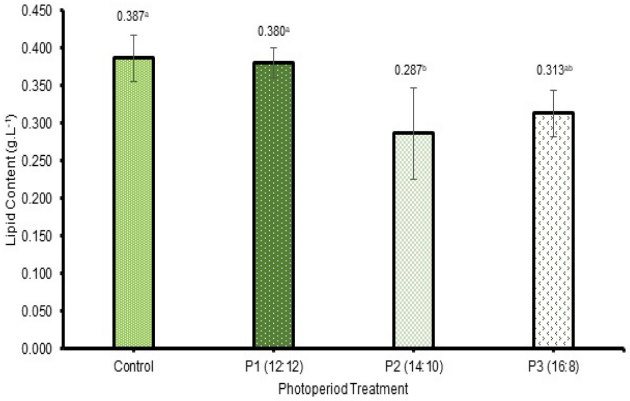

Figure 5. The content of lipid under different photoperiod cycle during cultivation of *Euglena* sp.

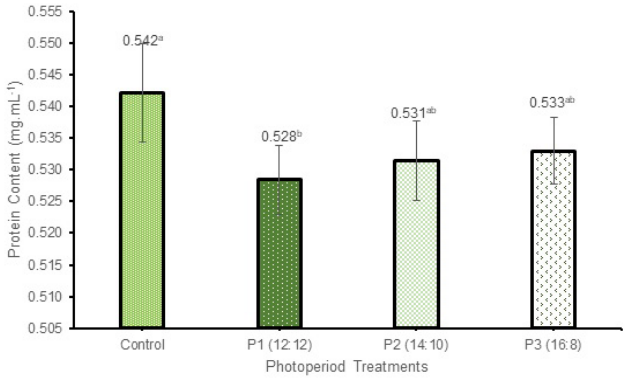

Figure 6. The content of protein under different photoperiod cycle during cultivation of *Euglena* sp.

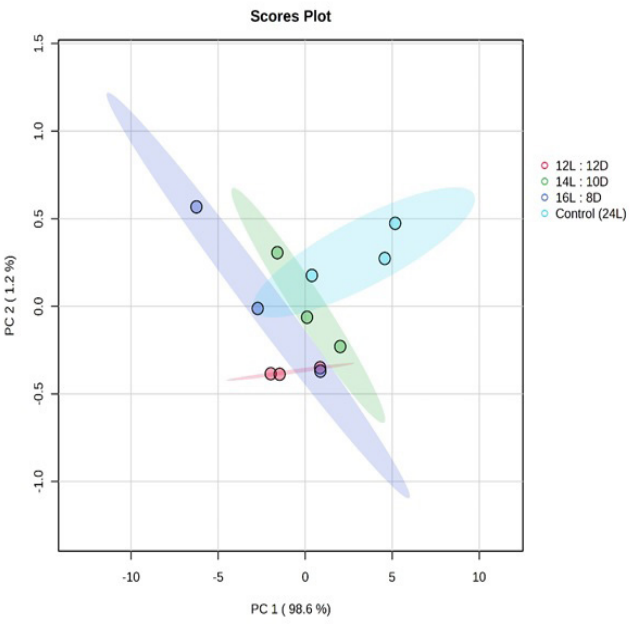

Figure 9. Principal component analysis (PCA) of the contents of the accessory pigments in photoperiod cycles at all experimental stages.

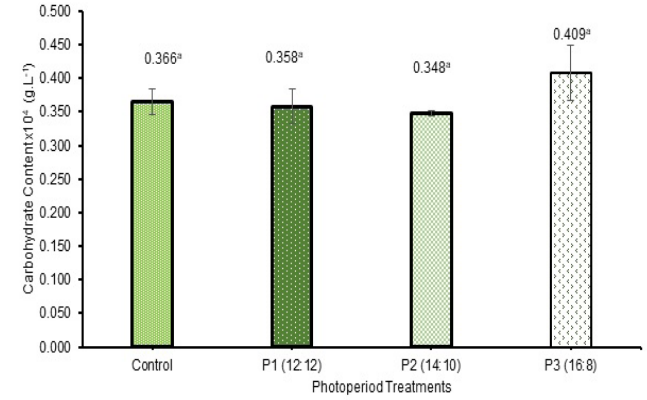

Figure 7. The content of carbohydrate under different photoperiod cycle during cultivation of *Euglena* sp.

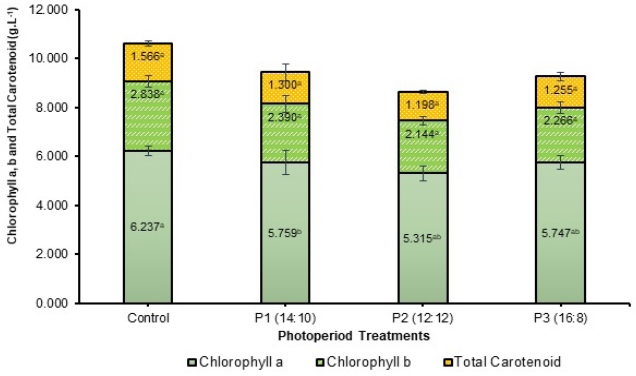

Figure 8. Chlorophyll a, b and total carotenoid under different photoperiod cycle during cultivation of *Euglena* sp.

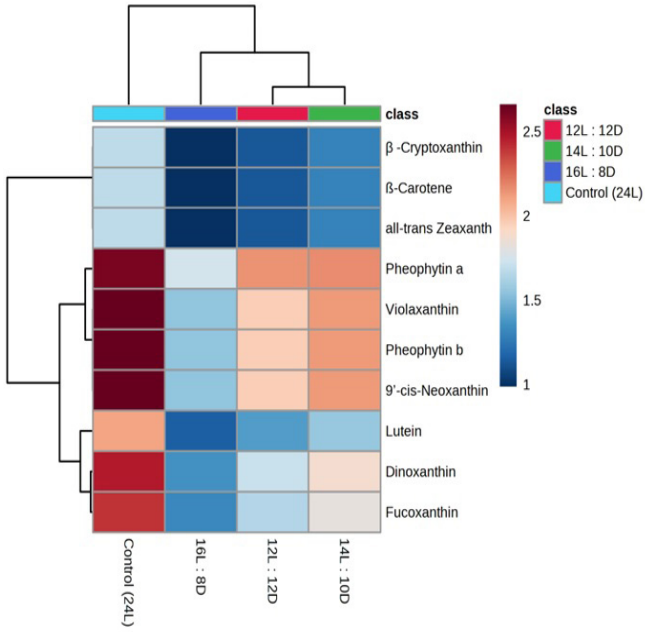

Figure 10. Heatmap of accessory pigments of *Euglena* sp. under different photoperiod cycles.

As presented in the study result, the total protein content values varied by 0.542 mg.mL^{-1}, 0.531 mg.mL^{-1}, and 0.528 mg.mL^{-1}, respectively (Figure 6). This result showed that the length of illumination (24 L:0 D) can significantly affect the protein content. Biosynthesis protein was decreased in the long dark period (12 L:12 D). According to the study result, the highest protein content was in the control treatment (24 L: 0 D), which reached 0.542 mg.mL^{-1}, followed by P3 (16 L:8 D), which reached 0.533 mg.mL-1, and P2 (14 L:10 D), which produced 0.531 mg.mL-1 (Figure 3). On the other hand, the lowest protein content was achieved by P1 (12 L:12 D) with a protein amount of 0.528 mg.mL^{-1}. This result indicated that the protein content was significantly affected by the length of illumination or light. Another research done by Seyfabadi *et al.* (2011) also reported that the protein content was the highest (54,92%) at a photoperiod of 20L:4D. Similar studies on *C. vulgaris* and *Ankistrodesmus falcatus* were conducted by George *et al.* (2014), who discovered that protein production increased with the increase in illumination duration and that the highest protein content was also obtained at high light intensity and long light exposure times.

Euglena sp. produced the highest carbohydrates during the half-period of light and dark (12 L:12 D). The range of carbohydrate content produced by *Euglena* sp. varied to 0.502x10^4 g.L-1, 0.498x10^4 g.L-1, 0.489x10^4 g.L-1, and 0.488x10^4 g.L-1 (Figure 7). P3 treatment (16 L: 8D) was the best treatment for producing carbohydrates, which reached 0.409x10^4 g.L-1 (Figure 7). This result indicated that the production of carbohydrates was affected by the ratio of dark and light conditions. According to the ANOVA test, there were no significant differences between the treatments. It showed that other photoperiod durations still need to be developed to evaluate this study. We found that the presence of carbohydrates and lipids in this study had different patterns, with higher lipid content detected in long periods of light, while carbohydrates grew higher in the 8 hours of darkness and 16 hours of light. The statistical analysis showed that there is no significant difference among treatments in terms of carbohydrate production. Subramanian *et al.* (2013) demonstrated that the biosynthesis of carbohydrates is a competitive process to that of lipids in algal cells, as less ATP and NAD(P)H per carbon are required for carbohydrate synthesis than lipid synthesis.

This study indicated that biochemical components such as lipids, proteins, and carbohydrates were slightly affected by the different light cycles (photoperiod). Lipid accumulation in this study was produced by the higher light cycle (24 L; 0 D) (Figure 5). However, 12 L:12 D also indicated higher lipid content than other treatments, just as in continuous illumination. This might be affected by nitrogen consumption, which accounts for a decrease in protein in response to an increase in illumination. It is also possible that the carbon skeleton required for the synthesis of amino acids and proteins is diverted to function as a carbon and energy source for TAG biosynthesis (He *et al.*, 2017). Higher lipid content correlates with lower protein content, indicating that lipid synthesis depends primarily on protein degradation or inhibition of protein synthesis (Nzayisenga *et al.*, 2020). As the light/dark cycle provides energy for the transfer of electrons from water to NADP, thereby forming NADPH (nicotinamide adenine dinucleotide phosphate) and producing ATP (Wahidin *et al.*, 2013), light intensity and exposure to light are the primary determinants of photosynthetic microalgae strain productivity. Continuous, high-intensity light and photoperiod duration cause photodamage to PSI. Thus, various strains respond differentially to high light intensity and photoperiod, resulting in either a low or high lipid yield (Nzayisenga *et al.*, 2020). This process can be regulated by subjecting algal cells to very brief light-dark cycles (Pulz, 2001).

In the present study, it was determined that the light/dark cycle had an effect on the metabolites of *Euglena* sp. The results represent that optimal growth, biomass content, and metabolite content were obtained with a 24 D:0 L lighting cycle. The most affected metabolites are lipid, protein, and chlorophyll a. This result is affected by the light and dark cultivation cycles, which is associated with the peculiarities of photosynthesis—a complex multistep process in which light-dependent photochemical reactions and dark reactions are distinguished, wherein the dark reaction of photosynthesis is not dependent on the presence of light (Vecchi *et al.*, 2020). According to the previous report, Maltsev *et al.* (2021) explained the effect of light conditions on the development of microalgae, the content of lipids, carotenoids, and the composition of fatty acids in their biomass, taking into account parameters such as the intensity, duration, and use of rays with different spectral compositions. In addition to chlorophyll a, microalgae contain the chlorophylls b, c, d, and f (Chen *et al.*, 2010). All chlorophylls are magnesium complexes of different tetrapyrroles. Chlorophylls absorb light in the red and blue spectral regions selectively. Moreover, each chlorophyll's absorption spectrum has unique characteristics. Microalgae cells either contain only chlorophyll an or chlorophyll an as the primary pigment and additional chlorophylls as the second pigment (Mulders *et al.*, 2014).

3.5 Pigment Content of Euglena sp. Under Different Photoperiod Cycles

This study showed that the production of chlorophyll a was higher in full-light illumination (6.237 g.L-1) than in other treatments (Figure 8). For chlorophyll b, the highest production of chlorophyll b was also achieved by full light (24 hours of light), which reached 2.838 $g.L^{-1}$. Total carotenoid was also higher when *Euglena* sp. was grown under full light illumination (1.566 $g.L^{-1}$). This finding showed that long illumination significantly affected the production of pigmen in microalgae, especially chlorophyll a, b, and total carotenoid. Several previous reports also showed that microalgae's production of carotenoids can be altered through manipulations of photoperiod length and light intensity. In *Nostoc calcicola*, an increase in light intensity from 21 to 63 mol photons m^{-2} s^{-1} and photoperiod duration from 8:16 to 16:8 hours (light:dark) led to an increase in carotenoids (Khajepour *et al*., 2015). In addition, high light intensity and extended photoperiods were conducive to increasing the astaxanthin concentration in *Haematococcus lacustris* cultures (Liyanaarachchi *et al*., 2020).

The principal component analysis (PCA) of the carotenoid contents in different photoperiod treatments indicated that PC 1, explaining 98.6% of the total variance, was separated by four photoperiod cycles, respectively. PC 2, accounting for 1.2% of the total variance, separated full-light treatment, and others (Figure 9). Accessory pigments were calculated using a spectrophotometer and its compositions (Figure 10). The cluster analysis of carotenoid and pigment contents revealed that all contents clustered into four clusters. As presented in the result, full light illumination (24 hours) was the best treatment for producing accessory pigments such as phaeophytin a, phaeophytin b, violaxanthin, 9'-cis-neoxanthin, dinoxanthin, and fucoxanthin. The control treatment (24 full light) produced higher accessory pigments, particularly phaeophytin a, phaeophytin b, 9'-cis-Neoxanthin, and violaxanthin (Figure 10). Moreover, 14L:10D treatment contained the second abundance of accessory pigments as we mentioned previously. It can be assumed that photoperiod might affect the compositions of these accessory pigments. This finding was in line with previous reports done by Minhas *et al.* (2023) that the proportions of total carotenoids and chlorophyll in both strains were influenced by photoperiod and light intensity. In comparison to *N.oculata* (2656.94 ± 8.22 µg/g) and *Dunaliella tertiolecta* (3635.16 ± 1.59 µg/g) under a 13:11h light/dark cycle and 250 $\mu E/m^2$ /s light intensity in SSM (NSW), the total carotenoids content of *Dunaliella tertiolecta* was marginally higher. Previous report by Tamaki *et al.* (2020) demonstrated that diadinoxanthin, diatoxanthin, β-carotene, and neoxanthin were identified as the primary carotenoids in *E. gracilis.* Diadinoxanthin, diatoxanthin, and β-carotene, carotenoids that are associated with phototaxis, were present in the stigma of *Euglena.*

Microalgae and cyanobacteria contain pigments classified as chlorophylls (*chls*), carotenoids, and phycobiliproteins. The distinctions between these categories are based on their chemical composition (Mulders, 2014). The PCA results statistically compared the impacts of different light and dark cycles in *Euglena* sp. (Figure 9). When the culture was under control treatment or continuous illumination, the results were different with the 12 L:12 D treatment. Our heatmap results indicated that continuous illumination (24 hours) produced the highest content of carotenoids such as 9'-cis-Neoxanthin, phaeophytin a, b, violaxanthin, dinoxanthin, and fucoxanthin. A previous study by Seyfabadi *et al.* (2011) reported that changing the light and dark cycles influences the chlorophyll and ß-carotene content of microalgae *C. vulgaris*.

Carotenoids consist of a single, long hydrocarbon chain made up of eight isoprene units. Carotenoids are comprised of a single long chain of eight isoprene units (Mulders, 2014). In microalgae, carotenoids are associated with the photosynthetic apparatus of microalgae and provide the assimilation of light energy. According to reports, their diversity is substantially less than that of secondary carotenoids, which are unrelated to the provision of photosynthesis and vary considerably between different taxonomic groups of algae (Solovchenko, 2015). Cells frequently produce secondary carotenoids in response to specific environmental conditions, such as high light. They accumulate in the cytoplasm or stroma of plastids (Novoveská *et al*., 2019); carotene and lutein, which are characteristic of the photosynthetic apparatus of algae, can also accumulate as secondary carotenoids (Shi *et al*., 2020).

4. Conclusion

In this research, the photoperiod cycle consisting of 24 L:0 D, 12 L:12 D, 14 L:10 D, and 16 L:8 D is a factor that supports the growth of *Euglena* sp. Control treatment or continuous illumination was a treatment with the best growth results, biomass, and metabolite content compared to other treatments. This shows that light is an indispensable component for *Euglena* sp., in addition to other environmental factors such as pH, temperature, salinity, CO_2, aeration, etc. that cannot be

explained clearly in this study.

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Authors' Contributions

The contribution of each author is as follows, Tia Erfianti; collected the data, drafted the manuscript, and designed the research. Arief Budiman and Budi Setiadi Daryono; devised the main conceptual ideas and critical revision of the article. Eko Agus Suyono: monitored the research and drafted the manuscript. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

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