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Role of Brassinosteroid on Growth, Metabolic Contents and Wax Ester Fermentation in *Euglena* sp.

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ABSTRACT

Euglena is one of the alternative natural resources for medicine, food, and energy, and it is important to develop its metabolic contents to fulfill human demands. Improvement of metabolic content in *Euglena* was conducted in several ways, such as by adding the phytohormone. Brassinolide is one of the phytohormones and is well-known for its ability to stimulate and protect the plant from stressful environments. The application of brassinolide is still lacking. In addition, previous studies have never applied this phytohormone to *Euglena* sp. cultures. This research aimed to analyze the effect of brassinolide on the growth, metabolic content and wax fermentation in *Euglena* sp. The growth rate was measured during cultivation, and the metabolic content was analyzed at the late exponential phase before entering the fermentation process. Gas Chromatography-Mass Spectrometry (GC-MS) was carried out to reveal the wax ester content after the fermentation process. The result showed that brassinolide significantly increased the growth rate and metabolic content at lower concentrations, while high concentration tends to inhibit the effect. The high metabolite content, including carbohydrate, lipid, protein, and paramylon, was 0.47 ± 0.02 g/L, 0.20 ± 0.01 g/L, 15.91 ± 1.21 × 10^{-3} g/L, and 145 ± 0.10 × 10^{-3} g/L, respectively. Interestingly, wax esters at lower brassinolide concentrations showed contrasting results compared to the control treatment. These findings provide information

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about the effect of brassinolide in *Euglena* sp., and advanced research is needed to reveal the mechanism of brassinolide in *Euglena* sp.

Keywords: Brassinolide, *Euglena* sp., fermentation, metabolite

INTRODUCTION

The exponential growth of the human population has led to a significant reduction in natural resources such as food, fuel, and medicine. This overconsumption and associated land deforestation and greenhouse gas emissions have directly contributed to global climate change (Maja & Ayano, 2021). The urgency of this issue is underscored by the fact that we need to explore and develop alternative natural resources that align with the Sustainable Development Goals (SDGs) set by the United Nations for 2030.

Microalgae, particularly *Euglena*, stands out as a promising candidate for future alternative natural resources. *Euglena* is easily found in fresh and brackish water (rich in organic matter), consists of single cells, and exhibits rapid growth (Borowitzka, 2018). *Euglena's* ability to thrive is not only under photoautotroph conditions, where it uses light as a proton source for photosynthesis and energy production but also under heterotrophic conditions (Leander et al., 2017). This unique adaptability makes *Euglena* a fascinating subject for further exploration. Additionally, *Euglena'*s metabolic content is diverse, encompassing protein, lipids, pigments, paramylon, wax ester, and more (Kottumparambil et al., 2019).

The metabolic content of *Euglena* holds immense promise for human life. For instance, the protein in *Euglena gracillis* can be harnessed as dietary protein (Gissbl et al., 2019). Conversely, paramylon and wax ester produced by *Euglena* are of particular interest. Paramylon in *Euglena* has numerous benefits; for example, in medicine, paramylon extracted from *E. gracillis* has shown an anticancer effect on *A4gnt* mice by reducing the gene expression of gastric dysplasia (Lida et al., 2021). Moreover, the conversion of paramylon to wax ester through fermentation could produce biofuel rich in 28 carbon chains (Inui et al., 2017). The diverse range of beneficial metabolites obtained from *Euglena* underscores the need for further research and improvement in its metabolic content, given its potential to support almost all aspects of human life.

Significant improvements in the metabolic content of various microalgae species have been demonstrated in previous studies. For instance, the protein content of *Chlorella vulgaris* was found to increase by up to 296% (Bajguz, 2000). Similarly, improvements in monosaccharide, chlorophyll a+b, and photosynthesis rate were 330%, 329%, and 298%, respectively, in *Scenedesmus quadricauda* (Bajguz & Czerpak, 1998). Moreover, *S. quadricauda* also demonstrated a significant enhancement, with the lipid and carotenoid content reaching 107.43 mg/g and over 140 ng/cell, respectively. These improvements were all observed under the addition of Brassinosteroids (BRs).

Brassinosteroids, which are steroidal hormones similar in structure to steroids in animals and insects (Divi & Krishna, 2009), have been widely used in plants as growth stimulators and stress protectants by regulating and enhancing the photosynthesis process (Liu et al., 2023) and regulating the stress defense mechanism both enzymatic and non-enzymatic (Vardhini & Anjum, 2015). For instance, adding brassinolide (BL), an active type of BRs, enhanced fruit yields of over 175 tons/ha in peaky pears (Atteya et al., 2022). Brassinolide also regulates the phenolic content and produces unsaturated fatty acids for cell membrane integrity in banana fruit under chilling injury (Zhang et al., 2022). Given these beneficial effects in plants, the potential application of BRs, particularly BL, in microalgae such as *Euglena* is worth exploring.

The role of BL in plants has demonstrated significant effects and is commonly used to enhance productivity under normal or stressful environments. However, its application in microalgae is relatively unexplored. As mentioned earlier, we have observed the application of BL in some microalgae species, such as *Chlorella*, which has been extensively studied. In this research, we delve into the potential of BL in *Euglena* sp. cultivation, inspired by the positive effects observed in other microalgae and the potential impact of BL on *Euglena* sp. We focus on the growth rate and metabolic production after and before fermentation. As we mentioned before, the metabolites and pigments produced by *Euglena* had a wide beneficial scope, including energy, food, and medicine. Thus, analyzing those parameters becomes our focus. Moreover, we have conducted growth kinetic modeling and cell morphology distribution in this study.

MATERIALS AND METHODS

Culture Condition

The culture of *Euglena* sp. was obtained from the Biotechnology Laboratory, Faculty of Biology Universitas Gadjah Mada, isolated from Dieng plateau, Indonesia, which was isolated from the result of research by Maghfiroh et al. (2023). The culture grew on Cramer and Myers (CM) medium at 5 liters volume, 2 liters of *Euglena* stock (OD_{650}: 0.370) and 3 liters of the medium for nine days or reached the late-log phase that was determined from preliminary results (unpublished data). Brassinolide was added in the early cultivation of *Euglena* sp. The aeration of culture was 4.25 LPM, containing 15% CO_2 (0.75 LPM), and the light was 3.600 lux with a white LED lamp (CRI index 80) for 24 h (Mardyansah, 2023). The pH culture during the cultivation process was controlled and adjusted to 3.50 by adding 1N KOH or 1N H_2SO_4—however, the temperature was adjusted to 24°C. After reaching the late-log phase, the culture was fermented by changing the culture condition to dark hypoxia for three days to generate wax fermentation. The dark condition was conducted by placing the culture in a dark place and wrapping it with three layers of aluminum foil. The hypoxia condition was performed by turning off the aeration during fermentation (low oxygen level).

Growth Rate Analysis

The growth rate of *Euglena* sp. under BL treatment was analyzed by observing the cell density and growth kinetic modeling. Cell density was monitored daily by taking 2 mL of culture sample and was read by spectrophotometer UV-Vis (Genesys 150 Thermo, USA) at 680 nm (Suzuki et al., 2015). The cell growth kinetic model was performed using Logistics and Gompertz. The logistic model will calculate using Equations 1 and 2 (Phukoetphim et al., 2017; Nurafifah et al., 2023).

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

$$x = \left(\frac{x_0 . exp\ (\mu_{max}\ .t)}{1-[(\frac{x_0}{x_{max}})(1-exp\ exp\ (\mu_{max}\ .t)\ ]}\right) \quad [2]$$

Where $\frac{dx}{dt}$ is growth rate, X is cell density, X_0 is initial cell, X_{max} is maximum cell density, and μ_{max} is maximum of specific growth rate.

The Gompertz model is calculated by using Equations 3 and 4 (Phukoetphim et al., 2017; Nurafifah et al., 2023).

$$x = x_0 + [X_{max} . exp\,[\,-exp\,(\left(\frac{r_m . exp\ exp\ (1)}{x_{max}}\right)(t_L - t) + 1] \quad [3]$$

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

Where r_m is maximum cell production, t_L is lag time, SSR is a sum of square residual, and SST is the total sum square.

Biomass Measurement

The biomass was measured and conducted by vacuum filtration every three days after fermentation (twelfth day). About 20 mL of sample (V) was poured into a vacuum filtered with filter paper (GF/C Whatman filter paper 47 mm, China), which had been weighed before (W1). The filtered biomass dried for 90 min at 100°C (OV-65 B-ONE, China). The dried biomass was weighed at an analytical balance (GR-200 AND, Japan) (W2). The biomass content was measured by using Equation 5 (Morais et al., 2021).

$$\text{Biomass (g/L)} = \frac{W2-W1}{V} \quad [5]$$

Cell Morphology Distribution

Cell morphology distribution was observed on the ninth day following Kim et al. (2022) with several modifications. Cell morphology was observed under an Inverted Microscope (BDS400 DRAWELL, China). Cells fixed with 1% Lugol solution. The ratio of cell and fixative was 1:1, given directly to avoid movement from the cell during the fixation process. The 100 cells in each sample were measured with Optilab Viewer v4 (Miconos, Indonesia). Cell shape was justified by comparing the aspect ratio (AR). Cells were categorized as spherical when AR was under 1.5, spindle when AR was between 1.5 and 5, and elongated when AR was more than 5 (Jeon et al., 2019).

Primary Metabolite Analysis

Primary metabolite was measured on the ninth day of cultivation. The pellet was collected from a centrifugation process (1327 × g; 10 min) (Ultra 8s LW Scientific, USA) of 10 mL culture used for primary metabolite determination, consisting of carbohydrates, lipids, and proteins. The Bradford method was used to determine the protein level (Bradford, 1976). The pellet was extracted by adding 1 mL of 10% sodium dodecyl sulfate (SDS) and continued with incubation at 95°C for 5 min and 4°C for 5 min, respectively. The sample took about 8µL into 95 well-plates, followed by the addition of 200 µL Bradford reagent. After that, the absorbance read at 595 nm wavelength using an ELISA reader (ELx800 BioTek, USA). The protein standard curve used bovine serum albumin (BSA). The blank solution consisted of 10% SDS and Bradford reagent.

The carbohydrate content was determined using the phenol-sulphuric acid method (Dubois et al., 1956; Suyono et al., 2016). The pellet was added by 1:5 of 5% phenol and H_2SO_4. The sample was incubated for 30 min at room temperature and homogenized before reading the absorbance at 490 nm wavelength using a UV-Vis spectrophotometer (Genesys 150 Thermo, USA). The carbohydrate standard was glucose. The blank solution was composed of 5% phenol and H_2SO_4.

The lipid was analyzed using the Bligh-Dyer method (Bligh & Dyer, 1959). The pellet was added with 1 mL of methanol and 1 mL of chloroform. The sample was mixed using a vortex until homogenized. Afterward, about 1 mL of chloroform and 1 mL of distilled water (Water One-ONEMED, Indonesia) were added and remixed. The sample was centrifuged at 1327 × g for 10 min and continued removing the polar solution. The non-polar solution was poured into a petri dish, which weighed before (M1). The lipid was incubated overnight at 33°C (OV-65 B-ONE, China) until the solvent perfectly evaporated and continued with measuring the petri dish (M2) using an analytical balance (GR-200 AND, Japan). The lipid content was obtained by reducing the weight of M2 and M1 divided by volume (V).

Pigment Analysis

The pigment was analyzed by following Pruvost et al. (2011) on the ninth day of cultivation. Ten mL of the sample was centrifuged (Ultra 8s LW Scientific, USA) at 1327 × g for 10 min. The supernatant was discarded, and the pellet was added with 2 mL of methanol. After that, the sample was homogenized by vortexing until well-mixed. The homogenate was incubated overnight at 4°C in a dark condition by covering it with aluminum foil. The sample was centrifuged (1327 × g; 10 min), and the supernatant read at 665, 662, and 480 nm at spectrophotometer UV-Vis (Genesys 150 Thermo, USA). The absorbance of the supernatant was converted by using Equations 6, 7 and 8 to determine the pigment content.

$$\text{Chlorophyll a } (\mu g/L) = -8.0962 \times \lambda_{652} + 16.5169 \times \lambda_{665} \quad [6]$$

$$\text{Chlorophyll b } (\mu g/L) = 27.4405 \times \lambda_{652} - 12.1688 \times \lambda_{665} \quad [7]$$

$$\text{Carotenoid Total } (\mu g/L) = 4 \times \lambda_{480} \quad [8]$$

Paramylon Analysis

According to Kim et al. (2020), Paramylon was analyzed with several modifications on the ninth day and after fermentation (twelfth day). The pellet from 20 mL of sample was collected through centrifugation (1327 × g; 10 min) (Ultra 8s LW Scientific, USA) and added with 3 mL of 1% SDS. The sample was incubated at 95°C for 30 min using a water bath (WNB-22 Memmert, Germany), then centrifuged (1327 × g; 10 min) to discard the supernatant. The sample was washed twice using distilled water (Water One-ONEMED, Indonesia). About 2 mL of 0.5N NaOH was added into the pellet and homogenized by vortexing. Paramylon was determined by taking 500 µL of aliquots and analyzed using the phenol-sulphuric acid (Dubois et al., 1956). The standard curve was made with D-glucose.

Productivity Measurement

The productivity of biomass, primary metabolite, pigment, and paramylon was calculated using Equation 9.

$$\text{Productivity (g/L/day)} = \frac{\Delta C}{\Delta T} \quad [9]$$

Where: ΔC is the difference in concentration between day-n and day-0, and ΔT is the time period.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC analysis was used to determine the organic compounds after fermentation, following Padermshoke et al. (2016) with several modifications. The pellet, harvested on the twelfth day, was collected from 2L culture centrifugation (Universal 320R Hettich, Germany) for 4 min, 2468 × g, 4°C, added with 100 µL pyridine followed by sonication (JPS-10A Digital Pro+, China) to dissolve the organic compound. About 1 µL chlorotrimethylsilane and 99 µL N, O-Bis(trimethylsilyl) were added and continued with sonification. The extract was dried for 30 min at 37°C using an oven and resuspended with 1 mL n-hexane as a solvent. After filtration, the dissolved extract was injected into GC-MS (QP2010 Shimadzu, Japan). The GC condition fixed: DB one capillary column (30 m × 0.25 mm I.D. and 0.25 µm); column temperature: 10°C; Injection temperature: 290°C; Programed temperature: early temperature was 70°C (held for 5 min) until end temperature 285°C in addition 6°C per min (held for 20 min); interface temperature: 270°C; split ratio was 1:0; pressure 108.1 kPA; helium gas was used as carrier gas. The database library on GC-MS (NIST II library Version) was used to identify detected organic compounds. The quantification of each compound was based on the percentage area of each GC-MS peak.

Statistical Data Analysis

The data was technically collected and analyzed with ANOVA One way (p-value: 0.05) continued with Duncan's multiple range test (DMRT). Microsoft Excel v.2013 (Microsoft Inc., USA), IBM SPSS Statistic v.26 (IBM, USA) and Origin Pro Student Version v.2023b (Origin Lab, USA) were used to perform and analyze the data. The data showed in means followed by standard deviation.

RESULTS

Growth and Biomass of *Euglena* sp.

In this research, the growth and biomass of *Euglena* sp. are positively affected by the presence of BL during the cultivation process. Figure 1a illustrates the growth curve of *Euglena* sp. under BL treatment at nine days of cultivation. The growth of *Euglena* sp. fluctuated, but 0.05 mg/L BL showed the highest cell density over other treatments after day sixth cultivation. In contrast, 0.10 and 1.50 mg/L BL treatments performed lower cell density than the control treatment. Moreover, a similar result was found in the biomass of *Euglena* sp. (Figure 1b). Before the seventh day of cultivation, the biomass of *Euglena* sp. under BL addition showed identical results among all BL treatments and became different from the seventh until the ninth day of cultivation ($p < 0.05$). The 0.05 mg/L BL showed the highest biomass and productivity for about 0.60 ± 0.01 g/L and 58.30 ± 1.47 g/L/day, respectively.

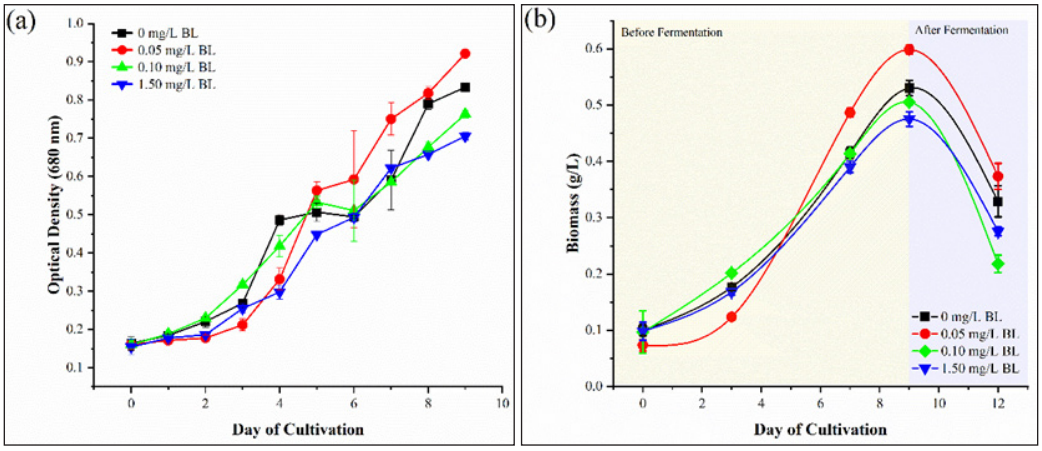

Figure 1. Growth curve (a) and biomass (b) of *Euglena* sp. under BL treatment. Error bars represent standard deviation (n=3)

The precision of our Gompertz and Logistic models in predicting *Euglena* sp.'s growth under BL treatment is noteworthy. Figure 2 illustrates the Gompertz model, with 0.05

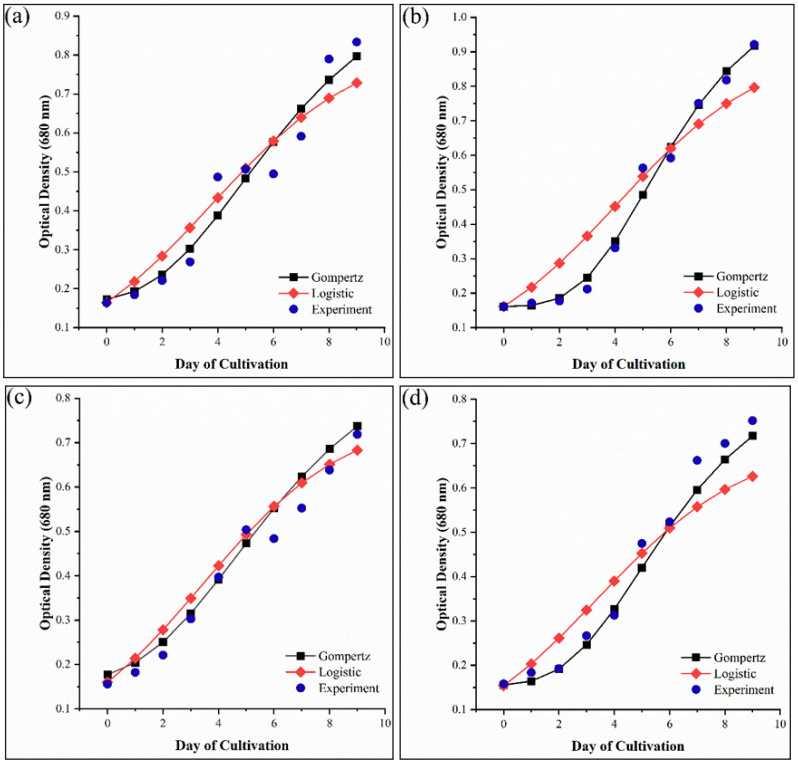

Figure 2. Growth kinetic modeling of *Euglena* sp. under BL treatment: 0 mg/L BL (a), 0.05 mg/L BL (b), 0.10 mg/L BL (c), and 1.50 mg/L BL (d), respectively

mg/L BL showing the highest r_m (0.14) and t_L (2.70). All BL treatments demonstrated an impressive R^2 (Table 1). The Logistic model, on the other hand, showed that 0.10 mg/L BL had the best μ_{max}. While the R^2 of the Logistic model was lower than the Gompertz model, it still scored above 0.900 in all models, instilling confidence in the accuracy of our predictions.

Table 1
The growth parameter of Gompertz and Logistic model in Euglena sp. under BL treatment

BL Concentration (mg/L)	Gompertz Model			Logistic Model	
	r_m	t_L	R^2	μ_{max}	R^2
0	0.10	1.68	0.947	0.37	0.912
0.05	0.14	2.70	0.988	0.38	0.901
0.10	0.08	1.16	0.977	0.39	0.961
1.50	0.10	2.21	0.992	0.37	0.916

Morphology Distribution of *Euglena* sp.

Morphology distribution of *Euglena* sp. under BL treatment was different on day 0 (early), day 9 (late exponential), and after fermentation. On day 0 cultivation, the cell morphology distribution was dominated by spindle shape. This condition changed at the end of cultivation, with cell morphology dominated by elongated shape in all BL treatments. After the culture condition changed to dark hypoxia, the observation of cell morphology distribution showed that the shape domination changed into spherical and spindle shapes. The most spherical shape was found in 0.05 mg/L BL and 0.10 mg/L BL treatments, while the spherical shape showed in 0 mg/L BL and 1.50 mg/L BL treatments (Figure 3).

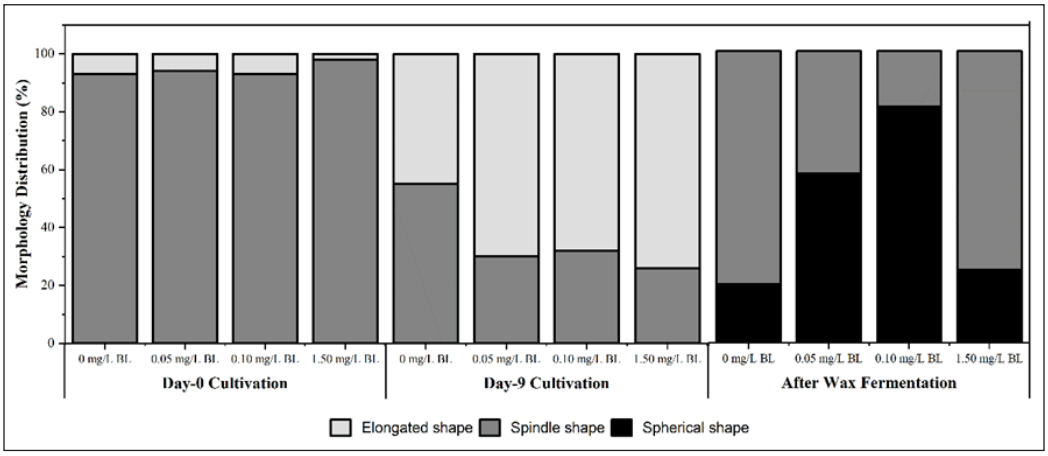

Figure 3. Morphology distribution of *Euglena* sp. under BL treatment on 0 days, 9 days, and after fermentation (12 days)

Primary Metabolites Content of *Euglena* sp.

Brassinolide in *Euglena* sp.'s culture affected the primary metabolite, as seen in Figure 4. Protein content showed a significant improvement at 0.05 mg/L BL up to $15.92 \pm 1.20 \times 10^{-3}$ g/L ($p < 0.05$). This condition was contrasted with 0.10 mg/L BL and 1.50 mg/L BL treatments where the protein content amount was lower than 0 mg/L BL. Besides BL enhancing the protein, the lipid under BL treatment significantly improved ($p < 0.05$). The high lipid content performed in 0.05 mg/L BL treatment was about 0.20 ± 0.01 g/L, but insignificant result with 0.10 mg/L BL based form Duncan test. The positive effect of BL addition also showed in carbohydrate content ($p < 0.05$). The 0.05 mg/L BL gave the highest carbohydrate content compared to other treatments. The carbohydrate content at 0.05 mg/L BL reached 0.47 ± 0.03 g/L. Based on Figure 4c, the lowest carbohydrate level performed at 0.10 mg/L BL, while the 0 and 1.50 mg/L BL showed insignificant results. According to Table 2, the productivity of primary metabolites was performed in high quantities at 0.05 mg/L BL treatment.

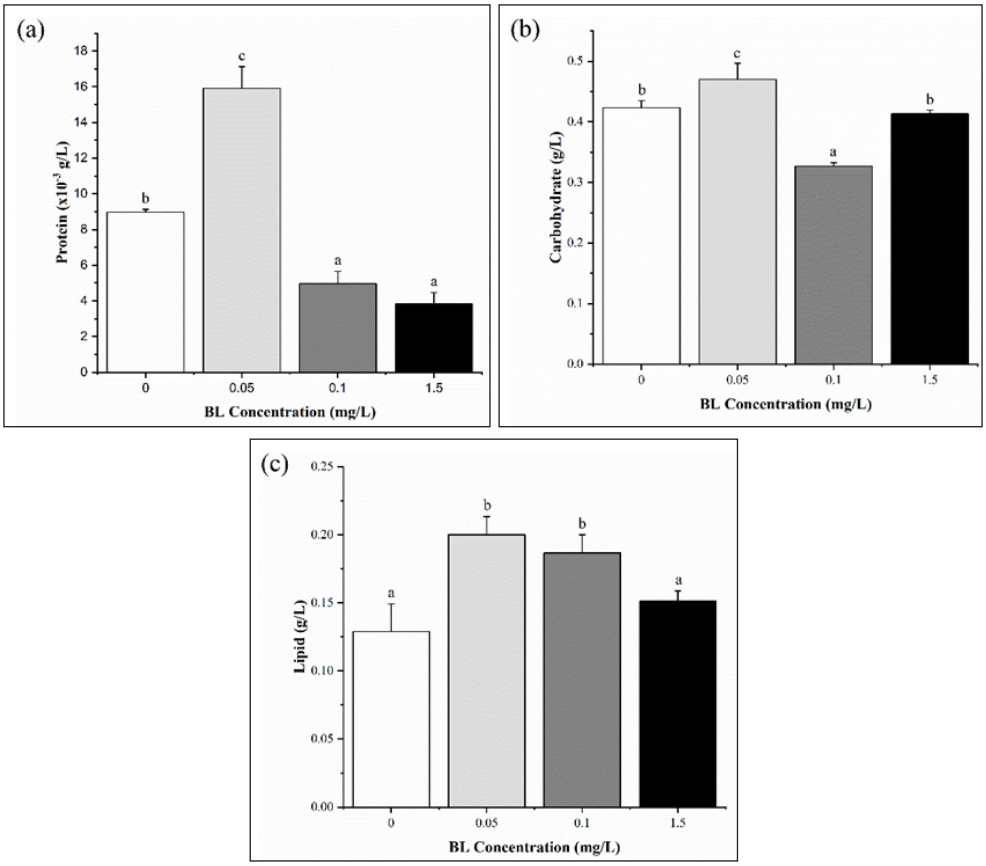

Figure 4. Primary metabolites content of *Euglena* sp. under BL treatment at ninth-day cultivation: protein content (a), carbohydrate content (b), and lipid content (c), respectively. The lowercase letter shows the significant differences based on Duncan's Test ($p < 0.05$). Error bars represent standard deviation (n=3)

Table 2
The productivity of biomass, primary metabolites, pigment, and paramylon in Euglena sp. under BL treatment

Productivity ($\times 10^{-3}$ g/L/day)	**BL Concentrations (mg/L)**			
	0	**0.05**	**0.10**	**1.50**
Biomass	47.80 ± 1.67^{b}	58.30 ± 1.47^{c}	45.40 ± 4.66^{ab}	41.90 ± 3.06^{a}
Primary metabolites				
Carbohydrate	40.70 ± 0.58^{b}	46.30 ± 2.89^{c}	31.70 ± 0.58^{a}	40.00 ± 1.00^{b}
Lipid	9.00 ± 0.00^{a}	15.00 ± 0.00^{bc}	12.70 ± 2.52^{bc}	11.00 ± 1.70^{ab}
Protein	0.82 ± 0.01^{b}	1.58 ± 0.14^{a}	0.34 ± 0.05^{c}	0.28 ± 0.07^{a}
Pigment				
Chlorophyll a	0.23 ± 0.03^{a}	0.23 ± 0.02^{a}	0.26 ± 0.01^{ab}	0.29 ± 0.02^{b}
Chlorophyll b	0.46 ± 0.01^{ab}	0.59 ± 0.01^{c}	0.48 ± 0.02^{b}	0.43 ± 0.04^{a}
Carotenoid Total	0.14 ± 0.01^{a}	0.17 ± 0.01^{b}	0.15 ± 0.01^{ab}	0.14 ± 0.01^{a}
Paramylon	10.70 ± 1.41^{b}	13.80 ± 0.12^{c}	6.40 ± 1.76^{a}	7.50 ± 1.01^{a}

Notes. The data is shown in the means followed by the standard deviation (n=3). The lowercase in the same row showed significant differences based on Duncan's Test ($p < 0.05$)

Pigment Content on *Euglena* sp.

The pigment content at the end of cultivation revealed a significant effect ($p < 0.05$) in BL treatments (Table 3). The 1.50 mg/L BL gave the highest chlorophyll a for about 4.14 ± 0.10 µg/mL, while the highest chlorophyll b and carotenoid total found in 0.05 mg/L BL, 5.43 ± 0.07 µg/mL and 1.83 ± 0.03 µg/mL, respectively. In addition, 0.05 mg/L BL exhibited a high amount of chlorophyll a+b but decreased the chlorophyll a/b. The chlorophyll a and b productivity significantly increased in 1.50 mg/L BL and 0.05 mg/L BL, respectively.

Table 3
Pigment content on Euglena sp. under BL treatment on the ninth day of cultivation

BL Concentration (mg/L)	**Chl a (µg/mL)**	**Chl b (µg/mL)**	**Car. Total (µg/mL)**	**Chl a+b (µg/mL)**	**Chl a/b**	**Car./Chl a+b**
0	4.00 ± 0.01^{b}	4.37 ± 0.02^{b}	1.70 ± 0.03^{b}	8.73 ± 0.03^{b}	0.92 ± 0.00^{b}	0.20 ± 0.00^{b}
0.05	3.73 ± 0.03^{a}	5.43 ± 0.07^{c}	1.83 ± 0.03^{c}	9.16 ± 0.05^{c}	0.69 ± 0.01^{a}	0.20 ± 0.00^{b}
0.10	4.02 ± 0.06^{b}	4.36 ± 0.23^{b}	1.70 ± 0.04^{b}	8.38 ± 0.17^{b}	0.92 ± 0.06^{b}	0.20 ± 0.00^{b}
1.50	4.14 ± 0.10^{c}	3.86 ± 0.36^{a}	1.54 ± 0.08^{a}	7.99 ± 0.27^{a}	1.08 ± 0.12^{c}	0.19 ± 0.00^{a}

Notes. The data shows in means followed by standard deviation (n=3). The lowercase in the same column showed significant differences based on Duncan's Test ($p < 0.05$)

Paramylon Content of *Euglena* sp.

Paramylon content of *Euglena* sp. under the presence of BL showed a significant effect ($p < 0.05$) (Figure 5). The concentration of BL at 0.05 mg/L gave the great result of paramylon

production at the end of cultivation, accounting for 145 ± 1.04 × 10^{-3} g/L. In contrast, the high BL concentration performed the lowest concentration of paramylon, where 0.01 mg/L BL and 1.50 mg/L BL were 92.7 ± 7.97 × 10^{-3} g/L and 55.3 ± 2.80 × 10^{-3} g/L, respectively. On the other hand, 0.05 mg/L BL showed the highest paramylon productivity than other BL treatments (Table 2). The depletion of paramylon occurred after the fermentation process, which was conducted for three days at dark-hypoxia conditions. All paramylon contents were depleted, and the highest depletion of paramylon was found at 0.05 mg/L BL. Due to the highest production and depletion of paramylon found at 0.05 mg/L BL, this treatment was chosen to determine the organic compound by GC-MS. Besides that, the 0 mg/L BL was used as a control treatment to compare with the selected treatment. Therefore, this treatment continued for further analysis through GC-MS.

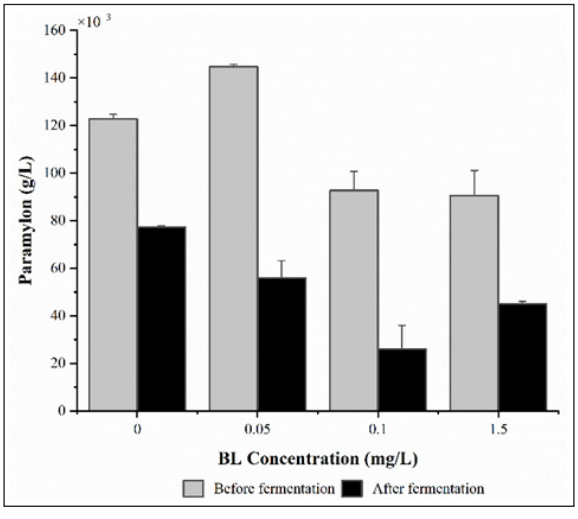

Figure 5. Paramylon content in *Euglena* sp. is under BL treatment on the ninth and twelfth days of cultivation. Error bars represent the standard deviation (n=3)

Wax Ester of *Euglena* sp.

The untargeted GC-MS results revealed the presence of 14 and 15 organic chemical compounds at 0 and 0.05 mg/L BL treatment, respectively. Among these, we detected the presence of a unique organic compound, which we assumed to be a wax ester, in both treatments after 40 min reading at GC-MS (Figure 6). The wax ester content at 0 mg/L BL remained higher than 0.05 mg/L BL treatment. We classified the wax ester species from carbon chain numbers 26 to 36 (Table 4). The wax ester concentration was dominated by carbon chain numbers 26 (lauryl myristate), 28 (myristyl myristate), and 30 (cetyl myristate), while the other wax ester species were present in low concentrations. Interestingly, at 0.05 mg/L BL, we found a menthol compound in higher concentration than a wax ester, namely $C_{10}H_{20}O$ or neomenthol, for about 18.75 ± 1.15 %.

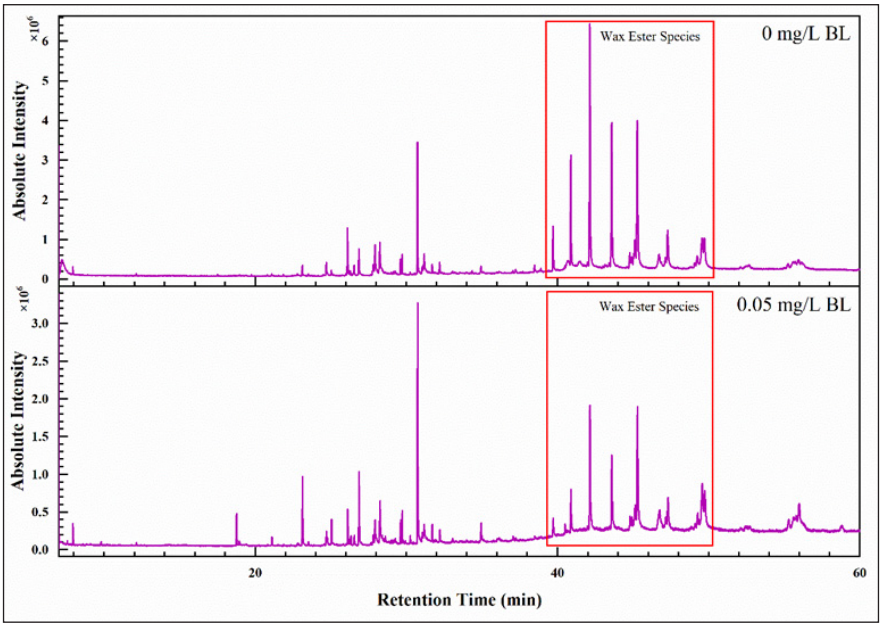

Figure 6. TIC graph from GC-MS analyses the most significant treatment in *Euglena* sp. after fermentation (twelfth day)

Table 4
Wax ester species of Euglena sp. under BL treatment after wax fermentation (twelfth day)

0 mg/L BL				
Ret. Time (min)	**Similarity Index**	**Name**	**Formula**	**Relative Abundance (%±SD)**
42.137	87	Myristyl myristate	$C_{28}H_{56}O_2$	24.74±1.54
43.579	89	Lauryl myristate	$C_{26}H_{52}O_2$	16.97±1.48
45.276	87	Cetyl myristate	$C_{30}H_{60}O_2$	19.76±0.89
47.289	88	Lauryl palmitate	$C_{28}H_{56}O_2$	5.34±0.04
49.721	89	Stearyl stearate	$C_{36}H_{72}O_2$	3.59±2.08
0.05 mg/L BL				
Ret. Time (min)	**Similarity Index**	**Name**	**Formula**	**Relative Abundance (%±SD)**
30.746	89	(1S, 2S, 5R)-(+)-Neomenthol	$C_{10}H_{20}O$	18.75±1.15
42.146	89	Myristyl myristate	$C_{28}H_{56}O_2$	13.67±1.17
43.590	88	Lauryl myristate	$C_{26}H_{52}O_2$	8.80±0.49
45.289	87	Cetyl myristate	$C_{30}H_{60}O_2$	15.76±0.49
47.308	88	Stearyl stearate	$C_{36}H_{72}O_2$	4.01±0.02
49.740	88	Cetyl palmitate	$C_{32}H_{64}O_2$	4.92±0.24

Notes. The data is shown in the means followed by the standard deviation (n=2)

DISCUSSION

Growth and Biomass of *Euglena* sp.

In this study, we presented the effect of BL on improving the growth rate and metabolic content in *Euglena* sp., where the lowest concentration gave the best result (0.05 mg/L BL) at the end of cultivation. Bajguz and Czerpak (1998) reported that a lower concentration of BRs gave insignificant results, while a higher concentration gave inhibition effects. Brassinolide affected the growth rate of *Euglena* sp. by increasing the cell number; this result was indicated by cell density. The promotion of cell numbers depends on the cell division process. Brassinolide addition promotes cell division by up-regulating the *CyD3*, a cyclin-dependent kinase (CDK) family, which has a role in the division of eukaryotic cells (Hu et al., 2000). Bajguz and Czerpak (1998) and Kozlova et al. (2017) demonstrated a similar result in improving cell numbers by adding BL. According to this result, adding BL could promote *Euglena* sp.'s growth rate. However, the difference between 0 mg/L BL and 0.05 mg/L BL was slightly different and less than the given impact of other phytohormones in *Euglena* sp., such as indole-3-acetic acid (IAA), which greatly enhanced the growth rate of *Euglena* sp. (Hakim et al., 2023). The life cycle of *Euglena* was varying. Patil et al. (2024) showed that on the tenth day of cultivation, the culture of *Euglena* entered the stationary phase. A similar result was shown in research by Erfianti et al. (2024) that the tenth day of *Euglena* cultivation undergoes the stationary phase. Moreover, Jung et al. (2021) exhibited *Euglena's* earlier late exponential phase, which was the seventh day of cultivation. It was indicated that the life cycle of *Euglena* was varying, and in this research, the presence of BL could not change the life cycle of *Euglena* but enhance the cell division during the cultivation process.

The cell biomass increased during the cultivation process. Cell biomass production is affected by the presence of BL through photosynthesis. BL could promote the photosynthesis process by affecting the pigment, photosynthesis rate, and fixation of CO_2. Singh et al. (2016) showed that the high photosynthesis rate resulted in a high accumulation of biomass. As a result of the high photosynthesis rate, the product of photosynthesis remains high and supports the cell development marked by the cell biomass. The cell biomass was positively correlated with the cell concentration (Lim et al., 2022). This was similar to this research, showing the improvement of cell concentration followed by the cell biomass.

The growth kinetic modeling was carried out to illustrate the suitable growth model for *Euglena* sp. under BL treatment. Gompertz and Logistic models, which are commonly used to demonstrate the growth kinetic model in microorganisms, especially in microalgae (Erfianti et al., 2023; Naser et al., 2023; Širi´c et al., 2023), were employed in this study. The R^2 value obtained from both models described how appropriate those models represented the data (Ajala & Alexander, 2020). For instance, the growth kinetic model of *C. vulgaris* performed extensively with an R^2 value of more than 0.98 in the Gompertz and Logistic

model (Kothari et al., 2023). According to the R^2 value, the Gompertz model is more appropriate to demonstrate *Euglena* sp.'s growth rate under BL treatment. Gompertz's model showed the r_m value, which corresponds to biomass production. Moreover, the t_L value also extensively describes the lag phase based on the cell density; for instance, in 0.05 mg/L, BL has the highest value of t_L, which means the time needed by the cell to continuously in log phase, and this result was appropriate with a growth curve that shows the time of lag phase is longer than other treatments.

Morphology Distribution of *Euglena* sp.

Cell elongation was one effect resulting from the presence of BL through the BL mechanism signaling process (Yang et al., 2011). Mumtaz et al. (2022) revealed that the absence of BL in tomato *abs 1 mutant* generated the smaller fruit size, which means the presence of BL was needed to avoid the dwarfism in tomato plants that caused the smaller fruit size. The role of BL in promoting cell elongation was also presented in Yayao (gourd cultivar) seeds grown at salt-stress conditions (Liu et al., 2023). In addition, the same result of BL in promoting cell elongation was shown in the sesame cultivar (Nasser & Sarhan, 2023). Besides BL promoting cell elongation through regular mechanisms described by Muller and Munne-Bosch (2021), BL could encourage the elongation of cells by interacting with some hormones. Improvement of *D18/GAox-2* gene transcription exhibited under the presence of BL (Tong et al., 2014). The *D18/GAox-2* gene was a family gene in gibberellic acid (GA_3), which has a role in the elongation of plant cells. The mechanism of how BL promotes cell elongation has not been well-observed. However, the mechanism of BL promoting cell elongation in plants illustrated how BL works. BL probably promoted cell elongation in *Euglena* by interacting with the GA_3, which could promote cell elongation in plant cells (Figure 3). Thus, it indicates that BL could promote cell elongation in *Euglena* sp.

Primary Metabolites Content of *Euglena* sp.

The primary metabolites had similar results to previous studies that revealed a significant improvement in primary metabolites. Research by Bajguz and Czerpak (1998) demonstrated the improvement of monosaccharides in *C. vulgaris*. As a result of the photosynthesis process, carbohydrates play crucial roles in cell activity in the case of energy supply. In photosynthesis, BL had a positive effect in light and dark cycles. The depletion of some carbohydrates resulted in the photosynthesis process, which would be converted into lipids, which is affected by some factors. A stressful environment could trigger lipid accumulation, for instance, lightning and CO_2 stress, nutrient modification, cultivation process, and chemical addition (Song et al., 2022). Moreover, Huang et al. (2022) reported that adding a phytohormone combination (GA_3, IAA, and BL) with turbulence intensity improved lipid content in *S. quadricauda*. Improvement in lipid content under BL was also found

at *S. quadricauda*, both at BL and epi-brassinolid (EBL) addition (Kozlova et al., 2017). Additionally, Pokotylo et al. (2014) and Liu et al. (2018) showed improved lipid content under BL treatment in a stressful environment.

Adding BL induced the related genes from *Euglena* sp. as a response to BL and affected the improvement of transcription and translation in *Euglena* sp. The presence of BL was verified to enhance the genetic material, DNA and RNA in *C. vulgaris*, improving protein level by up to 296% (Bajguz, 2000). In addition, a similar result was also performed in *Wolffia arrhiza*, showing that protein levels were elevated by about 25% (Chmur & Bajguz, 2021). Moreover, free amino acids and soluble protein improvement were performed under BL treatment on *Pinnela ternate* (Guo et al., 2022), showing that BL could affect protein levels.

All the primary metabolites, including carbohydrates, lipids, and proteins, were significantly enhanced under the presence of BL. This result was similar to cell growth and cell biomass, which significantly improved. In this result, BL is enhanced in photosynthesis, resulting in a high accumulation of glucose that will break down into some metabolites, such as lipids. In addition, the protein content is elevated by cell response, DNA and RNA under BL. Further analysis of the effect of BL in the photosynthesis process completely describes pigment content.

Pigment Content on *Euglena* sp.

Brassinolide's role in regulating chlorophyll synthesis, a process it tends to suppress, has significant implications. Tachibana et al. (2022) demonstrated this, reporting that the BRASSINOZOLE insensitive mutant 1 in Arabidopsis exhibited a light-green leaf color. Intriguingly, the Arabidopsis BRASSINOLIDE insensitive mutant 1 showed a dark green leaf morphology and high chlorophyll content (Zhang et al., 2021). Even in the presence of BL, chlorophyll biosynthesis was suppressed, but BL could also promote chlorophyll biosynthesis by interacting with certain phytohormones. The addition of exogenous BL was found to induce GA_3 synthesis (Peres et al., 2019). Furthermore, the mutation in tomato *abs 1* led to a lower expression of *GA20ox1*, GA_3 gene, and chlorophyll genes (Mumtaz et al., 2020). The role of GA_3 in improving chlorophyll content was found to have a significant effect (Khandaker et al., 2015; Iftikhar et al., 2019). Additionally, BL was found to interact with cytokinin (CK), further enhancing chlorophyll content (Bajguz & Potrowska-Niczyporuk, 2014; Peres et al., 2019).

The addition of BL did not improve pigment content but improved photosystem II (PSII) efficiency and CO_2 assimilation. PSII efficiency increased after the addition of BL, even in normal and stressful conditions, and enhanced net photosynthesis (Yuan et al., 2012; Ma & Guo, 2014; Lima & Lobato, 2017; Zhao et al., 2019; Liu et al., 2022; Sun et al., 2022; Liu et al., 2023). The high chlorophyll b content indicated the stress condition

and caused the lower ratio of chlorophyll a/b (Yuan et al., 2012). Overexpression of chlorophyllide an oxygenase (CAO) affected the reduction of chlorophyll a because the chlorophyll a converted into chlorophyll b, causing the depletion of chlorophyll a/b ratio but enlarging the antenna size (Tanaka et al., 2014). Furthermore, photosynthetically effective radiation (PAR) had a crucial role in the ratio of chlorophyll a/b (Kume et al., 2018). In a similar study, Amelia et al. (2023) found that light shade adaptation to light intensity exhibited lower chlorophyll a, supporting our findings. Following this research, adding a lower concentration of BL probably promotes *Euglena* sp. to respond in cell adaptation to light by regulating the CAO activity and improving the PSII efficiency. Because of this, net photosynthesis increased and was marked by high biomass accumulation (Yuan et al., 2012). Moreover, the accumulation of primary metabolites was high under lower BL treatment, affecting photosynthesis. At the same time, high photosynthesis activity will produce more glucose than used to, for example, lipid biosynthesis (Li et al., 2020).

Assimilation of CO_2 was assumed to improve the chlorophyll content by an unknown mechanism - BL addition has proven to enhance CO_2 assimilation (Yuan et al., 2012; Bajguz & Potrowska-Niczyporuk, 2014). Xia et al. (2009) showed the improvement of six genes related to carbon fixation by the addition of EBL and improved the maximum Rubisco carboxylation rate (V_{cmax}) and maximum RuBP regeneration rate (J_{max}). A similar result was presented on maize seedlings added by EBL that up-regulates eight genes that worked on the dark cycle of photosynthesis (Gao et al., 2021). The great Rubisco activity illustrated that the CO_2 absorption elevated and produced glucose as the main product of the dark cycle. Consequently, carbohydrate content in this research reached a significant result at 0.05 mg/L BL treatment. A similar result was shown in *W. arrhiza*, where monosaccharides were elevated under BL addition (Chmur & Bajguz, 2021).

Paramylon Content of *Euglena* sp.

Paramylon, the energy storage from *Euglena* sp., is formed in granules composed of β-1,3-glucan (Zakryś et al., 2017), catalyzed by paramylon synthase (β-1,3-glucan synthase or callose synthase) from uridin diphosphate (UDP) (Marechal & Goldemberg, 1964; Yoshida et al., 2016). Production of paramylon reached the highest content at the late exponential phase, remained constant, and tended to decrease while entering the stationary and death phases (Kim et al., 2019). Thus, the analysis of paramylons is conducted at the late exponential phase, which is the ninth day before the wax fermentation process and at the twelfth day after the wax fermentation process. The addition of phytohormones into *Euglena* culture has been found to play a crucial role in increasing the production of paramylon. This intriguing phenomenon was observed in a study by Kim et al. (2019), where the production of IAA by bacteria significantly enhanced paramylon production. Furthermore, this study also revealed that BL promotes the production of paramylon under low concentrations. As

mentioned earlier, a lower concentration of BL significantly increased the protein content. This may correlate with paramylon production in *Euglena* sp. by regulating gene-coded protein (enzyme) that works in the biosynthesis of paramylon.

Wax Ester of *Euglena* sp.

Fermentation was conducted to produce wax ester through a mechanism described by Inui et al. (2017). After the fermentation process, the depletion of biomass and paramylon content occurred in all treatments. This result was similar to Jung et al. (2021) in that the depletion of both parameters occurred after fermentation and increased in fatty acid, rich in 14 carbon chains (myristic acid), which is useful for biofuel. Moreover, Padermshoke et al. (2016) and Ogawa et al. (2022) showed the domination of 28 carbon chains (myristyl myristate) after wax fermentation. According to this result, the wax ester on BL treatment showed lower wax ester content than the control treatment.

On the other hand, the presence of neomenthol (a cyclic monoterpene) in BL treatment remained high. This is significant as terpenoids are synthesized through the mevalonate acid (MEV) pathway and methylerythritol phosphate (MEP) pathway (Harrewijin et al., 2000; Kang & Lee, 2016). The formation of monoterpenoids and their subsequent conversion into neomenthol, as described by Croteau et al. (2005), is a key process. Chizzola et al. (2004) demonstrated the presence of a menthol derivative after fermenting the *Thuja orientalis* into a rusteic system. Moreover, BL plays a pivotal role in the stress environment response by regulating the stress defense mechanism, whether enzymatic or non-enzymatic (Vardhini & Anjum, 2015). Dark hypoxia, a stress condition, may induce the stress defense mechanism in *Euglena* sp. The presence of BL probably affected *Euglena* by stimulating the stress defense mechanism. The change in cell morphology, particularly the drastic shift to a spherical shape at 0.05 mg/L BL compared to 0 mg/L BL after fermentation, may be correlated with this result. Consequently, the lower wax ester content on BL treatment was likely due to neomenthol, which uses the same substrate as wax ester during the fermentation process as a stress defense mechanism generated by the presence of BL under dark hypoxia conditions.

CONCLUSION

Adding BL has a positive effect on *Euglena* sp., especially in terms of growth rate, primary metabolite, and pigment content. The positive result was shown at lower BL concentration, whether high concentration tends to give a contrast result. Moreover, BL also promoted cell elongation on *Euglena* sp. After wax fermentation, the BL treatment showed a contrasting result, which produced lower wax ester content. This improvement made BL one of the chemical stimulators that enhance the productivity of *Euglena* sp. as a candidate for alternative natural resources. Research about the effect of BL in *Euglena* sp.

remained rare and has yet to be studied. As a result, advanced research on the BL mechanism affecting *Euglena* sp.'s growth rate and metabolic content is still needed to give more precise information.

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