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The effect of photoperiodism on nutritional potency of *Euglena* sp. Indonesian strains

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ABSTRACT

Introduction: Biomass microalgae functional materials, such as drugs and food supplements, have recently received much attention. *Euglena* sp. is a particularly appealing microalgae because of its simplicity of culture and capacity to produce a wide range of bioactive compounds. Moreover, it is one of the few microorganisms that produces carbohydrate, lipid, protein, ß-1,3-glucans, antioxidants, phytotoxins, wax esters, and polyunsaturated fatty acids that can be used to make nutraceuticals, pharmaceuticals, and cosmeceuticals. However, the potential utilisation of *Euglena* sp. for production of food supplements has been exploited only on a limited basis. **Methods:** This study was modified by adding protocatechuic acid and photoperiodism for 12:12; 14:10; 16:8; and full dark to affect the metabolite content of *Euglena* sp. **Results:** Results showed that the photoperiod had significant effect on lipid, chlorophyll-a, and carotenoid levels in the control treatment, with the highest levels as follows: 0.52±0.03 g/L, 1.20±0.01 $x10^{-2}$ g/L, 0.30±0.02 $x10^{-2}$ g/L; while the others were not significantly affected by the treatment, with the highest protein content at full dark 3.10±0.2 $x10^{-2}$ g/L; chlorophyll-b at photoperiod 14:10 0.70±0.03 $x10^{-2}$ g/L; paramylon at photoperiod 12:12 1.90±0.02 $x10^{-1}$ g/L. The highest carbohydrates were found in control, with a level of 1.20±0.02 g/L. **Conclusion:** Photoperiodism is recommended to enhance productivity of protein, paramylon, and chlorophyll-b, while full light is recommended to enhance carbohydrate, lipid, chlorophyll-a, and carotenoid production in *Euglena* sp. to improve the quality of food nutrition.

Keywords: *Euglena* sp., photoperiod, protocatechuic acid

INTRODUCTION

In recent years, it has been known that scientists are interested in developing the use of microalgae biomass as a source of component for producing bioactive metabolites that are environmentally friendly and renewable. In addition, recent advances in biological engineering and multi-omics have revealed numerous potentials for microalgae bioproducts in the nutrition, media, pharmaceutical, and commodity industries (Bajhaiya,

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Moreira & Pittman, 2017; Stengel & Connan, 2015). *Euglena* (protist) is a flagellated microfactory organism living in autotrophic, heterotrophic, or mixotrophic environments. It can grow heterotrophically with various carbon sources and pH levels in wastewater (Mahapatra, Chanakya & Ramachandra, 2013). *Euglena* sp. is not like bacteria or protozoa, so it does not have a dangerous or pathogenic risk to other living things, especially humans, and their numbers can be increased easily.

Euglena is high in carbohydrate-active enzymes, with an exceptional ability to synthesise complex carbohydrates for a unicellular organism. Furthermore, *Euglena* cells contain many of nutrients like fatty acids, such as eicosapentaenoic acid, docosahexaenoic acid, and vitamins, implying the genus is a valuable and potential resource for food supplement. *Euglena* sp. also contains many nutrients, such as carbohydrates, lipids, proteins (primary metabolites), ß-1, 3-glucans (paramylon), carotenoids, tocopherol, essential amino acids, minerals, phycobiliproteins (PBPs), phytohormones, phytosterols, phenolic compounds, and mycosporine-like amino acids (secondary metabolites), that have been shown to possess nutritional, antioxidant, neuroprotective, anti-inflammatory, antimicrobial, anti-angiogenic, and anti-cancer properties (Haque *et al.,* 2014; Rico *et al.,* 2017; Singh *et al.,* 2017; Nakazawa, 2017). Therefore, the use of *Euglena* sp. as a healthy supplementary food is promising.

In the cultivation of *Euglena* sp., dark:light cycle influences its spectral composition, oscillation pattern, and photoperiod, all of which contribute significantly to the microalgae metabolic process. Optimal irradiance, oscillation pattern, and dark length all influence phytoplankton metabolic activity (Oostlander *et al.*, 2020). Changes in photoperiod can affect the production of total pigment, fatty acid, and protein content in *Chlorella vulgaris*, lipid formation and growth in *Porphyridium cruentum* (Khoyi *et al.*, 2009; Oh *et al.*, 2009), as well as nutrient utilisation and biomass in *Chaetoceros muelleri* production (Minggat, Roseli & Tanaka, 2020). The duration of photoperiod significantly impacts diatom cell development (dark: light cycle) (Palanisamy *et al.*, 2022). Light intensity greater than 150 E $m^{-2}s^{-1}$ is thought to inhibit cell development; it may oversaturate cell growth under more extended irradiation, as indicated by the difference between the dark:light cycles of 8:16 and 16:8 (Li, Talmy & Campbell, 2017). Therefore, to confirm the potential of *Euglena* sp. as a source of various bioactive for human nutrition, various cultivation techniques can be carried out, one of which is by using different photoperiods.

MATERIALS AND METHODS

Isolation & cultivation

Water samples for microalgae isolation were collected from the Dieng Plateau, Wonosobo, Middle Java, Indonesia and isolated using capillarity pipette methods in aseptic laminary air flow. Cramer–Myers (CM) medium was used as a growth medium, with the composition ($g.L^{-1}$): 1 gram NH_4SO_4, 1 gram KH_2PO_4, 0.2 gram $MgSO_4.7H_2O$, 0.02 gram $CaCl_2.2H_2O$, 100 µL trace metal mix, 100µL $Na_2MoO_4.2H_2O$ solution, 20 µL vitamin B1 solution, 25 µL vitamin B12 solution, with a pH of 5.5 and an additional carbon source of 0.8 g protocatechuic acid (PCA). The CM medium was previously sterilised using an autoclave at 121°C for 20 minutes. After making the media, the starter was left for two weeks to equalise the age of *Euglena* sp. The starter was then transferred to a 50 mL culture bottle and

the volume of CM was 450 mL; thus, the total in one culture bottle was 500 mL.

Identification and screening strain

Samples from various strains that were successfully isolated were observed under the Olympus CX22LED1000x magnification light microscope, with added immersion oil and connected with Optilab Advance Observer (MTN034). Strain screening was done by cultivating isolates that have successfully grown without contamination, namely seven strains out of a total of 35 strains. The seven lines were IDN 23, IDN 29, IDN Mix, IDN 33 A Aerobic, IDN 33 A Anaerobic, IDN 33 B Aerobic, and IDN 33 B Anaerobic. Then, their growth rates were compared, and the highest growth rate was selected as the best candidate line. Microalgal growth was measured every day. In addition, the concentration of microalgae cells was determined by measuring the culture's optical density (OD) with the Thermo Scientific Evolution 201 UV-Vis spectrophotometer at an absorbance of 680 nm in three replicates. Calculation of cell-specific growth rate was done using the following formulas (1,2):

$$\mu = \frac{Ln\,(Nt - N0)}{t1 - t0} \quad (1)$$

$$Dt = \frac{Ln2}{24x\mu} \quad (2)$$

μ=Specific growth rate; Dt=generation time (hours); Nt=cell population on the t-day exponential phase (cells mL^{-1}); N0= cell population on day 0 of the exponential phase (cells mL^{-1}); *t*1-*t*0 = time interval in the exponential phase (days).

Growth kinetics modelling

The Logistic and Gompertz models were used to model the growth kinetics of *Euglena* sp. First, the Logistics model was calculated using the following formulas (equations 3 and 4), where X is cell density, X0 is initial cell density, Xmax is maximum cell density, and max is maximum specific growth rate (Phukoetphim *et al.,* 2017).

$$\frac{dx}{dt} = \mu_{max}\left(1 - \frac{x}{\mu_{max}}\right)x \quad (3)$$

$$x = \left(\frac{xo.\exp(\mu_{max}t)}{1 - [(\frac{xo}{x_{max}})\,(1 - \exp(\mu_{max}t))]}\right) \quad (4)$$

The parameters in the Gompertz model were maximum cell production (rm) and lag time (tL). The model was determined using the following formulas (equations 5 and 6), where SSR is the sum square residual and SST is the sum square total (Phukoetphim *et al.,* 2017).

$$x = Xo + [X_{max}.\exp[-\exp((\frac{r_m.\exp(1)}{x_{max}})\,(t_l - t) + 1] \quad (5)$$

$$R^2 - (1 - \frac{SSR}{SST}) \quad (6)$$

Determination of cell growth

Cell growth in different photoperiods was compared by counting cells every 24 hours using a light microscope and Haemocytometer Neubauer 1 mm. After shaking the sample to homogenise it, 100 μL was pipetted into a 2 mL microtube using a micropipette. The model was then transferred to a haemacytometer and the cells were counted using a light microscope linked to a computer running optilab software. The number of cells in each of the four corners was calculated and the total number of cells was computed.

Biomass calculation of *Euglena* sp.

Biomass production was calculated every three days using the dry weight of cells. Two mL of sample culture was transferred into a 2 mL microtube. For

10 minutes, the sample was centrifuged at 4000 rpm. The supernatant was removed and washed with distilled water. Cell suspensions at the bottom of microtubes were dried in an incubator oven at 37°C until they had a constant weight. The final biomass was calculated by subtracting the sample's final weight from its initial weight and then dividing it by its initial volume.

Carbohydrate estimation of *Euglena* sp.

Dubois's method quantified the carbohydrate content in microalgae biomass using phenol-sulfuric acid. After creating a standard curve of carbohydrate concentrations, the sample's absorbance was measured using a spectrophotometer at 490 nm to determine carbohydrate concentration.

Lipid estimation of *Euglena* sp.

The Bligh and Dyer method was used to quantify the lipid content of *Euglena* sp. Extraction method that included a 1:2 ratio of chloroform and methanol, followed by a 1:1 ratio of chloroform and aquades was used. The solution was then centrifuged until three layers were formed, and the bottom layer was removed and incubated in an oven at 30°C for 24 hours.

Protein estimation of *Euglena* sp.

The Bradford method was used to determine the protein content of *Euglena* sp. This method was carried out by centrifuging the supernatant from the separation process and adding in a SDS solution. Following a 5-minute incubation at 95°C, a 5-minute incubation at 4°C was performed. Bradford's solution was then added to the incubation samples. Absorbance was then measured at 595 nm using an eLISA Reader Biotech. Protein content was calculated using standard linear curve regression equations from standard Bovine Serum Albumin (BSA) protein solutions from Abbkine kit at 20, 50, 75, and 100 µg/ml concentrations.

Percentage and productivity determination of *Euglena* sp. primary metabolites

Productivity measurements of primary and secondary metabolites were calculated based on the following equations (8,9) (Chen *et al.*, 2020).

$$\% \text{ cell compound} = \frac{\text{Total cell compound}}{\text{Biomass}} \times 100\% \quad (8)$$

$$\text{Productivity} = \text{Biomass productivity} \times \% \text{ cell compound} \quad (9)$$

Pigmentation analysis

A spectrophotometric method was used to determine pigment content. Two ml culture was centrifuged at 4000 rpm for 5 minutes. Then, the supernatant was removed and the pellet was extracted overnight in the dark at 4°C with 1.5 mL methanol (99.9%). The use of waves on the spectrophotometer ranged from 400-750 nm. The concentrations of chlorophyll-a (Chl-a), chlorophyll-b (Chl-b), and photoprotective carotenoid (PPC) were calculated using the following equations:

$$\text{Chl a } (\mu g/mL) = -8.0962 \times \lambda_{652} + 16.5169 \lambda_{665};$$

$$\text{Chl b } (\mu g/mL) = 27.4405 \times \lambda_{652} - 12.1688 \times \lambda_{665};$$

$$\text{Total Carotenoids } (\mu g/mL) = 4 \times \lambda_{480}$$

Paramylon extraction and analysis

A culture of 10 mL *Euglena* sp. was taken and centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded to obtain pellets. The pellets were dissolved in 1% (w/v) SDS and 5% (w/v) Na2EDTA, then incubated in a water bath at 37°C for 30 minutes. The treatment was repeated without incubation in SDS-Na2EDTA solution

and then the paramylon was washed twice with distilled water. Paramylon pellets were dissolved in 2 mL NaOH. The phenol sulfuric acid method was used to determine paramylon concentration. The extraction solution was mixed in a test tube with 5% phenol and H_2SO_4 (sulfuric acid). The test tube was allowed to stand for 10 minutes in a standing position. Next, the solution was vortexed for 30 seconds before being allowed to stand at room temperature for another 20 minutes. Then, a 490 nm spectrophotometer solution was used. The paramylon standard was used to create standard curves. Paramylon productivity ($g\ L^{-1}\ day^{-1}$) = last exponential phase-first exponential phase (Zhu & Wakisaka, 2018).

Statistical analysis

All experiments were done in triplicates and data were shown as mean values of the three replicates. The various experiments' mean values and standard deviations were evaluated using Microsoft Excel 2007. Using IBM SPSS Statistics for Windows version 26.0 (IBM Corp., New York, United States), the results of each analysis were analysed using one-way analysis of variance (ANOVA) by rank at a 95% confidence level.

RESULTS

There were many species of *Euglena* in the Dieng plateau. Erfianti *et al.* (2023) stated that *Euglena* sp. is a member of the *Euglena* genus that has been successfully isolated under extreme conditions. The pH level of Dieng Peatland is 2.0-3.5, suitable for the growth of *Euglena*. The special characteristics of *Euglena* are elongated oblong or spherical shaped cells, green in colour because they contain the pigment chlorophyll-a and -b, and has a pellicle structure that allows its cells to make changes called euglenoid movement, as well as having eye spots (stigmas) containing carotenoids that control the intensity of light (Erfianti *et al.*, 2023). *Euglena* also has a cell size ranging from 31-68 µm, flagella for swimming, and a reservoir (Al-Ashra, Abiad & Allahem, 2014). Therefore, *Euglena* sp. was isolated and identified using a light microscope from all strains (Figure 1). The solitary *Euglena* sp. measured ±50 µm in length. Microscopical analysis revealed that *Euglena* cells were solitary and free to swim. They typically lacked a cell wall and were elongated and spindle-shaped with tapering ends. Seven different strains (IDN 23, IDN 29, IDN Mix, IDN 33 A Aerob, IDN 33 A Anaerob, IDN 33 B Aerob, and IDN 33 B Anaerob) were obtained based on the isolation process, which were then screened by growing the isolation results in laboratory-scale CM medium to get the strain type with the best growth rate.

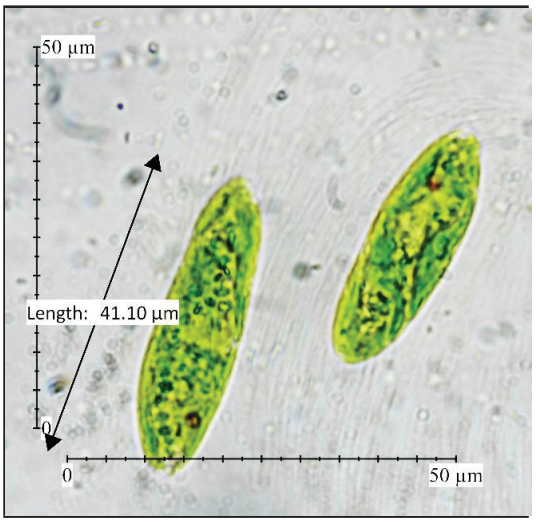

Figure 1. Cell of *Euglena* sp isolated from Dieng Plateau. Magnification 100 x

Table 1 and Figure 2 showed that the highest biomass productivity was found in strain IDN 33 A Aerobic, with a value of 0.76 g/L/day. The same results

Table 1. Screening productivity of biomass *Euglena* sp.

Strain	*SGR (μ)*	*DT (day^{-1})*	*Productivity biomass (g/L/day)*
IDN 23	0.149	0.193	0.614
IDN 29	0.153	0.189	0.668
IDN MIX	0.125	0.230	0.459
IDN 33 A Aerobic	0.168	0.172	0.764
IDN 33 A Anaerobic	0.122	0.238	0.450
IDN 33 B Aerobic	0.158	0.183	0.636
IDN 33 B Anaerobic	0.155	0.187	0.591

were obtained in measuring cell density using the spectrophotometric method. The results showed that strain IDN 33 A Aerobic had a growth phase close to the previously optimised IDN 29 strain. It is important to note that variation in microalgae can be seen between distinct genera, various species, and even strains of the same genus (Taleb *et al.,* 2016). From the two measurement processes, IDN 33 A Aerobic was then declared to be the selected strain that was to be used for the cultivation stage, with the highest specific growth rate value of 0.168 μ and the lowest doubling time of 0.172 day^{-1}.

The optical density of *Euglena* sp. cultivated in CM medium and the addition of PCA combined with specific photoperiod modes is presented in Figure 2. Optical density (OD) formed a different pattern between the control group without adding PCA and using the full light treatment with the addition of PCA and photoperiod. On day 2, the treatment group experienced a relatively rapid increase in OD compared to the control group. The treatment group entered the log phase faster than the control on day 2; the control group entered the log phase on day 6. Then, the treatment and control groups entered the stationary phase simultaneously on day 9. This meant that the photoperiod treatment group had a longer log phase, around seven days. The initial death phase in the treatment group began on day 13, while the control group started on day 15.

Based on the one-way ANOVA test, the photoperiod treatment of the control *Euglena* sp. had no statistically significant effect on the growth rate of *Euglena* sp. (p=0.440). In this experiment, higher carbohydrate content was obtained using a medium with dark condition and control treatment on day 9 at 1.10±0.02 g/L and 1.20±0.02 g/L, respectively (Figure 3a), with carbohydrate productivity of 0.64x10^{-1} g/L/day and 1.11x10^{-1} g/L/day, respectively. Moving to the following graph (Figure 3b), higher lipid content was obtained using a medium with dark condition on day 12 and control treatment on day 15 at 0.49±0.05 g/L and 0.52±0.03 g/L, respectively. Total lipid content of *Euglena* sp. in full dark treatment increased from 14.47% to 62.98%, while lipid content of *Euglena* sp. control increased from 10.70% to 43.94%, with lipid productivity of 0.32 x10^{-1} g/L/day and 0.26x10^{-1} g/L/day, respectively. A higher protein content was obtained using a medium with dark condition on day 15 and photoperiod 16:8 treatment on day 3 at 3.1±0.2 x 10^{-2} g/L and 2.6±0.1x10^{-2} g/L, respectively (Figure 3c). *Euglena* sp. in full dark treatment increased total protein content from 0.24% to 0.52%, and *Euglena* sp. in photoperiod 16:8 treatment increased protein content from 3.25% to 4.60%, with protein

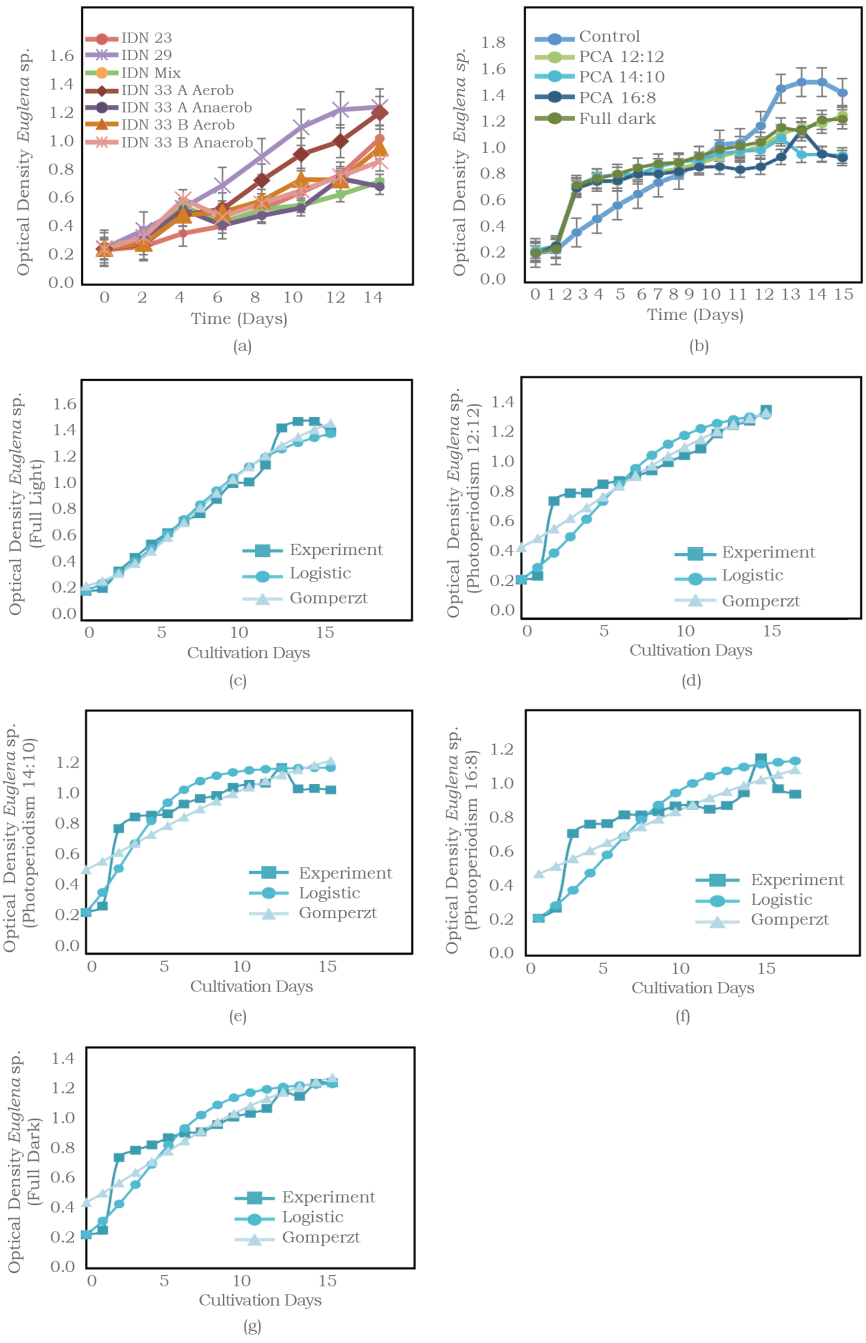

Figure 2. Growth of *Euglena* sp. (a) Screening potential strain of *Euglena* sp.; (b) Growth of *Euglena* sp. in photoperiod treatment and PCA addition; (c) Growth modelling of *Euglena* sp. (Full light); (d) Growth modelling of *Euglena* sp. (Photoperiodism 12:12); (e) Growth modelling of *Euglena* sp. (Photoperiodism 14:10); (f) Growth modelling of *Euglena* sp. (Photoperiodism 16:8); (g) Growth modelling of *Euglena* sp. (Full dark)

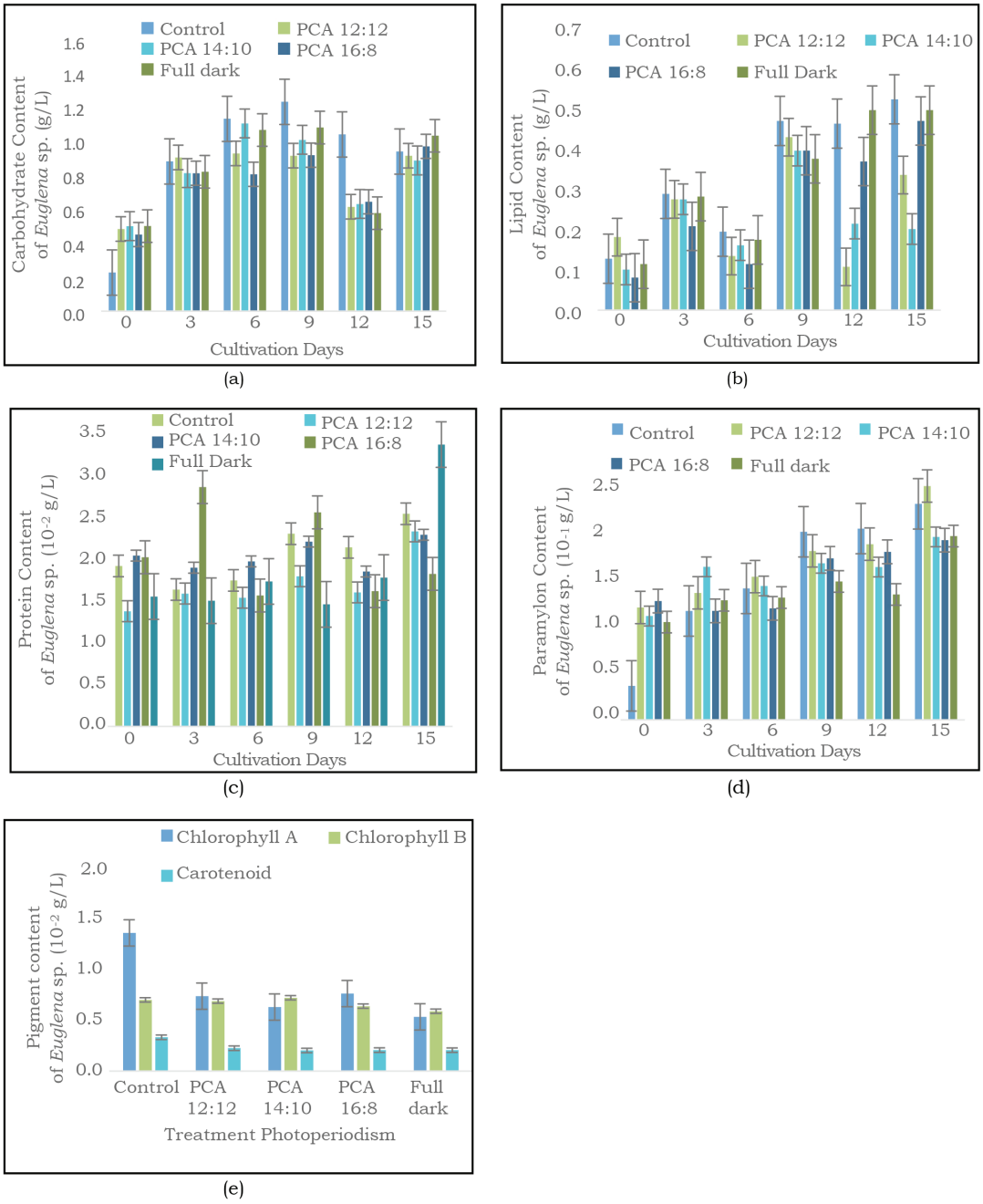

Figure 3. Metabolite content of *Euglena* sp (a) carbohydrate content; (b) lipid content; (c) protein content; (d) paramylon content; (e) pigment content.

productivity of 1.1 $x10^{-3}$ g/L/day and 2.5 $x10^{-3}$ g/L/day, respectively. Based on one-way ANOVA test, the photoperiod treatment of control *Euglena* sp. had no statistically significant effect on carbohydrate content (p=1.310) and protein content (p=0.060). In contrast, the result had a significantly different effect on lipid content (p<0.05); and from the Duncan post-hoc test, there was a significant difference between treatment and control groups.

In this experiment, higher pigment content was obtained. The overall type of pigment chlorophyll-a and carotenoid (Figure 3e) produced were highest in the control treatment, as extensive as 1.2±00.01 $x10^{-2}$ ±g/L and 0.30±0.02 $x10^{-2}$ g/L, respectively. The other most increased production was chlorophyll-b on the photoperiod treatment 14:10, as large as 0.70±0.03$x10^{-2}$ g/L. Different from pigment, higher paramylon content was obtained using a medium with photoperiod 12:12 treatment on day 15 at 1.90±0.02 x10-1 g/L (Figure 3d). Total paramylon content of *Euglena* sp. in photoperiod 12:12 treatment increased from 45.87% to 95.47%, with a productivity of 6.613 µg/ml/day. Based on the one-way ANOVA test, the photoperiod treatment of control *Euglena* sp. had no statistically significant effect on paramylon (p=0.457) and chlorophyll-b (p=0.192) content. However, the result had a significant effect on chlorophyll a content (p<0.05) and carotenoid content (p<0.05); and from the Duncan post-hoc test, there was a significant difference between treatment and control groups in chlorophyll-a and carotenoid content (Table 2).

DISCUSSION

In the initial phase of microalgae growth, cells adjust to the new media so that *Euglena*, with the addition of PCA and photoperiod treatment, requires a lag phase, namely the first and second days, while the control *Euglena* requires a longer time, namely the sixth day. Rapid exponential development in treatment culture begins on the second day of cultivation, reaches a stationary phase on day 9, and then begins to diminish. More extended periods of light exposure create photoinhibition in the cells and a lack of nutrients in the culture, causing growth to slow down. As a result, cells could not complete photosynthesis to multiply (Palanisamy *et al.,* 2022). In the control treatment, the death phase began on day 15. Due to decreased photosynthesis, continuous light generates the lowest cell multiplication number (373±104) and biomass productivity (0.371±21 g/L) on day 15 of cultivation (Palanisamy *et al.,* 2022). Based on ANOVA test, it was found that the photoperiod treatment did not significantly affect the growth rate of *Euglena*. This was possibly because some needed light, so *Euglena* became more adaptive. Microalgae requires light to produce adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH2), as well as critical chemicals for growth (Xie, Lin & Luo, 2021). Besides that, light is the source of energy that drives this process, and it must be considered in terms of intensity, spectrum quality, and photoperiod (Park & Craggs, 2011) so that a certain level of intensity or quality of light may have an impact on the growth rate when combined with a photoperiod.

Logistic and Gompertz were two non-linear models that were suitable for the rapid population growth of organisms such as microalgae (Lam *et al.,* 2017). Not limited by substrate type and consumption, the Logistic and Gompertz models (Figure 2c-g) were the simplest models and could be used for general microalgae growth rate. This research showed that the best pattern based on the Logistic and Gompertz kinetics models was on control cultivation. The resulting

regression treatment was below 0.9, which deviated from the model presented. Based on Logistic modelling, the maximum specific growth rate (μmax) of *Euglena* sp. was 0.2985/day. For the Gompertz modelling, the maximum cell production rate (rm) of *Euglena* sp. was 0.108 x106 cells/mL. The lag time (tL) of *Euglena* sp. was 1.428/day. Each of the *R* square error value for the Logistic and Gompertz models were 0.966 and 0.974, respectively. Therefore, based on the *R* square error values, the Gompertz model indicated a better-fit model compared to the Logistic model.

The proportions of chemical components (for example, carbohydrates, lipids, proteins, and pigments) in algal cells are closely related to cultivation conditions such as photoperiod and light intensity (Juneja, Ceballos & Murthy, 2013). Proteins are the most abundant component of dry microalgae biomass, accounting for 6-52% of the total; Zhu (2015) and Deng *et al.* (2018) found that protein content was similar (44.7-50.7%) when *C. kessleri* was grown in a mixotrophic condition. The highest carbohydrate production was on day 9, while the most increased lipid production was on day 12. As seen in Figure 3, productivity of carbohydrates on days 6, 12, and 15 were inversely proportional to the productivity of lipids. Carbohydrate biosynthesis is a competitive process in algal cells, requiring less ATP and NADPH per carbon than lipid synthesis (Subramanian *et al.,* 2013). Furthermore, microalgae can adjust their carbon partitioning programmatically in response to changes in culture conditions and environment (Wang *et al.,* 2013).

The effect of photoperiod on the chemical compositions of *C. sorokiniana* grown in CCW in a bubble-column bioreactor were investigated. Protein content increased with increasing illumination time, reaching a peak of

Table 2. Effects of photoperiod and addition of PCA on metabolite productivity of *Euglena* sp.

Treatment	*Biomass (g/L)*	*Productivity (g/L/day)*	*Percent protein (%)*	*Percent para-mylon (%)*	*Percent chlorophyll a (%)*	*Percent chlorophyll b (%)*	*Percent carotenoid (%)*
Control (Full light)	1.18	0.08	1.96±0.02	75.62±0.00	1.47±0.01^{a}	0.76±0.00	0.60±0.01^{b}
PCA-12:12	1.12	0.07	1.90±0.00	95.47±0.00	0.60±0.00^{ab}	0.70±0.00	0.26±0.00^{a}
PCA 14:10	1.03	0.07	2.02±0.00	56.04±0.01	0.97±0.00^{b}	0.58±0.01	0.30±0.00^{a}
PCA 16:8	3.27	0.22	4.60±0.00	67.74±0.00	0.21±0.00^{b}	0.64±0.00	0.30±0.00^{a}
PCA-Full dark	5.90	0.40	0.52±0.00	50.03±0.00	0.06±0.01^{c}	0.68±0.03	0.03±0.00^{a}

Note: Numbers followed by a different letter indicated significant differences between treatments and were calculated by one-way ANOVA followed by Duncan multiple range test (DMRT) (p<0.05)

54.92% at a photoperiod of 20L:4D (light:dark) (Gao *et al.,* 2022). George *et al.* (2014) also conducted similar experiments on *Ankistrodesmus falcatus* and discovered that protein production increased with increased illumination duration, light intensity, and prolonged exposure time. As for lipid content, it increased first, then decreased, reaching a maximum of 24.56% at 16:8. At the same time, carbohydrate content decreased first and then increased, reaching a minimum of 19.03% at 20:4. As photoperiods increased from 8:16 to 24:0, pigment content increased from 1.06 to 2.77% (Gao *et al.,* 2022). As previously stated, lipids and carbohydrates have two competing pathways for storing production in microalgae.

Short light exposure in microalgae can form stress signals, thereby encouraging cells to convert excess glucose into lipids and reduce cell division (Mitra, Leeuwen & Lamsal, 2012). This stress adaptation event is analogous to the modifications caused by hyperostosis or nitrogen limitation, which causes a rise in lipid synthesis (Hirai *et al.,* 2016). As a result, the principal chemical compositions of algae change as light conditions change, demonstrating that photoperiod is a significant factor in influencing the levels of chemical compositions in algal cells.

Cell wall resistance, solvent type, and extraction procedures all impact pigment extraction. To extract chlorophyll and fucoxanthin, methanol is used (Palanisamy *et al.,* 2022). Because chlorophyll accessory antenna transfers photosynthetic chemical energy from 10 NADPH2 to lipid, protein, and nucleic acid synthesis, its content must be quantified (Palanisamy *et al.,* 2022). Several studies have discovered that the type of solvent, photoperiod, wavelength, nitrogen starvation, and other physico-chemical properties significantly impact pigment content (Bhattacharjya *et al.*, 2020). In this research, the chlorophyll content was calculated and showed that the control of cultivation produced the highest quantities of chlorophyll and carotene pigments compared to treatment. This indicates that continuous light conditions are the optimum conditions for producing chlorophyll and carotenoids. However, in conditions of excess light, microalgae cells reduce the production and accumulation of chlorophyll to avoid excessive energy requirements, thereby reducing photodamage or photoinhibition due to too much light (Li *et al.,* 2017).

Paramylon production can be increased during the exponential growth phase (Mahapatra *et al.*, 2013), as well as by co-culture of *Pseudoalteromonas* sp. or marine microbes during the logarithmic phase. Another element that influences the existence of paramylon is light circumstances which affect the accumulation time of paramylon when grown in the dark versus grown in the light (Zeng *et al.,* 2016). In this research, the highest concentration of paramylon obtained at treatment photoperiod 12:12 was 95.47% for 15 days (Table 2). As a result, *Euglena* sp. produced high amounts of paramylon with a combination of light and darkness. As stated in a previous study, dark cultivation is an excellent strategy to accumulate paramylon until 80% of total biomass (Kim *et al.,* 2021). Besides that, higher irradiation levels, on the other hand, contribute to carbohydrate build up when grown in the light (Matsuda, Hayashi & Kondo, 2011). So, for the formation of paramylon, which is a carbohydrate derivative, it requires a combination of light and darkness.

Paramylon is an insoluble, linear, high-molecular-weight 1,3-glucan that occurs naturally in crystalline form. The euglenoids accumulate paramylon in granules that may be widely dispersed throughout the cytoplasm, form caps over

the pyrenoids or be packed. The various types of paramylon are classified into six morphological categories, all of which are present in various euglenids. Uridine diphosphate glucose (UDP-Glc) donates the glucose moiety that is subsequently linked to form the expanding polysaccharide (Skodová-Sveráková *et al.,* 2020). The sugar nucleotide formation depends on the enzymes UTP (D-glucose uridylyltransferase) or UDP-Glc pyrophosphorylase (Muchut *et al.,* 2018). In euglenoids, the appearance of paramylon requires the activity of paramylon synthase, a membrane-bound enzyme complex of approximately 670 kDa belonging to the eukaryotic glycosyltransferase 48 (GT48) family (Skodová-Sveráková *et al.,* 2020). In *Euglena gracilis*, the glucan synthase-like genes EgGSL1 and EgGSL2 have been identified. They encode 304 kDa and 258 kDa proteins with 15 and 19 transmembrane domains, respectively (Tanaka *et al.,* 2017). Tanaka *et al.* (2017) reported that paramylon synthesis depends on the activity of EgGSL2, which forms complexes with 37 and 54 kDa UDP-binding proteins. The membrane fraction surrounding the paramylon granules is associated with the activity of paramylon synthase (Skodová- Sveráková *et al.,* 2020).

CONCLUSION

This study found that photoperiod treatment had a significant effect on lipid, chlorophyll-a, and carotenoid levels of *Euglena* sp. in the control treatment (full light), with the highest total levels as follows: 0.52±0.03 g/L, 1.2±0.01 x 10^{-2} g/L, 0.3±0.02 x10^{-2} g/L, respectively; while the others were not significantly affected by the treatment. Photoperiod was able to increase the levels of metabolites when compared to control conditions, namely the highest protein content at full dark was 3.1±0.2x 10^{-2} g/L; chlorophyll-b at photoperiod 14:10 was 0.7±0.03 x10^{-2} g/L; and paramylon at photoperiod 12:12 was 1.9±0.02x10^{-1} g/L. The highest carbohydrate content was found in control conditions, with levels of 1.2±0.02 g/L. *Euglena* is a protist with high adaptability because it can live as an autotroph, heterotroph, or mixotroph, and the presence of a photoperiod does not significantly influence the accumulation of *Euglena* metabolites. Therefore, it is necessary to carry out further studies related to *Euglena*'s limitations in adapting to the environment so that it triggers stress and increases the targeted metabolisms.

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Authors' contributions

Khusnul QM, principal investigator, conceptualised and designed the study, prepared the draft of the manuscript and reviewed the manuscript; Tia E, advised on data analysis and interpretation, and reviewed the manuscript; Istini N, led the data collection and reviewed the manuscript; Ria A, reviewed the manuscript; Dedy K, reviewed the manuscript; Brilian RS, led the data analysis and reviewed the manuscript; Revata M, conducted the study; Bambang RA, conducted data analysis and interpretation; Arief B, assisted in drafting of the manuscript, Eko AS reviewed the manuscript.

Conflict of interest

Authors declare no conflict of interest. Authors have received a research grant from the Indonesian Ministry of Research, Technology and Higher Education (Ristekdikti).

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