TRENDS IN SCIENCES 2026; 23(12): 13335
https://doi.org/10.48048/tis.2026.13335
RESEARCH ARTICLE

Growth Kinetics, Crude Paramylon-Enriched Carbohydrate Fraction Production, and *Glucan Synthase-Like* Gene Expression Associated with Protocatechuic Acid Supplementation in Local Strain of *Euglena* sp. IDN5 Isolated from Glagah Beach, Kulon Progo, Indonesia

Dita Aulia Yulyanita, Woro Anindito Sri Tunjung, Angga Puja Asiandu, Renata Adaranyssa Egistha Putri and Eko Agus Suyono*

Faculty of Biology, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia

(*Corresponding author's e-mail: eko_suyono@ugm.ac.id)

Received: 12 January 2026, Revised: 22 May 2026, Accepted: 29 May 2026, Published: 15 July 2026

Abstract

Paramylon, one of the active compounds produced by *Euglena* sp., has broad application in biofuel, cosmetics, nut raceuticals, and food supplements. One potential strategy to increase paramylon production involves the administration of protocatechuic acid (PCA). Previous studies demonstrated that PCA modulates EgGSL2 expression, a key gene involved in paramylon biosynthesis. However, the expression of this gene had not been evaluated in the *Euglena* culture when cultivated in combination between photoperiod and macronutrient composition (glucose as carbon sources and KNO_3 as nitrogen sources) in variations of 0, 0.01 and 0.03 g L^{-1}, along with PCA addition in variations of 0, 0.01, 0.2 and 0.8 g L^{-1}, especially in local strain from Indonesia. Therefore, in this study, we modify glucose:KNO_3 ratio, PCA supplementation in various concentration, added with 16:8 h light–dark photoperiod to evaluate the effect of PCA on gene expression of *EgGSL* in *Euglena* sp. IDN5 (Indonesian local strain) that is related in crude paramylon-enriched carbohydrate fraction production. The addition of glucose, KNO_3, and PCA in high concentration along with photoperiod was hoped to have correlation in crude paramylon-enriched carbohydrate fraction, pigment, and growth of *Euglena* sp. IDN5. Gene expression was done through qPCR analysis. Metabolite production of *Euglena* sp. was measured through analysis of paramylon and pigments measurement. While the growth of microalgae was analysed through the cell count, biomass, and optical density analysis. Our study revealed that the combination of glucose:KNO_3:PCA 0.03:0.03:0.8 g L^{-1} probably associated with several parameters, such as specific growth rate (0.17 ± 0.003 day^{-1}), chlorophyll-a and chlorophyll-b (11.92 ± 1.22 × 10^{-3} g L^{-1}) and (11.17 ± 0.99 × 10^{-3} g L^{-1}) respectively, crude paramylon-enriched carbohydrate fraction productivity (0.23 ± 0.003 g L^{-1} day^{-1}), crude paramylon-enriched carbohydrate fraction percentage (84.90 ± 6.54%), and *EgGSL2* gene expression (20.19 ± 2.27 -fold increase). Thus, it shows the correlation of this strategy to the growth performance and paramylon productivity in *Euglena* sp. culture. However, for further exploration and application, detailed research about the mechanism needs to be done. This research serves as the base for potential further research of the effect of combination of glucose, KNO_3, and PCA in *Euglena* sp.

Keywords: Biofuel production, *EgGSL2*, Indonesian local strain, C:N supplementation, Mixotrophic cultivation, Crude paramylon-enriched carbohydrate fraction, Protocatechuic acid

Introduction

Euglena sp. is a unicellular microalga species with significant potential for numerous applications in functional foods, feeds, biofuel, and bioremediation supported by its remarkable ability to change their morphology in response to circadian rhythms, environmental changes, and external stimuli [1]. It produces a variety of bioactive compounds, including paramylon, fatty acids, vitamin E/tocopherol, proteins,

and pigments (chlorophyll and carotenoids) [2] that contribute to diverse bioactivities, such as maintaining prebiotic bacteria [3], renoprotective effects [4], improves gut immunity and enhances host protection from coccidiosis [5], and exhibits antidiabetic effects by reducing lipid accumulation and LDL levels [6].

Moreover, its biomass comprises approximately 34% carbohydrates, of which 70% - 80% consists of paramylon [7]. Paramylon, under hypoxic conditions, can be directly converted into lipids through wax ester fermentation used as bio-jet fuel precursor [8]. Several strategies have been developed to increase paramylon production, such as the application of phytohormones (indole-3-acetic acid) that is derived from *Vibrio natriegens*. This bacteria was inducted with ferulic acid from rice husks [9]. Electrical stimulation techniques[10] and the use of a protocatechuic acid also allow the increase of paramylon from *Euglena gracilis* [11].

In order to increase its biomass production, it is also essential to provide sufficient macronutrients, such as nitrogen and carbon sources in the culture [13]. Nitrogen (N) is a fundamental macronutrient required for cellular growth and metabolism, as it plays an important role in biochemical reactions and is essential for the synthesis of biomolecules such as chlorophyll, proteins, carbohydrates, and lipids [14]. Carbon (C) also plays an essential role as the building block of proteins, lipids, and carbohydrates [15,16]. Therefore, the concentration of organic carbon may exert a hormetic effect on microalgal metabolite accumulation [17].

As interest in microalgae as sustainable biofuel sources continues to increase due to fossil fuel depletion, rising costs, and associated greenhouse gas emissions, there is a need to optimize its cultivation strategies [18]. IDN 5 is a microalgae strain isolated from Dieng plateau, Indonesia. Out of many strains isolated from the area (IDN1 until IDN40), IDN 5 had limited exploration, especially in the terms of genetic exploration. PCA combined with glucose and KNO_3 as carbon and nitrogen sources was known to increase paramylon productivity individually. However, the combination between both treatments is not yet explored to increase both paramylon productivity and gene expression. Therefore, this study aimed to evaluate the combined effects of protocatechuic acid supplementation and glucose:KNO_3 ratio modulation under a 16:8 h light-dark cycle on growth performance and crude paramylon-enriched carbohydrate fraction productivity in *Euglena* sp. IDN5.

Materials and methods

Culture conditions

Euglena sp. IDN5 was previously isolated from Glagah Beach, Kulon Progo, Yogyakarta, Indonesia, while protocatechuic acid (PCA) ($(HO)_2C_6H_3CO_2H$) was purchased from Merck with purity of ≥ 98.00% as can be seen from batch number of 132003 certificate of analysis from article 89766 by PhytoLab. The starter of *Euglena* sp. IDN5 was previously cultivated using sterilized CM media (Free of NH_4SO_4) for about 7 days. Three biological replications (each with different flask) were made in the same condition, placement for flask was changed after each sampling condition (Rotating along all repetition) to make sure the equal light intensity and temperature condition. The flask was all handled consistently. Each treatment also had parallel sacrificial cultures to maintain the equal and representative biological condition during cultivations despite the use of destructive analysis method. The details can be seen in **Table 1**.

Table 1 Sampling design for each replication from each treatment.

Day	Sacrificial flask number	Amount of volume taken for sampling (mL)						Total volume taken for each sampling (mL)
		Cell Density (mL)	Optical Density (mL)	Biomass (mL)	Pigments (mL)	Crude paramylon-enriched carbohydrate fraction (mL)	qPCR (mL)	
0	A	5	2	10	-	-	15	22
1	B	5	2	10	5	10	-	32
2	C	5	2	10	-	-	-	17
3	D	5	2	10	5	10	-	32

Day	Sacrificial flask number	Amount of volume taken for sampling (mL)						Total volume taken for each sampling (mL)
		Cell Density (mL)	Optical Density (mL)	Biomass (mL)	Pigments (mL)	Crude paramylon-enriched carbohydrate fraction (mL)	qPCR (mL)	
4	E	5	2	10	-	-	-	17
5	F	5	2	10	5	10	-	32
6	G	5	2	10	-	-	15	32
7	H	5	2	10	5	10	-	32
8	I	5	2	10	-	-	15	32
9	J	5	2	10	5	10	-	32
10	K	5	2	10	-	-	-	17
11	L	5	2	10	5	10	-	32
12	M	5	2	10	-	-	-	17

Then, 50 mL of starter culture were transferred to 450 mL CM media, aseptically, with the total volume of 500 mL. The culture was incubated for 12 days (until stationary phase) under irradiation of under a cool daylight white lamp of Ecofit LED 6500K; T8; 16W; 1600 lm; 220 - 240 V; 50/60 Hz; 130 mA that is manufactured by Koninklijke Philips N.V. with length of 120 mm (light intensity of average of 8,500 lux or 114.063 PAR (µmol m^{-2} s^{-1}) on the culture's surface (above) (30 cm away from the source of light) and average of 6000 lux or 80.515 PAR (µmol m^{-2} s^{-1}) on the culture's surface (side) (40 cm away from the source of light)). Light intensity measurement was done using Apogee MQ-510 Full Spectrum Meter and Hanna HI-97500 Portable Lux Meter. Photoperiod was set on a 16 h light period / 8 h dark period. Aeration rate was set on 0.8 LPM using ambience air filtered with 0.22 µm syringe filter in the cultivation flask of 500 mL (Duran Laboratory Bottle). Headspace volume at the beginning of cultivation was 100 mL. Bubbling became the sole source of culture mixing. LP-100 Resun low noise air pump was used to give aeration from ambience air to the cultivation bottles with output of 140L/min and pressure 0.042 MPa. The rate of CO_2 ambience was measured to be around 700 ppm using CO_2-mini carbon dioxide monitor from Custom Tokyo Japan. The aeration was flowed through Trilliun PVC hose with diameter 3/16 inch (Production code SIMS18311) with length of one meter. In the middle (50 cm) of the tubing, 0.22 µm syringe filter MCE Biosharp (Cat No. BS-MCE33-22-S) was placed to prevent contamination from aeration. Bubbling was flowed directly from the tubing to the culture without airstone. The mouth of the hose in the culture was placed 2.5 cm from the base of cultivation flask. The mouth of cultivation flask was covered with sterile (autoclaved) cotton roll from Masa Husada Klaten, Indonesia with the bubbling hose in the middle and the cotton covered in tight plastic wrap to prevent contamination from outside of the culture. Evaporation rate was at 2% (v/v) per day of cultivation. pH of culture was maintained every sampling time at pH 3.5 using 1N NaOH and 1N H_2SO_4. Cultivation temperature was maintained in 27 °C. Sample collections were done on parallel cultures to avoid the bias of all kinetic and productivity calculations.

The Modified Cramer-Myers (CM) medium was prepared by following the composition prepared for 1 L of distilled water: 1 g KH_2PO_4 (7.34×10^{-6} M), 0.2 g $MgSO_4.7H_2O$ (811.4×10^{-6} M), 0.02 gram $CaCl_2{\cdot}2H_2O$ (136.04×10^{-6} M), 100 µL Trace metal mix solution, 100 µL $Na_2MoO_4{\cdot}2H_2O$ Solution (235.32×10^{-6} M), 20 µL Vitamin B_1 Solution (14.82×10^{-6} M), 25 µL Vitamin B_{12} Solution (14.75×10^{-6} M), with a pH of 5.5. NH_4SO_4 (7.56×10^{-6} M) that is in the original media recipe was removed (not added) to make sure the only nitrogen source come from KNO_3. The initial stock culture used also cultured in the media without NH_4SO_4. Stock solution was only made for trace metal and vitamins, while other chemicals was measured with AL204 Mettler Toledo Analytical balance for every time the media was made. Trace metal mix was made from 3 mg L^{-1} $FeSO4{\cdot}7H_2O$ (278.01×10^{-6} M), 1.8 mg L^{-1} $MnCl_2{\cdot}4H_2O$ (9.09×10^{-6} M), 1.5 mg L^{-1} $CoSO_4{\cdot}7H_2O$ (5.34×10^{-6} M), 0.4 mg L^{-1} $ZnSO_4{\cdot}7H_2O$ (1.39×10^{-6} M),

0.02 mg L^{-1} $CuSO_4 \cdot 5H_2O$ (80.10×10^{-6} M) in 1 L of distilled water. $Na_2MoO_4 \cdot 2H_2O$ Solution was made by dissolving 2 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$ in distilled water. Vitamin B_1 Solution was made from 0.1 mg/L thiamine in double distilled water. Vitamin B_{12} Solution was made from 0.0005 mg/L of cobalamin in double distilled water. All stocks were stored in 4 °C refrigerator. All chemicals used was stored in 25 °C room (Vitamins were stored in dark condition in 4 °C refrigeration). Meanwhile, nitrogen source was in the form of potassium nitrate (KNO_3) and carbon source was in the form of glucose ($C_6H_{12}O_6$). Potassium nitrate, glucose and PCA was added during the media making and sterilized along with the media. The composition of each treatment is presented in **Table 2**. Media and stock cultures was sterilized using filtration through Biosharp 0.22 µm syringe filter added with UV exposure using Evaco UV light GL 220V 50 Hz T8 30W for 30 min.

Table 2 Concentration of glucose, KNO_3 and protocatechuic acid.

Code	Carbon (Glucose) (g L^{-1})	Nitrogen (KNO_3) (g L^{-1})	C-mol ($\times 10^{-3}$ mol/L)	N-mol ($\times 10^{-3}$ mol/L)	PCA concentration (g L^{-1})
Control	0	0	0.00	0.00	0
T1	0.01	0.01	0.33	0.09	0.01
T2	0.01	0.01	0.33	0.09	0.2
T3	0.01	0.01	0.33	0.09	0.8
T4	0.03	0.03	1.00	0.29	0.01
T5	0.03	0.03	1.00	0.29	0.2
T6	0.03	0.03	1.00	0.29	0.8

Growth measurement

Cell density was calculated daily based on the formula Megarani [19], and cells were counted used a hemocytometer under a light microscope and optical laboratory observations. The 5 mL of cells were taken using a micropipette, dropped on a hemocytometer and covered with cover glass. The cell density was then calculated using the following formula:

$$\text{Number of cell}\left(\frac{\text{cell}}{\text{mL}}\right) = \frac{\text{Number of cells counted in 5 count boxes}}{5} \times 25 \times 10^4 \quad (1)$$

In addition to used a hemocytometer, the calculation of cell density was measured using the Optical Density (OD) method to confirmed the cell density obtained used a spectrophotometer at a wavelength of 680 nm [20]. Samples were taken at 2 mL and the absorbance was observed using a UV-Vis spectrophotometer (Genesys 150, Thermo Scientific). Media from each treatment was used as blanks. Quartz cuvette from Mettler Toledo was used with cuvette path length of 10 mm. The results were crosschecked and standardized with biomass and cell count results as defined in **Figures 2** and **3** along with **Tables 3** and **4**. The specific growth rate was calculated by using the following formula [11].

$$\mu = \frac{\text{Ln(Nt)} - \text{Ln (N0)}}{t_1 - t_0} \quad (2)$$

$$\text{Dt} = \frac{\text{Ln 2}}{\mu} \quad (3)$$

where, µ = Specific growth rate (day^{-1}); Dt = generation time (h); 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).

Specific growth rate was calculated from the exponential phase of the culture (Day 4 of cultivation as the beginning and Day 9 as the end of calculation time). The results of optical density were calibrated with the result of biomass and cells density measurement. Ten biological replicates were used to made the calibration curve.

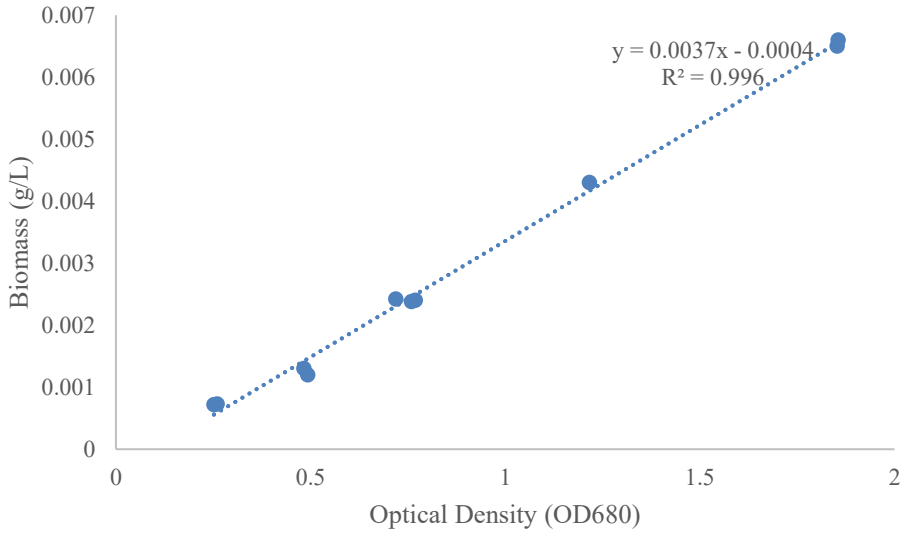


Figure 1 Calibration curve of biomass and optical density.

Table 3 Regression statistical analysis of biomass and optical density.

Regression Statistics								
Multiple R					0.997994			
R Square					0.995992			
Adjusted R Square					0.995491			
Standard Error					0.039715			
Observations					10			
	df	SS	MS	F	Significance F			
Regression	1	3.135758	3.135758	1988.066	7.07E-11			
Residual	8	0.012618	0.001577					
Total	9	3.148376						
	Coefficients	Standard Error	t Stat	*p*-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	0.105573	0.021172	4.986427	0.001071	0.05675	0.154396	0.05675	0.154396
X Variable 1	266.1985	5.970217	44.58774	7.07E-11	252.4311	279.9658	252.4311	279.9658
Observation		Predicted Y		Residuals		Standard Residuals		
1		1.862483		−0.00758		−0.20252		
2		1.835863		0.016637		0.444309		
3		1.250227		−0.03403		−0.90874		
4		0.739126		0.019774		0.528106		
5		0.749774		−0.03117		−0.83255		
6		0.425012		0.067388		1.799724		
7		0.451631		0.030569		0.816387		
8		0.297236		−0.04574		−1.22147		
9		0.299898		−0.0401		−1.07089		
10		0.74445		0.02425		0.647646		

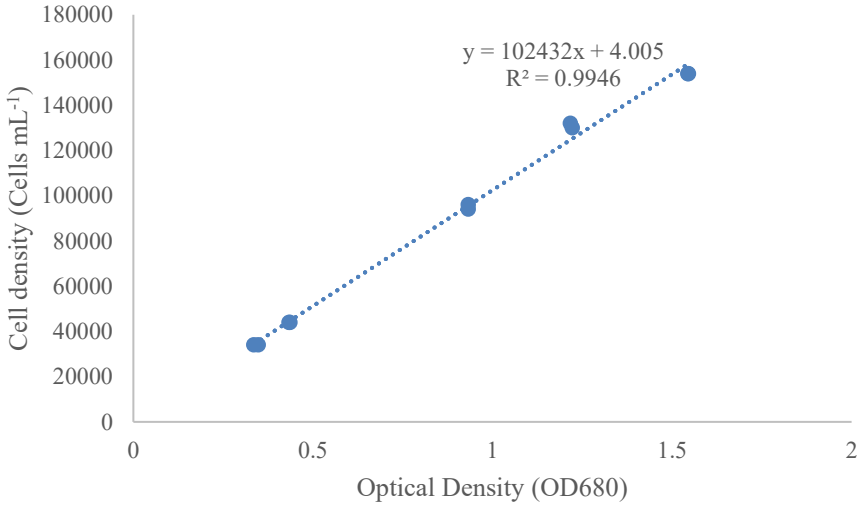


Figure 2 Calibration curve of cells density and optical density.

Table 4 Regression statistical analysis of cells density and optical density.

Regression Statistics							
Multiple R				0.997312			
R Square				0.994631			
Adjusted R Square				0.99396			
Standard Error				0.037485			
Observations				10			
Observation		Predicted Y		Residuals		Standard Residuals	
1		0.334906		0.000394		0.011136	
2		0.334906		0.011994		0.339369	
3		0.432008		0.001192		0.033736	
4		0.432008		0.003792		0.107305	
5		0.936935		−0.00493		−0.13963	
6		0.917514		0.014586		0.412716	
7		1.286499		−0.0703		−1.98918	
8		1.267079		−0.04488		−1.26989	
9		1.500122		0.043078		1.218927	
10		1.500122		0.045078		1.275519	
	df	SS	MS	F	Significance F		
Regression	1	2.082453	2.082453	1482.077	2.28E-10		
Residual	8	0.011241	0.001405				
Total	9	2.093693					
	Coefficients	Standard Error	t Stat	*p*-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	0.004762	0.025967	0.183384	0.859059	−0.05512	0.064643	−0.05512	0.064643
X Variable 1	9.71E-06	2.52E-07	38.49776	2.28E-10	9.13E-06	1.03E-05	9.13E-06	1.03E-05

Growth kinetics modelling

The growth kinetics model of *Euglena* sp. IDN5 was performed using the Logistic, Gompertz, and Richards models [21-23] by using the following formula:

Logistic model

$$\frac{dx}{dt} = \mu_{max}\left(1 - \frac{x}{x_{max}}\right)x \quad (4)$$

$$x\,(t) = \frac{Xmax}{1+(\frac{Xmax-X0}{X0})e^{-\mu t}} \quad (5)$$

where, X(t) = cell density at time *t* (cells mL^{-1}), X_0 = initial cell density (cells mL^{-1}), X_max = maximum cell density or carrying capacity (cells mL^{-1}), μ_{max} = maximum specific growth rate (day^{-1}), and t = cultivation time (days). Application used in this analysis is Spyder Anaconda Python version 3.

Gompertz model

$$X(t) = Xmax\ Exp[-exp(\frac{\mu\, max\, e}{Xmax}(\lambda - t) + 1)] \quad (6)$$

$$R^2 = \left(1 - \frac{SSR}{SST}\right) \quad (7)$$

where, X(t) = cell density at time *t* (cells mL^{-1}), X_0 = initial cell density (cells mL^{-1}) , X_max = maximum cell density (cells mL^{-1}), r_m = maximum growth rate parameter, t_l = lag duration (days), t = cultivation time (days), e = Euler's number (≈ 2.71828).

Richards model

$$y(t) = A(1 + v\ exp[k(\tau - t])^{-1/v} \quad (8)$$

where, X(t) = cell density at time *t* (cells mL^{-1}), A = asymptotic maximum cell density (carrying capacity) (cells mL^{-1}), k = growth rate constant (day^{-1}), τ = inflection time, ν = shape parameter controlling curve asymmetry, and t = cultivation time (days) [24,25].

Biomass measurement

Initial weight of Whatman GF/C glass microfiber filters was measured using AND GR-200 Analytical Balance after drying the filter in biomass for 2 h in 100 °C temperature. The biomass was measured by taking 10 mL of *Euglena* sp. culture and centrifuged for 10 min at 3,000 rpm. Supernatant was discarded. Then, the pellet was added with 20 mL of 0.5 M ammonium formate. The mixture was then mixed with vortex. This process was repeated for 4 times [26]. The mixture was then filtered through vacuum filtration using Whatman GF/C glass microfiber filters. The filter paper was then dried using an oven at 80 °C overnight. Filter paper was cooled in vacuum desiccator for 2 h. The filter paper will be dried again in the oven and cooled in desiccator if the measurement shows differences. This process repeated until the constant amount of biomass was found. Biomass measurement was carried out every day for 12 days. Blank (correction factor) was made by filtering media from each treatments using the same method as biomass measurements to make sure the measured factors in the filter is only the microalgae biomass. The blanks was made every biomass measurement. Biomass was measured by using the following formula [11]:

$$\text{Biomass titer } (g\ L^{-1}) = (\frac{B_x - B_0 - B_l}{V}) \quad (9)$$

where, B_x = Final weight of filter paper after biomass filtration (g); B_0 = Initial weight of filter paper before filtration (g); B_l = Blank weight from the respective treatment (g); V = Culture volume used in biomass measurement (0.01 L)

$$Biomass\ productivity\ (\text{g L}^{-1}\ \text{day}^{-1}) = \frac{M_x - M_0}{t_x - t_0} \quad (10)$$

where, M_x: Biomass amount in the last day of exponential phase (g L^{-1}); M_0: Biomass amount in the first day of exponential phase (g L^{-1}); t_x: Last day of exponential phase (day); t_0: first day of exponential phase (day)

Analysis of photosynthetic pigments

Chlorophyll and carotenoid contents were measured using the method by Dere *et al.* [27] by taking 5 mL of the *Euglena* sp. sample. The measurement was done once every 2 days for 12 days of cultivation. Then, it was centrifuged at 3,300 rpm for 15 min and the supernatant was discarded. Absolute methanol was added as solvent, then vortex for 10 - 15 s. Samples were put to a cuvette as much as 2 mL, and the absorbance

was measured at wavelengths of 470, 653 and 666 nm. Methanol was put into the cuvette as a blank solution. The absorbance results were recorded and entered into the calculation as follows:

$$\text{Chlorophyll-a (g/L)} = 15.65\ \text{Abs}\ 666 - 7.340\ \text{Abs}\ 653 \quad (11)$$

$$\text{Chlorophyll-b (g/L)} = 27.05\ \text{Abs}\ 653 - 11.21\ \text{Abs}\ 666 \quad (12)$$

$$\text{Carotenoid (g/L)} = \frac{(1000\ \text{Abs}\ 470 - 2.860\ \text{chlorophyll-a} - 129.2\ \text{chlorophyll-b})}{245} \quad (13)$$

Extraction and measurement of crude paramylon-enriched carbohydrate fraction

Crude paramylon-enriched carbohydrate fraction was measured once every 2 days. 10 mL *Euglena* sp. culture was centrifuged for 15 min at 4,000 rpm. The pellets were dissolved with 1% (w/v) SDS and 5% (w/v) Na_2EDTA and then incubated at 37 °C for 30 min in a water bath. The supernatant was discarded and then added with 1% (w/v) SDS and 5% (w/v) Na_2EDTA solution without incubation. The pellet was washed twice using distilled water. The resulting crude paramylon-enriched carbohydrate fraction pellets were dissolved in 2 mL NaOH 0.5N [28]. The measurement of crude paramylon-enriched carbohydrate fraction concentration was carried out used the phenol-sulfuric acid method [29]. Crude paramylon-enriched carbohydrate fraction pellet was mixed with 5% phenol and H_2SO_4 1N with a ratio of 1:1 (v/v) in a test tube. The test tube was incubated for 10 min. The solution was vortexed for 30 s and then incubated again for another 20 min at room temperature. The concentration was measured through measuring the solution in a spectrophotometer with a wavelength of 490 nm. Standard curves were made used paramylon standard from Sigma MCLS (89862-1G-F). Crude paramylon-enriched carbohydrate fraction was calculated with y = 0.906x + 0.3749 formula through standard calculation (R^2 = 0.9975). Crude paramylon-enriched carbohydrate fraction productivity was measured using formula as stated in formula 13:

$$Crude\ Paramylon\ titer\ (\text{g L}^{-1}) = \frac{(y - 0.3749)}{0.906} \quad (14)$$

where, y = results of OD490 nm

$$Crude\ Paramylon\ productivity\ (\text{g L}^{-1}\ \text{day}^{-1}) = \frac{P_x - P_0}{t_x - t_0} \quad (15)$$

where, P_x: Crude paramylon-enriched carbohydrate fraction amount in the last day of exponential phase (g L^{-1}); P_0: Crude paramylon-enriched carbohydrate fraction amount in the first day of exponential phase (g L^{-1}); t_x: Last day of exponential phase (day); t_0: first day of exponential phase (day)

Quantitative real-time PCR analysis

RNA isolation is a major step in gene expression QPCR was used to determine the relative gene expression of *EgGSL* in each lag phase (day 0), late exponential (dx/dt > 0; day 6), stationary phase (dx/dt ≈ 0; day 8), and decline phase (dx/dt < 0; day 12). This RNA kit isolation protocol that effectively removed this contaminant contains beta-mercaptoethanol by used the FavorPrep Plant, Total RNA Mini Kit follows some protocols. Macerated centrifuge in liquid nitrogen is an efficient method for *Euglena* sp. RNA extraction. The RNA isolated by this protocol was of sufficient quality for molecular applications and this technique can be applied to other organisms with similar substances that inhibit RNA extraction. The room and equipment were ensured free of RNAse and then 15 mL of *Euglena* sp. from each treatment was taken and crushed using a physical method with a cold centrifuge at 4 °C. After being separated into 2 phases, the supernatant was discarded, and the pellet was taken and then added with 500 µL of FARB buffer, which had been added with beta-mercaptoethanol. The samples were homogenized by vortex and allowed to stand at room temperature for 5 min.

The RNA samples obtained were then measured for concentration and purity used the nanodrop spectrophotometer in OD260 and OD280. The isolated RNA sample was synthesized into cDNA. Genomic RNA contaminants were digested using 5 µL of DNase I per 25 µg RNA. Started with 2 µg of the RNA sample and negative controls used RNase-free water instead of the RT/RNase mixture were also prepared. Three sets of primers were designed and used for the reactions *EgGSL1, EgGSL2* and one for the 18S rRNA as the housekeeping gene. The forward and reverse primers for *EgGSL1* F: 5'-AGCGCATCCCGGTCACAATG-3' and

R: 5'-TTGTCCCACGTGTACGCCAC-3', *EgGSL2* F: 5'-CAT AGG GCT ACT TGC CAC-3' and R 5'-TGT TTG TGC ATT TGA CAG AT -3' and for the 18S rRNA they were 5'-CTT AGA TCG CTG CCA GAT CC-3' and 5'-GGC TGT GGA TTT CTC GTT GT-3', respectively.

The 2× Fast Q-PCR Master Mix (SYBR, no ROX) was used for qPCR as per the protocol, with a total reaction volume of 20 µL. Three technical replicates and 2 biological replicates from each time point were analysed. Before synthesizing cDNA, the RNA concentration was first equalized to reduce bias between treatments when calculating gene expression levels. The calculation uses the formula:

$$V_1 \times M_1 = V_2 \times M_2 \quad (17)$$

where, V_1: Starting volume (µL); V_2: Final volume (µL); M_1: Starting molarity (M); M_2: Final molarity (M);

$$V_{RNA} = \frac{n}{M_2} \quad (18)$$

where, n: mol number (mol); V_{RNA}: Volume RNA

If final volume decided to be 10 µL, volume of nuclease-free water (NFW) can be calculated by:

$$NFW\ Volume = 10 - V_{RNA} \quad (19)$$

cDNA was synthesized according to the ExcelRT™ series Reverse Transcription Kit II (Smobio) protocol. Mixture A was made with a composition of 1 µL Oligo (dT) that is added with 8 µL DEPC-Treated H_2O along with 1 µL of total RNA. Mixture B (buffer First-strand cDNA) was made with the composition of 4µL 5× RT Buffer (DTT/dNTPs), 5 µL DEPC-Treated H_2O and 1 µL RTase/RI Enzyme Mix. Mixture A and B then mixed. The mixture was incubated at 37 °C for 50 min and heated at 85 °C for 30 min used a PCR machine with one cycle.

The qPCR used the ExceltaqTM 2× Fast Q-PCR Master Mix (SYBR, no ROX). The initial denaturation was carried out at 95 °C for 2 min, followed with 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing/extension). The threshold cycle (CT) for each reaction was retrieved from the machine, and the data were analysed used the $2^{-\Delta\Delta CT}$ method [30]. The placement of the sample followed the guidance in **Figure 3**.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk GSL2 Kontrol	Unk GSL2 Kontrol	Unk GSL2 Kontrol	Unk GSL2 Kontrol					Unk 18S Kontrol	Unk 18S Kontrol	Unk 18S Kontrol	Unk 18S Kontrol
B	Unk GSL2 0.01:0.01	Unk GSL2 0.01:0.01	Unk GSL2 0.01:0.01	Unk GSL2 0.01:0.01					Unk 18S 0.01:0.01	Unk 18S 0.01:0.01	Unk 18S 0.01:0.01	Unk 18S 0.01:0.01
C	Unk GSL2 0.01:0.2	Unk GSL2 0.01:0.2	Unk GSL2 0.01:0.2	Unk GSL2 0.01:0.2					Unk 18S 0.01:0.2	Unk 18S 0.01:0.2	Unk 18S 0.01:0.2	Unk 18S 0.01:0.2
D	Unk GSL2 0.01:0.8	Unk GSL2 0.01:0.8	Unk GSL2 0.01:0.8	Unk GSL2 0.01:0.8					Unk 18S 0.01:0.8	Unk 18S 0.01:0.8	Unk 18S 0.01:0.8	Unk 18S 0.01:0.8
E	Unk GSL2 0.03:0.01	Unk GSL2 0.03:0.01	Unk GSL2 0.03:0.01	Unk GSL2 0.03:0.01					Unk 18S 0.03:0.01	Unk 18S 0.03:0.01	Unk 18S 0.03:0.01	Unk 18S 0.03:0.01
F	Unk GSL2 0.03:0.2	Unk GSL2 0.03:0.2	Unk GSL2 0.03:0.2	Unk GSL2 0.03:0.2					Unk 18S 0.03:0.2	Unk 18S 0.03:0.2	Unk 18S 0.03:0.2	Unk 18S 0.03:0.2
G	Unk GSL2 0.03:0.8	Unk GSL2 0.03:0.8	Unk GSL2 0.03:0.8	Unk GSL2 0.03:0.8					Unk 18S 0.03:0.8	Unk 18S 0.03:0.8	Unk 18S 0.03:0.8	Unk 18S 0.03:0.8
H	NTC GSL2	NTC GSL2									NTC 18S	NTC 18S

Figure 3 Sample arrangements for plate in qPCR analysis.

Statistical analysis

The data were analyzed using IBM SPSS. Data originating from the same day (biomass, crude paramylon-enriched carbohydrate fraction productivity, and daily result of pigmentations) were analyzed for significance using one-way ANOVA, after ensuring that the normality and homogeneity of the data were met. Kolmogorov-Smirnov and Shapiro-Wilk was used to check the normality of the data. Meanwhile, data related to the time series (chlorophyll-a, chlorophyll-b, total chlorophyll, and carotenoids) were analyzed for significance using Repeated Measures ANOVA, considering data homogeneity, normality, and Sphericity. Repeated-measures ANOVA was used to examine the effect of cultivation day, the interaction between day and treatment, and treatment in each test.

Results and discussion

Growth curve profile of *Euglena* sp.

The growth of *Euglena* sp. under mixotrophic culture conditions during 12-day cultivation is shown in **Figure 4**. Started on day 6, the growth pattern differed, especially in the control group which exhibited slower growth rate. On the 10th day, the cell density in all treatments began to decline due to the nutrient depletion. This result also consistent with the growth dynamics of *E. gracilis* over time as presented in **Figure 4**. A where the late exponential phase determined by $dx/dt > 0$, stationary phase with $dx/dt \approx 0$, and the final decline phase with $dx/dt < 0$.

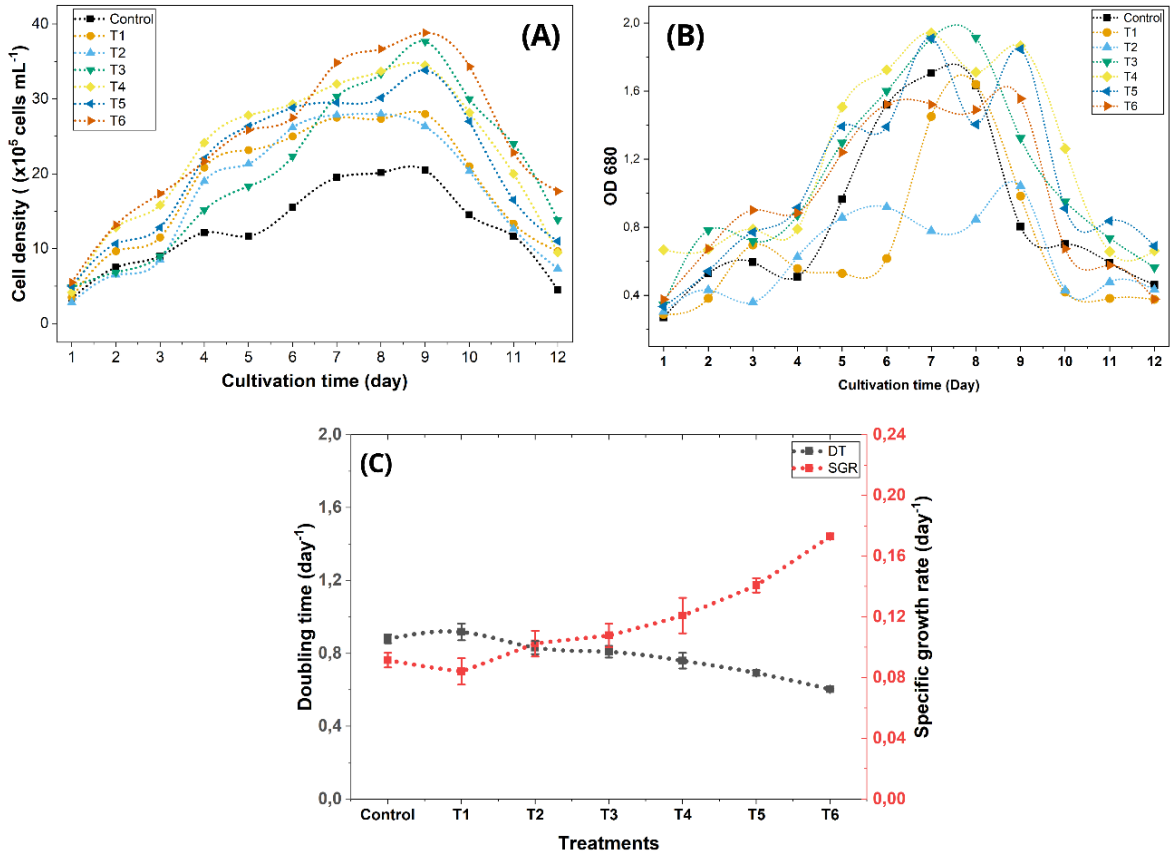


Figure 4 Growth of *Euglena* sp. IDN5 under different treatments. (A) cell density, (B,C) optical density at 680 nm, and (D) doubling time and specific growth rate. Where T1: 0.01:0.01:0.01, T2: 0.01:0.01:0.2, T3: 0.01:0.01:0.8, T4. 0.03:0.03:0.01, T5. 0.03:0.03:0.2 and T6. 0.03:0.03:0.8. The bar represents standard deviation from 3 replications (1-way ANOVA; Doubling time $p = 0.000$, df = 6, F = 35.866).

The treatment of T6 produced the highest cell density compared to other treatments. This suggests that PCA, along with addition of glucose and KNO_3 effectively estimated to alter cell density. This is in line

with Zhu and Wakisaka [9], where ferulic acid extracted from rice grains also increased 1.4 times the growth of *Euglena* sp. under phototropic conditions. A previous study also reported that its growth was dependent on PCA concentration. The addition of 800 mg/L PCA resulted in a cell density of more than 120×10^4 cells mL^{-1}.

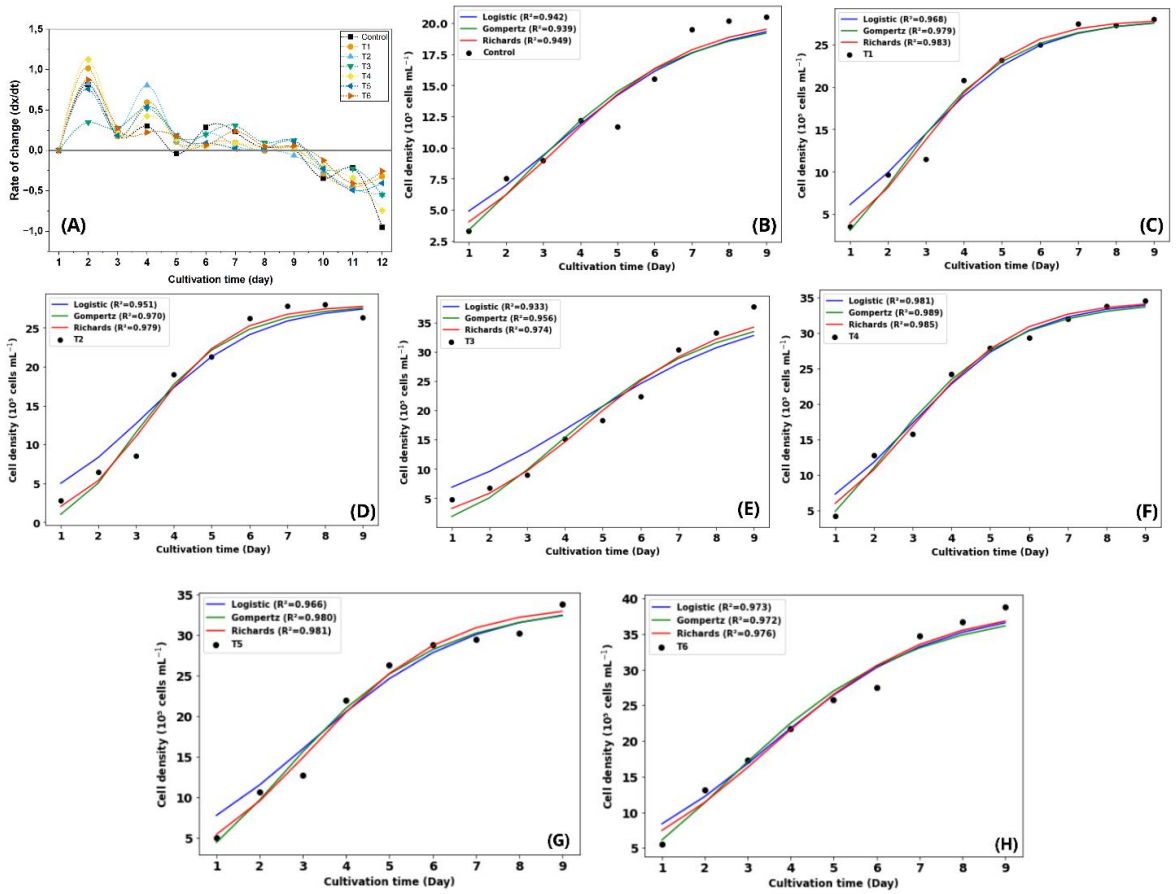


Figure 5 Growth kinetics of *Euglena* sp. IDN5 under various treatments. (A) Growth dynamics over time, (B) Logistics, Gompertz, and Richards modelling of control, (C) T1, (D) T2, (E) T3, (F) T4, (G) T5, and (H) T6; T1: 0.01:0.01:0.01, T2: 0.01:0.01:0.2, T3: 0.01:0.01:0.8, T4. 0.03:0.03:0.01, T5. 0.03:0.03:0.2 and T6. 0.03:0.03:0.8.

Additionally, the addition of glucose and KNO_3 also influenced the growth of *Euglena*. This unique organism is capable of mixotrophic growth [34], allowing the utilization of different nutrient uptake modes [35]. A previous study reported that under a mixotrophic state, *Euglena* absorbs additional carbon (glucose) in addition to photosynthesis. This state successfully increased biomass accumulation up to 38 g L^{-1} [36]. Glucose also might alter *Euglena* metabolism through the pentose phosphate pathway as well as upregulated some related metabolites [37]. Moreover, nitrate supplementation to the media also supports its mixotrophic state, as nitrate improves its carbohydrate and lipid content [38]. However, to determine the exact effect of glucose, KNO_3, and PCA to *Euglena* sp. growth in the culture, expanded tested range to demonstrate that the reported condition need to done and detailed biochemical analysis from many other angles need to be tested.

Growth kinetics

Based on growth kinetics modelling (**Figure 5** and **Table 5**), in the logistic model, the highest maximum growth potential (μ_{max}) was found in the T1 and T2 treatments with μ_{max} values of 0.67 day^{-1}, respectively. The lowest maximum growth potential was found in the control (0.49 day^{-1}). Based on the Gompertz model, the T4 treatment had the highest maximum growth potential (6.85 day^{-1}), followed by the T2 (0.68 day^{-1}) and T1 (6.22 day^{-1}) treatments. Meanwhile, the lowest growth

potential was also found in the control, although the λ value in the control was lower (0.01) than in the other treatments. The λ value itself indicates how long the adaptation time required by the cells is to enter the log phase. In the control, the λ value is 0.01, meaning that the adaptation phase is much faster than the other treatments. However, due to its low maximum growth potential, the cell density was lower than that of the treatment group. The treatment with a longer adaptation period is T2, which is about 1.30 days. However, the high maximum specific growth rate (μ_{max}) resulted in a high cell density in this study. The treatment with the highest cell density of T6 (almost reaching 40×10^5 cells mL^{-1}) has an adaptation period that is also relatively short, only 0.05 day^{-1}, with a maximum growth potential of 5.85 day^{-1}. Meanwhile, based on the Richards model, the v values of all treatments and controls were < 1, indicating a growth curve that begins with a high growth rate until it reaches the maximum logarithmic phase, then ends with a slow decline in growth rate. The use of these 3 models was not intended to compare the model itself, but to strengthen the interpretation of growth pattern and dynamics by incorporating different parameters in within each formula.

Table 5 Growth kinetic modelling of *Euglena* sp. IDN5 under different treatments; T1: 0.01:0.01:0.01, T2: 0.01:0.01:0.2, T3: 0.01:0.01:0.8, T4. 0.03:0.03:0.01, T5. 0.03:0.03:0.2 and T6. 0.03:0.03:0.8.

Model	Treatments	Parameters				
		μ_{max} (d^{-1})	R^2	RMSE	AIC	BIC
Logistic model	Control	0.49	0.94	1.39	7.89	8.09
	T1	0.67	0.97	1.54	9.75	9.94
	T2	0.67	0.95	2.09	15.26	15.46
	T3	0.42	0.93	2.93	21.38	21.57
	T4	0.66	0.98	1.37	7.64	7.84
	T5	0.55	0.97	1.78	12.38	12.58
	T6	0.51	0.97	1.74	11.96	12.16
Gompertz model		μ_{max}	λ	R^2	RMSE	AIC	BIC
	Control	3.11	0.01	0.94	1.43	10.38	10.77
	T1	6.22	0.66	0.98	1.25	7.96	8.35
	T2	6.82	1.30	0.97	1.62	12.64	13.04
	T3	5.57	1.24	0.96	2.36	19.47	19.86
	T4	6.85	0.39	0.99	1.07	5.18	5.57
	T5	6.01	0.39	0.98	1.34	9.31	9.70
	T6	5.82	0.05	0.97	1.78	14.38	14.73
Richard Model		μ_{max}	v	R^2	RMSE	AIC	BIC
	Control	0.62	0.86	0.95	1.31	8.79	9.19
	T1	1.05	0.74	0.98	1.11	5.88	6.27
	T2	1.34	0.61	0.98	1.35	9.37	9.77
	T3	0.77	0.66	0.97	1.81	14.67	15.06
	T4	0.82	0.87	0.99	1.21	7.36	7.75
	T5	0.81	0.77	0.98	1.33	9.13	9.52
	T6	0.58	0.92	0.98	1.66	13.15	13.55

Note: RMSE: Root Mean Square Error; AIC: Akaike Information Criterion; BIC: Bayesian Information Criterion.

Light intensity and photoperiod are among the most important environmental factors influenced growth rate and biomass composition in microalgae [39]. When the PCA concentration was increased to 0.8 g L^{-1}, the cell-specific growth rate and doubling time were 0.17 and 0.60 day^{-1}, much higher than the control group 0.09 and 0.88 day^{-1}, respectively. Tan *et al.* [11] stated that the highest specific growth rate and doubling

time were obtained in the 0.8 treatment 0.23 and 3.07, respectively. Meanwhile, Wang [40] found that the higher yield was 1.02 day^{-1} in the mixotrophic treatment at 27 ℃. The treatment is due to several factors, including macronutrients, pH, temperature, aeration rate, and light intensity.

Also, doubling time (**Figure 4(C)**) is closely correlated with nutrient concentration and cell growth rate [41]. The results show that the T1 treatment had the highest doubling time value compared to others, while the lowest doubling time was T6 treatment. Doubling time refers to the time needed for a certain population to double in numbers. The higher the numbers, the slower the growth of those populations [42]. İn this study, the provision of PCA reduced the doubling time. For example, in the treatment of T6, its doubling time was the lowest among all, therefore exhibiting a higher growth rate. This phenomenon is not only associated to PCA provision, but also the addition of glucose:KNO_3 (0.03:0.03:0.03 g L^{-1}), as described by [43] that fulfils the required macronutrients aimed to alter microalgal growth.

Biomass productivity

Under 16/8 h of irradiation, the highest biomass productivity was achieved in the treatment T3, which producing 1.21 g L^{-1} day^{-1} of biomass, followed by T6 treatment which produced more than 0.8 g L^{-1} day^{-1}. Meanwhile, the lowest biomass productivity was in the control (~0.5 g L^{-1} day^{-1}).

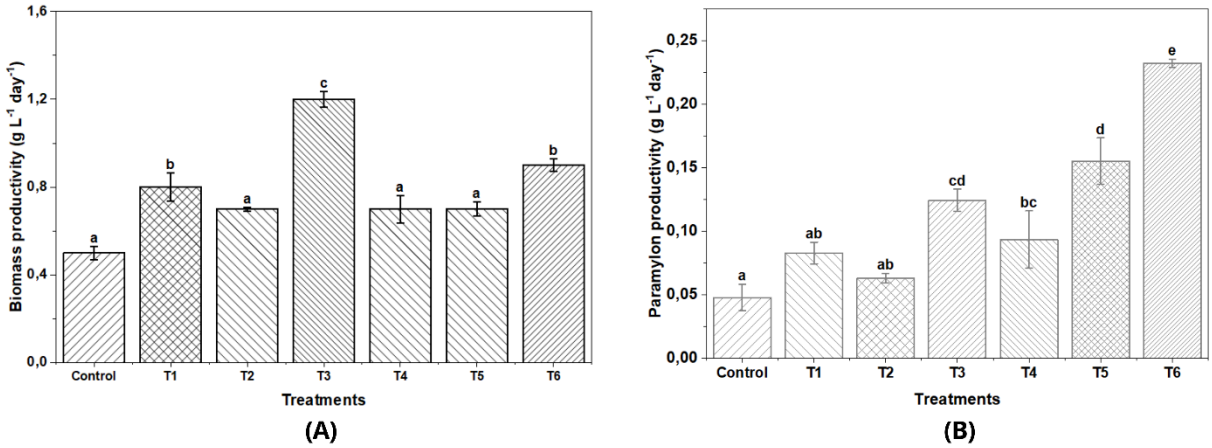


Figure 6 Productivity of biomass and crude paramylon-enriched carbohydrate fraction from *Euglena* sp. IDN5 under different treatments. (A) biomass productivity, (B) crude paramylon-enriched carbohydrate fraction productivity. The bar represents standard deviation from 3 replications; different letters above the bar represent significant differences between each treatment (1-way ANOVA; paramylon productivity $p = 0.000$, df = 6, F = 111.293; Biomass productivity $p = 0.000$, df = 6, F = 60.460). T1: 0.01:0.01:0.01, T2: 0.01:0.01:0.2, T3: 0.01:0.01:0.8, T4. 0.03:0.03:0.01, T5. 0.03:0.03:0.2 and T6. 0.03:0.03:0.8.

Biomass productivity in **Figure 6(A)** showed biomass changes over the 12-day cultivation until the stationary phase; with additional PCA, significant results were obtained. The treatment with the addition of PCA of 0.8 g L^{-1}produced higher yield in the treatment of T3 and T6, 1.21 and 0.90 g L^{-1} day^{-1}, respectively. According to LSD (Least Significant Difference), PCA at 0.8 g/L significantly affected biomass productivity. This result is lower compared to the previous research conducted by Tan *et al.* [11], where the maximum biomass was peaked at 1.32 g L^{-1}. However it is higher compared to another study that reported the highest biomass productivity at 115 ± 1.4 mg L^{-1} day^{-1}in the culture with the indicated nitrogen concentration (400 mg $L^{-1}(NO_3^-)^{-1}$), temperature (36 °C), pH (9.8), and light intensity (23,500 lux) [44]. Also higher compared to the study by Farjallah and Guéguen [37], where the mixed microalgae culture from Glagah beach with seawater medium produced highest biomass productivity for only about 3.42 g L^{-1} day^{-1}.

This biomass productivity is associated with the ratio of glucose and KNO_3 along with PCA concentration in the media [45-47]. Therefore, increasing external glucose concentration in the media

most likely affect its biomass productivity [17]. Previous research found that under glucose concentration up to 20 g L^{-1}, the biomass concentration increased gradually, and the maximum biomass concentration peaked at 6.93 g L^{-1}[17].

Crude paramylon-enriched carbohydrate fraction productivity

Under mixotrophic conditions, organic carbon (the type and concentration of carbon sources) will affected the paramylon accumulation in *Euglena* sp. [10]. The supplementation of glucose as carbon and YE and peptone as nitrogen sources in the culture of *Euglena* sp. could improve its biomass titer to 6.4 g L^{-1} with paramylon percentage reached more than 50% [3].

In this study (**Figure 6 (B)**), the addition of PCA 0.8 g L^{-1} had a significant effect on the average daily paramylon, in particular on day 9 (the end of the logarithmic phase). The highest crude paramylon-enriched carbohydrate fraction content was found in the treatment of T6 (1.21 ± 0.04 g L^{-1}), followed by T3 (0.90 ± 0.04 g L^{-1}). The result also consistent with crude paramylon-enriched carbohydrate fraction percentage. The control showed the lowest crude paramylon-enriched carbohydrate fraction percentage (45.77%), while T6 produced the highest crude paramylon-enriched carbohydrate fraction productivity, almost 85%. From the result, it is known that PCA had correlation in enhancing crude paramylon-enriched carbohydrate fraction production. Our finding produce higher crude paramylon-enriched carbohydrate fraction compared to the previous study, where paramylon percentage of *Euglena* sp. in sewage effluent containing waste wine was only 67.7%, with the amount of titer is about 44.1 mg L^{-1} [48]. Other study also reported that in order to achieve paramylon percentage more than 80%, it was needed 15 g L^{-1} glucose concentration in supplemented in the media [10]. Compared to that study, our findings appear to need substantially lower glucose concentration used. Moreover, the addition of PCA resulted in a higher paramylon percentage than that reported in studies employing higher glucose concentrations.

The administration of protocatechuic acid might correlate with the production of paramylon. In a previous study, the addition of 800 mg L^{-1} PCA resulted in 1.7-fold higher paramylon production [10]. In another study, a phenolic compound in the form of guaiacol 10 mg L^{-1} (phenolic acid) was also reported to increase paramylon production by 16.67% [58]. In this research, ratio of glucose and KNO_3 in this study did not significantly affect crude paramylon-enriched carbohydrate fraction accumulation in *Euglena* sp.

Pigment productivity

Microalgae pigments are eye-catching natural colours that have high-value components with health-promoting qualities including antioxidants, vitamin precursors, neuroprotective, and immunological boosters. Different environmental parameters, such as temperature, radiation, coloured wavelength, photoperiod, pH, nutrient limitation, nitrogen supplementation, salinity, pesticides, and heavy metal stress, greatly affect the productivity of microalgae pigments [60].

According to Repeated Measures ANNOVA of chlorophyll-a, the pigmentation data were normally distributed, and the assumption of sphericity was met as indicated by Mauchly's Test of Sphericity ($p = 0.911$). A repeated measures ANOVA revealed a significant effect of days of incubation ($p < 0.001$), along with a significant interaction between days of cultivation and treatments ($p < 0.001$). Furthermore, the between-subjects analysis demonstrated a significant effect of treatment ($p < 0.001$). According to Post hoc comparisons, there were significant differences among treatments, with higher treatment levels (T4 - T6) showing significantly greater responses compared to the control group, while T2 exhibited intermediate values. Furthermore, the other pigmentations (chlorophyll-b, total chlorophyll, and carotenoids) were also normally distributed, and the assumption of sphericity was met in chlorophyll-b as indicated by Mauchly's Test of Sphericity ($p > 0.05$), while in the total chloropyll and carotenoids Mauchly's Test of Sphericity was violated as $p < 0.05$. Therefore, the Greenhouse-Geisser correction was applied for both pigmentations ($p > 0.05$). However, there is still a significant effect of days of cultivation on the measured variable ($p < 0.05$), along with a significant interaction between days and treatment ($p < 0.005$), and demonstrated a significant effect of treatment ($p < 0.005$). Post hoc comparisons using Bonferroni correction revealed significant differences among treatments, which control exhibited

the lowest value that grouped in the separated group (a) in all the pigmentation analysis. Meanwhile, the other treatments exhibited higher values (T1 - T5) with T6 consistently demonstrated the highest response (group e).

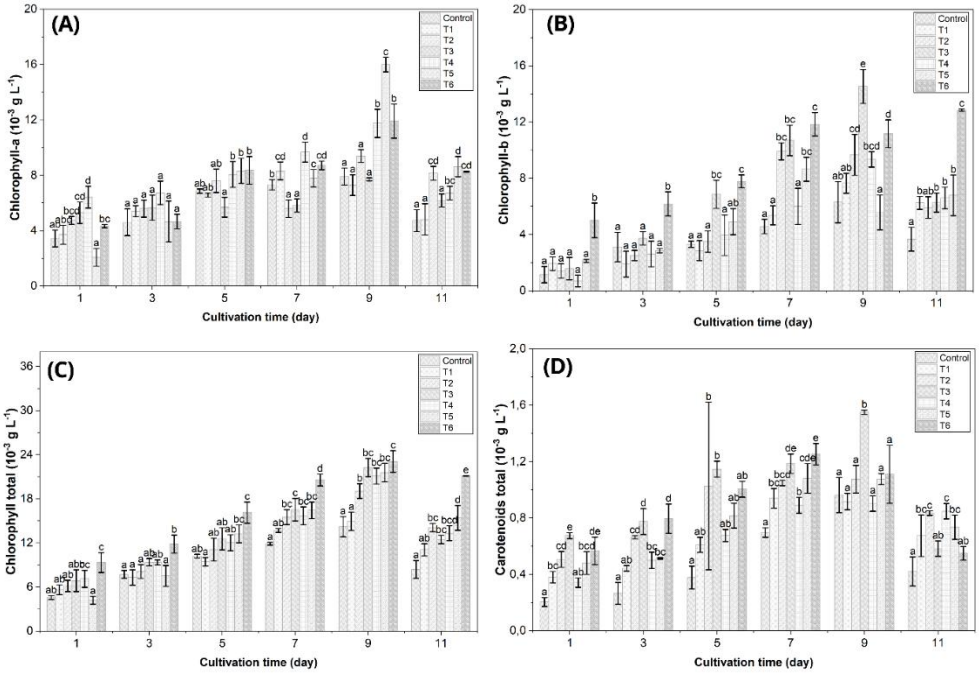


Figure 7 Pigment productivity of *Euglena* sp. IDN5 under different treatments. (A) chlorophyll-a, (B) chlorophyll-b, (C) chlorophyll total, and (D) carotenoids total. Where T1: 0.01:0.01:0.01, T2: 0.01:0.01:0.2, T3: 0.01:0.01:0.8, T4. 0.03:0.03:0.01, T5. 0.03:0.03:0.2 and T6. 0.03:0.03:0.8. The bar represents standard deviation from 3 replications; different letters above the bar represent significant differences between each cultivation day (1-way ANOVA; Chlorophyll-a $p = 0.000$, df = 6, F = 88.128; Chlorophyll-b $p = 0.000$, df = 6, F = 212.702; Total Chlorophyll $p = 0.008$, df = 6, F = 7.060; Carotenoid $p = 0.000$, df = 6, F = 22.952).

Based on the **Figure 7**, the highest content of chlorophyll-a was in the T6 treatment on day 9th, which was 11.92×10^{-3} g L^{-1}, and the highest chlorophyll-b found in the T3 treatment, which was 14.54×10^{-3} g L^{-1}. According to LSD (Least Significant Difference) at $p < 0.05$, the combination of PCA and glucose along with KNO_3 were significantly affected pigment productivity.

Chlorophyll is an important component of the light-harvesting pigment-protein complex, and photosynthetic capacity was enhanced when chlorophyll content was correlated with the increased microalgae growth [60]. Higher Chlorophyll a/b ratio indicated higher photosynthetic activity and faster cell growth. In this study, higher Chlorophyll a/b than carotenoids indicated that photosynthetic efficiency was improved after treatment with different PCA concentrations. This result is consistent with the previous study conducted by Tan *et al.* [11], where PCA increased pigments content. Under the supplementation of 0.5 and 0.8 g L^{-1} PCA, chlorophyll-a and chlorophyll-b were peaked at 5.3×10^{-3} and 5.5×10^{-3} g L^{-1}, respectively. Meanwhile, the carotenoids content was ranged between 2.9 mg/L under the same PCA concentration [11] compared to the aforementioned study, our finding produce higher chlorophyll concentrations. However, further detailed analysis of HPLC, LC-MS, UV-Vis stability profiling, or another validated analytical method need to be done to actually address the effect of glucose, KNO_3 and PCA to the paramylon and pigments quantification of *Euglena* sp.

***Glucan synthase like* gene expression**

Cell intracellular components, such as RNA, DNA, and proteins, will undergo changes through

various environmental stimulations. The RNA concentration (RNA/cell weight) varied significantly during the batch growth cycle; however, the concentration of DNA and protein remains relatively constant [62].

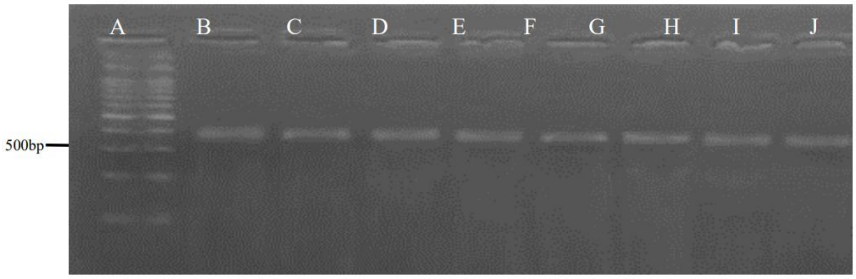
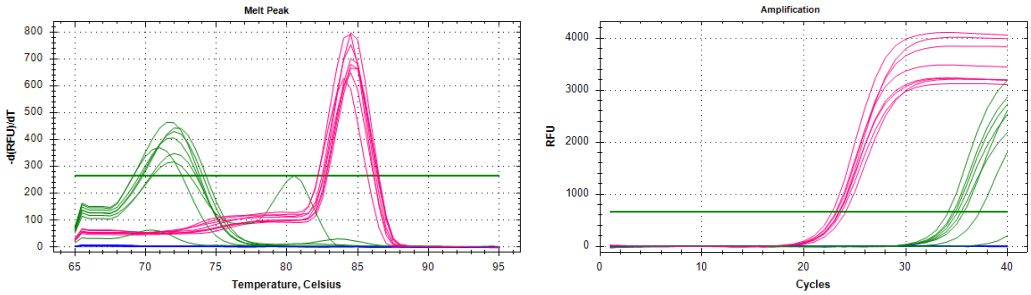


Figure 8 cDNA validation on temperature of (A = 55 ℃; B = 55.7 ℃; C = 57.0 ℃; D = 59.0 ℃; E = 61.4 ℃; F = 63.3 ℃; G = 64.5 ℃; H = 65.0 ℃).



(A) (B)

Figure 9 (A) Melt Peak and (B) Amplification of qPCR analysis, where green line represents *EgGSL2* and red line represent 18s.

Table 6 Cq quantification of qPCR analysis. T1: 0.01:0.01:0.01, T2: 0.01:0.01:0.2, T3: 0.01:0.01:0.8, T4. 0.03:0.03:0.01, T5. 0.03:0.03:0.2 and T6. 0.03:0.03:0.8.

Well	Treatments	Cq Mean	Cq Std. Dev.	End RFU
A01	Control	4.74	0.000	3203
A02	Control	17.82	0.000	1.83
A03	Control	26.96	0.000	1013
A04	Control	27.62	0.000	3120
B01	T1	9.75	0.000	1866
B02	T1	7.76	0.000	0.837
C01	T2	27.32	0.000	4002
C02	T2	10.65	0.000	77.3
D01	T3	28.86	0.000	3840
D02	T3	28.97	0.000	2052
E01	T4	38.93	0.000	4077
E02	T4	35.59	0.000	2179
F01	T5	14.70	0.000	3460
F02	T5	6.67	0.00	2530
G01	T6	24.63	0.000	3207
G02	T6	31.39	0.000	1720
H01	NTC	11.96	0.000	3211
H02	NTC	11.56	0.000	1598

As the culture in this study was conducted under 16:8 photoperiodism, the *EgGSL1* gene was not detected, because it is only expressed when cultured placed in the complete dark place without any light during cultivation period [62]. Gene regulation can occur at any time during gene expression but generally occurs at the transcriptional level where signals from the environment or from other cells activate proteins called transcription factors. This protein binds to the regulatory region of the gene and increases or decreases the level of transcription (Switch on-off). By controlling the level of transcription, this process can determine when and how much protein product a gene makes [64]. **Figure 8** shows the temperature used synthesize cDNA. Defining the size of cDNA to be around 500 bp. **Figure 9** define the quality of the qPCR. The melt peak and amplification show the good quality of qPCR. The Cq in **Table 6** indicates the number of cycles used in amplifying the targeted genes. The results show similar number in the same treatments. Indicating the results to be justified.

The results of the *EgGSL2* gene expression study showed changes in gene expression in each lag, log, stationary, and death phase. In the lag phase or on day 0, almost all treatments had gene expression close to the control as shown in **Figure 10**. This indicated that there have been no significant changes in *EgGSL2* gene expression in the lag phase. During the initial log phase, the treatment of T6 exhibited highest gene expression fold-changes, followed by T3 and T2. In the peak log phase, the expression of *EgGSL2* in those treatments was exceeded 15-fold changes. In the final log phase, PCA 0.8 g L^{-1} treatment had a significant effect on the increase in gene expression. The glucose:KNO_3:PCA treatment of 0.03:0.03:0.03 g L^{-1} showed a higher gene expression than other results. This phase also indicated that gene expression was still high because it was still in the log phase. On day 12 or the stationary phase, the expression of the *EgGSL2* gene began to decrease. This phase can be seen by the gene expression value closed to the control value.

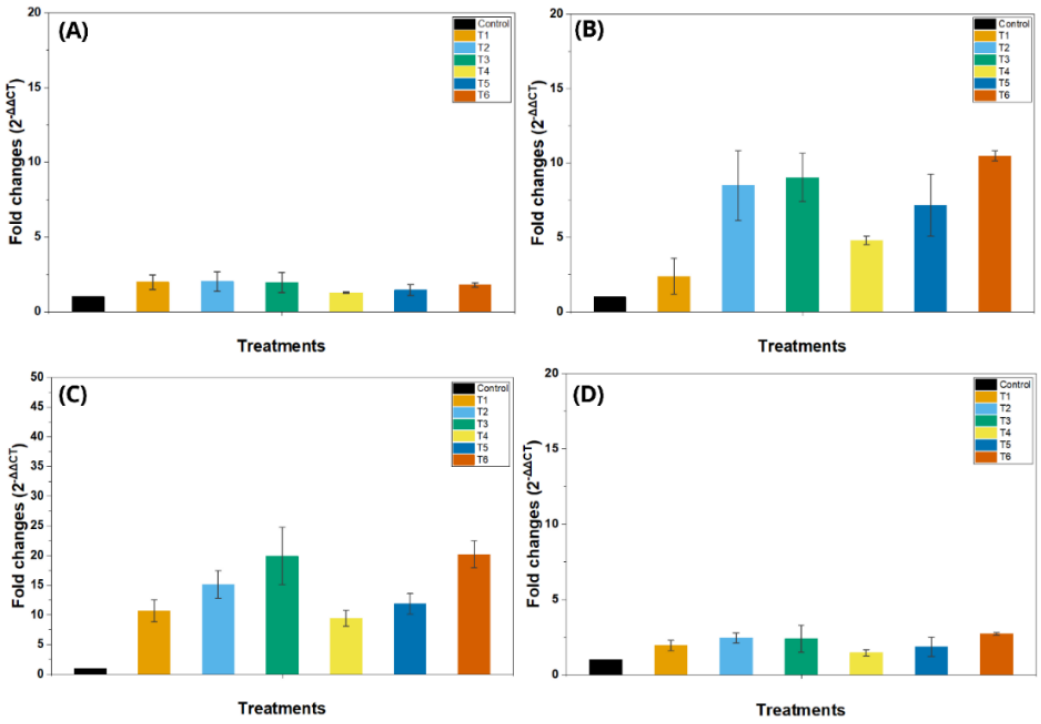


Figure 10 Relative Gene Expression of *EgGSL2* under different treaments. (A) lag phase (day 0), (B) late exponential (dx/dt > 0; day 6), (C) stationary phase (dx/dt ≈ 0; day 8), and (D) decline phase (dx/dt < 0; day 12). T1: 0.01:0.01:0.01, T2: 0.01:0.01:0.2, T3: 0.01:0.01:0.8, T4. 0.03:0.03:0.01, T5. 0.03:0.03:0.2 and T6. 0.03:0.03:0.8. The bar represents standard deviation from 3 replications (1-way ANOVA; lag phase p = 0.021, df = 6, F = 11.469, late exponential p = 0.000, df = 6, F = 7.521; stationary phase p = 0.000, df = 6, F = 23.411; decline phase p = 0.036, df = 6, F = 2.816.

availability, carbon consumption, dissolved oxygen, nitrogen consumption, pH, FTIR, GC-MS, and LC-HRMS need to be done to validate the claims.

Therefore, the addition of PCA changes crude paramylon-enriched carbohydrate fraction yield. Even though further mechanism needs to be confirmed through analysis of enzyme activity, substrate

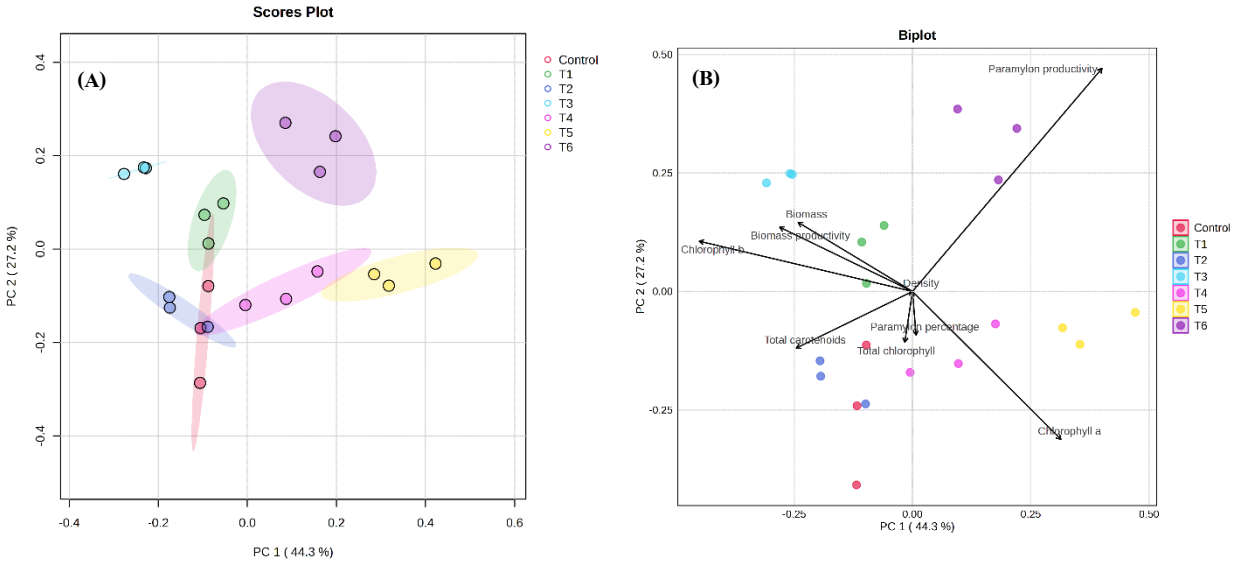


Figure 11 Principal Component Analysis of *Euglena* sp. IDN5 under different treatments. (A) Score plots, and (B) biplot. T1: 0.01:0.01:0.01, T2: 0.01:0.01:0.2, T3: 0.01:0.01:0.8, T4. 0.03:0.03:0.01, T5. 0.03:0.03:0.2 and T6. 0.03:0.03:0.8.

Principal Component Analysis (PCA) was conducted using primary data, including density, biomass, biomass productivity, crude paramylon-enriched carbohydrate fraction productivity, crude paramylon-enriched carbohydrate fraction percentage, chlorophyll-a, chlorophyll-b, and total carotenoids. The data were processed using mean scaling, log transformation (base 10), and sample normalization using sum normalization. Based on Principal Component Analysis, the PERMANOVA f-value was 35.9, R^2 0.94, and p-value 0.001; it is quite reliable and representative of the data. Meanwhile, according to **Figure 11**, the PC1 (44.3%) and PC2 (27.2%) values represent 71.5% of the data variation, so it is strong enough to present the result of this. Based on the score plot, there are 7 treatment groups marked with different color groups that are separated from each other. Each treatment cluster is well separated from the others with a considerable distance from the base. Based on the biplot, crude paramylon-enriched carbohydrate fraction productivity and chlorophyll-a were associated positively with PC1; biomass, biomass productivity, and chlorophyll-b were associated negatively with PC1; crude paramylon-enriched carbohydrate fraction productivity was associated positively with PC2; while total chlorophyll and crude paramylon-enriched carbohydrate fraction percentage were associated negatively with PC2. Based on these results, T6 is associated with higher crude paramylon-enriched carbohydrate fraction productivity than other treatments. Moreover, PCA suggests that paramylon production in *Euglena* in this study is associated with chlorophyll-a, as it is needed in paramylon synthesis. Further comparison of this research with previous research results can be seen in **Table 7**.

Table 7 Research benchmark of paramylon and biomass productivity in *Euglena* sp.

Source	Species	Method	Paramylon productivity	Biomass Productivity
This Research	*Euglena* sp. IDN5	Glucose:KNO^3:PCA 0.03:0.03:0.8 g L^{-1},	0.23±0.003 g L^{-1} day^{-1}	1.21 g L^{-1} day^{-1}
[11]	*Euglena gracilis*	800 mgL^{-1} of PCA	0.055 g L^{-1} day^{-1}	1.3 g L^{-1} day^{-1}
[64]	*Euglena gracilis*	White light	0.000261 g L^{-1} day^{-1}	0.813 ± 0.261 g L^{-1} day^{-1}
[65]	*Euglena gracilis*	Nitrogen concentration of 500 mg L^{-1}	0.151 g L^{-1} day^{-1}	3.4 g L^{-1} day^{-1}
[66]	*Euglena gracilis*	5% CO_2	-	2.3 g L^{-1} day^{-1}
[67]	*Euglena* sp. IDN 33	photoperiod 12:12; Aerobic cultivation	0.19 g L^{-1} day^{-1}	0.764 g L^{-1} day^{-1}
[68]	*Euglena* sp.	Photoautotrophic without CO_2	0.229 g L^{-1} day^{-1}	0.361 g L^{-1} day^{-1}

Conclusions

This study demonstrated the conditions for *Euglena* sp. IDN5 strain local of Indonesia crude paramylon-enriched carbohydrate fraction enhancement. It is found that the optimal condition in the present study is T6 (0.03:0.03:0.8 g L^{-1} (Glucose:KNO_3: PCA)) with photoperiodism 16:8 (light:dark). This treatment shows the highest results of several parameters such as specific growth rate, biomass, crude paramylon-enriched carbohydrate fraction, chlorophyll-a, chlorophyll-b, and carotenoids compared to other treatments. This treatment also exhibited the highest *EgGSL2* gene expression compared to other treatments. Therefore, this approach is recommended to be further explored to understand the underlying mechanism and produce paramylon from *Euglena gracilis* species.

Acknowledgements

This manuscript was a part of the first author's thesis, supported by Lembaga Pengelola Dana Pendidikan (LPDP), Indonesia.

Declaration of Generative AI in Scientific Writing

The authors acknowledge the use of generative AI tools, app.grammarly.com, in the preparation of this manuscript, specifically for language editing and grammar correction. No content generation or data interpretation was performed by AI. The authors take full responsibility for the content and conclusions of this work.

CRediT Author Statement

Dita Aulia Yulyanita: Conceptualization; Methodology; Software; Formal analysis; Investigation; Resources; Writing - Original Draft; Writing - Review & Editing; Visualization; Writing - Review & Editing. **Woro Anindito Sri Tunjung:** Conceptualization; Methodology; Validation; Resources; Supervision. **Angga Puja Asiandu:** Software; Formal analysis; Writing - Original Draft; Visualization; Writing - Review & Editing. **Renata Adaranyssa Egistha Putri:** Writing - Original Draft; Visualization; Writing - Review & Editing. **Eko Agus Suyono:** Conceptualization; Methodology; Validation; Resources; Writing - Review & Editing; Supervision

References

[1] YJ Chen and GB Huang. Flagellum malfunctions trigger metaboly as an escape strategy in Euglena gracilis. *Journal of Eukaryotic Microbiology* 2021; **68(3)**, e12842.

[2] J He, C Liu, M Du, X Zhou, Z Hu, A Lei and J Wang. Metabolic responses of a model green microalga Euglena gracilis to different environmental stresses. *Frontiers in Bioengineering and Biotechnology* 2021; **9**, 662655.

[3] T Bhattad, A Koradiya and G Prakash. Prebiotic activity of paramylon isolated from heterotrophically grown Euglena gracilis. *Heliyon* 2021; **7(9)**, e07884.

[4] Y Nagayama, N Isoo, A Nakashima, K Suzuki, M Yamano, T Nariyama, M Yagame and K Matsui. Renoprotective effects of paramylon, a β-1,3-D-Glucan isolated from Euglena gracilis Z in a rodent model of chronic kidney disease. *PLoS One* 2020; **15(8)**, e0237086.

[5] R Levine, G Horst, R Tonda, B Lumpkins and G Mathis. Evaluation of the effects of feeding dried algae containing beta-1, 3-glucan on broilers challenged with Eimeria. *Poultry Science* 2018; **97(10)**, 3494-3500.

[6] S Aoe, C Yamanaka, K Koketsu, M Nishioka, N Onaka, N Nishida and M Takahashi. Effects of paramylon extracted from *Euglena gracilis* EOD-1 on parameters related to metabolic syndrome in diet-induced obese mice. *Nutrients* 2019; **11(7)**, 1674.

[7] S Kottuparambil, RL Thankamony and S Agusti. Euglena as a potential natural source of value-added metabolites. A review. *Algal Research* 2019; **37**, 154-159.

[8] S Nagappan, P Das, M AbdulQuadir, M Thaher, S Khan, C Mahata, H Al-Jabri, AK Vatland and G Kumar. Potential of microalgae as a sustainable feed ingredient for aquaculture. *Journal of Biotechnology* 2021; **341**, 1-20.

[9] J Zhu and M Wakisaka. Growth promotion of Euglena gracilis by ferulic acid from rice bran. *AMB Express* 2018; **8(1)**, 16.

[10] S Kim, R Wirasnita, D Lee, J Yu and T Lee. Enhancement of growth and paramylon production of *Euglena gracilis* by upcycling of spent tomato byproduct as an alternative medium. *Applied Sciences* 2021; **11(17)**, 8182.

[11] X Tan, J Zhu and M Wakisaka. Effect of protocatechuic acid on Euglena gracilis growth and accumulation of metabolites. *Sustainability* 2020; **12(21)**, 9158.

[12] WJ Lee and SH Lee. Protocatechuic acid protects hepatocytes against hydrogen peroxide-induced oxidative stress. *Current Research in Food Science* 2022; **5**, 222-227.

[13] BS Yu, YJ Sung, ME Hong and SJ Sim. Improvement of photoautotrophic algal biomass production after interrupted CO_2 supply by urea and KH_2PO_4 injection. *Energies* 2021; **14(3)**, 778.

[14] W Lathifah, RA Fikri, NA Hidayati, ID Anggraini, N Putri, B Prabowo and S Marno. Effect of commercial NPK fertilizer on growth and biomass of *Navicula* sp. and Nannochloropsis sp. *IOP Conference Series: Earth and Environmental Science* 2021; **762**, 012060.

[15] SJ Daines, JR Clark and TM Lenton. Multiple environmental controls on phytoplankton growth strategies determine adaptive responses of the N:P ratio. *Ecology Letters* 2014; **17(4)**, 414-425.

[16] ZV Finkel, MJ Follows, JD Liefer, CM Brown, I Benner and AJ Irwin. Phylogenetic diversity in the macromolecular composition of microalgae. *PLoS One* 2016; **11(5)**, e0155977.

[17] MK Chai, YH Tan and LS Wong. Macronutrient effect on biomass of Microalgae in biofuel production: A review. *Indian Journal of Science and Technology* 2020; **13(22)**, 2245-2263.

[18] YS Pradana, H Sudibyo, EA Suyono and A Budiman. Oil algae extraction of selected microalgae species grown in monoculture and mixed cultures for biodiesel production. *Energy Procedia* 2017; **105**, 277-282.

[19] S Rahmadi. Algae to energy - using and re-using a hemocytometer to count algae cells, Available at: https://www.scribd.com/document/780868586/e-Algae-to-Energy-Counting-Algae-Cells, accessed February 2026.

[20] C Camacho-Fernández, D Hervás, A Rivas-Sendra, MP Marín and JM Seguí-Simarro. Comparison of six different methods to calculate cell densities. *Plant Methods* 2018; **14(1)**, 30.

[21] N Phukoetphim, A Salakkam, P Laopaiboon and L Laopaiboon. Kinetic models for batch ethanol production from sweet sorghum juice under normal and high gravity fermentations: Logistic and modified Gompertz models. *Journal of Biotechnology* 2017; **243**, 69-75.

[22] S Hanief, L Prasakti, YS Pradana, RB Cahyono and A Budiman. Growth kinetic of *Botryococcus braunii* microalgae using Logistic and Gompertz Models. *AIP Conference Proceedings* 2020; **2296**, 020065.

[23] AP Asiandu, AP Nugroho, AS Naser, BR Sadewo,

MD Koerniawan, A Budiman, EA Suyono and EA Zulaika. The effect of tofu wastewater and pH on the growth kinetics and biomass composition of *Euglena sp*. *Current Applied Science and Technology* 2023; **23(2)**, 1-16.

[24] MK Lam, KT Lee, CG Khoo, Y Uemura and JW Lim. Growth kinetic study of *Chlorella vulgaris* using lab-scale and pilot-scale photobioreactor: Effect of CO_2 concentration. *Journal of Engineering Science and Technology* 2016; **11**, 73-87.

[25] FJ Richards. A flexible growth function for empirical use. *Journal of Experimental Botany* 1959; **10(2)**, 290-301.

[26] F Wang, X Yu, Y Cui, L Xu, S Huo, Z Ding, Q Hu, W Xie, H Xiao and D Zhang. Efficient extraction of phycobiliproteins from dry biomass of *Spirulina platensis* using sodium chloride as extraction enhancer. *Food Chemistry* 2023; **406**, 135005.

[27] Ş Dere, T Güneş and R Sivaci. Spectrophotometric determination of chlorophyll-A, B and totalcarotenoid contents of some algae species using different solvents. *Turkish Journal of Botany* 1998; **22(1)**, 13-18.

[28] L Barsanti, R Vismara, V Passarelli and P Gualtieri. Paramylon (β-1, 3-glucan) content in wild type and WZSL mutant of Euglena gracilis. Effects of growth conditions. *Journal of Applied Phycology* 2001; **13(1)**, 59-65.

[29] M DuBois, KA Gilles, JK Hamilton, PA Rebers and F Smith. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* 1956; **28(3)**, 350-356.

[30] KJ Livak and TD Schmittgen. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 2001; **25(4)**, 402-408.

[31] RB Cain. The metabolism of protocatechuic acid by a vibrio. *Biochemical Journal* 1961; **79(2)**, 298-312.

[32] S Kakkar and S Bais. A review on protocatechuic acid and its pharmacological potential. *International Scholarly Research Notices* 2014; **2014(1)**, 952943.

[33] J Cai, L Sun and Q Wang. Antibacterial activity and mechanism of Protocatechuic acid against bacterial canker of tomato fruits caused by *Clavibacter michiganensis* subsp. michiganensis. *Food Bioscience* 2024; **60**, 104437.

[34] A Khanra and MP Rai. Evaluation of mixotrophic cultivation of *Euglena gracilis* for lipid synthesis and FAME characterization towards biodiesel application. *Journal of Scientific & Industrial Research* 2018; **77(6)**, 359-364.

[35] M Ma, C Wei, M Chen, H Wang, Y Gong and Q Hu. Effects of nutritional mode on the physiological and biochemical characteristics of the mixotrophic flagellate *Poterioochromonas malhamensis* and the potential ecological implications. *Microorganisms* 2022; **10(5)**, 852.

[36] KT Yan, IS Hie, EA Samaranayake, JL Chang and AZ Wang. Medium and process optimizations for *Euglena gracilis* with high biomass production enriched with protein. *Algal Research* 2023; **75**, 103265.

[37] A Farjallah and C Guéguen. Untargeted metabolomic analysis of heterotrophic *Euglena gracilis* cells in response to the organic carbon source. *Algal Research* 2023; **76**, 103305.

[38] X Li, W Li, J Zhai, H Wei and Q Wang. Effect of ammonium nitrogen on microalgal growth, biochemical composition and photosynthetic performance in mixotrophic cultivation. *Bioresource Technology* 2019; **273**, 368-376.

[39] MA Borowitzka. *Chapter 3 - Biology of microalgae. In*: IA Levine and J Fleurence (Eds.). *M*icroalgae in health and disease prevention. Academic Press, London, 2018, p. 23-72.

[40] Y Wang, T Seppänen-Laakso, H Rischer and MG Wiebe. Euglena gracilis growth and cell composition under different temperature, light and trophic conditions. *PLoS One* 2018; **13(4)**, e0195329.

[41] N Hossain and TMI Mahlia. Progress in physicochemical parameters of microalgae cultivation for biofuel production. *Critical Reviews in Biotechnology* 2019; **39(6)**, 835-859.

[42] WT Godbey. *Chapter 5 - cell growth. In*: WT Godbey (Ed.). *Biotechnology and its applications: Using cells to change the world*. Academic Press, London, 2022, p. 117-150.

[43] M Ibrahim, A Al-Thukair, S Abdur Razzak, W Farooq and I Ahmad. Isolation of indigenous

microalgae: Nitrogen/phosphorous removal and biofuel production. *Biofuels* 2017; **11(3)**, 269-276.

[44] EA Suyono, W Haryadi, M Zusron, M Nuhamunada, S Rahayu and AP Nugroho. The effect of salinity on growth, dry weight and lipid content of the mixed microalgae culture isolated from Glagah as biodiesel substrate. *Journal of Life Sciences* 2015; **9**, 229-233.

[45] NR Boyle and JA Morgan. Flux balance analysis of primary metabolism in *Chlamydomonas reinhardtii*. *BMC Systems Biology* 2009; **3(1)**, 4.

[46] F Martinez and MI Orus. Interactions between glucose and inorganic carbon metabolism in *Chlorella vulgaris* strain UAM 101. *Plant Physiology* 1991; **95(4)**, 1150-1155.

[47] M Khan, R Karmakar, B Das, F Diba and MH Razu. *Heterotrophic growth of micro algae. In*: J Liu, Z Sun and H Gerken (Eds.). *Recent advances in microalgal biotechnology*. OMICS Group eBooks, California, United States, 2016.

[48] T Matsui, K Mori and T Toyama. Paramylon production by *Euglena gracilis* via mixotrophic cultivation using sewage effluent and waste organic compounds. *Bioresource Technology Reports* 2021; **15**, 100735.

[49] X Yan, H Xu, Z Yang, Y Yin, W Fang, M Wakisaka and J Zhu. Synergistic enhancement of paramylon production in edible microalga *Euglena gracilis* via ethanol-guaiacol co-regulation. *Foods* 2025; **14(14)**, 2457.

[50] Y Huang, X Wan, Z Zhao, H Liu, Y Wen, W Wu, X Ge and C Zhao. Metabolomic analysis and pathway profiling of paramylon production in *Euglena gracilis* grown on different carbon sources. *International Journal of Biological Macromolecules* 2023; **246**, 125661.

[51] X Xiao, R He, X Guo, X Zhao, Z Yang, Y Yin, M Wakisaka and J Zhu. Strategic carbon source selection enhances biomass and paramylon yields in mixotrophic *Euglena gracilis* cultivation. *Microorganisms* 2025; **13(10)**, 2339.

[52] Y Yoshida, T Tomiyama, T Maruta, M Tomita, T Ishikawa and K Arakawa. De novo assembly and comparative transcriptome analysis of *Euglena gracilis* in response to anaerobic conditions. *BMC Genomics* 2016; **17(1)**, 182.

[53] Q He, X Zhang, H Xu, X Wang, X Zhang, Y Hao, X Song and X Cao. Cultivation of high-protein *Euglena gracilis* for enhanced protein production under inorganic nitrogen sources: Mechanisms revealed by proteomics. *Bioresource Technology* 2025; **430**, 132560.

[54] MS Jeon, SI Han, JY Kim and YE Choi. Co-cultivation of *Euglena gracilis* and *Pseudoalteromonas* sp. MEBiC 03607 for paramylon production. *Journal of Applied Phycology* 2020; **32(6)**, 3679-3686.

[55] M Zeng, W Hao, Y Zou, M Shi, Y Jiang, P Xiao, A Lei, Z Hu, W Zhang, L Zhao and J Wang. Fatty acid and metabolomic profiling approaches differentiate heterotrophic and mixotrophic culture conditions in a microalgal food supplement "Euglena". *BMC Biotechnology* 2016; **16(1)**, 49.

[56] JS Rodríguez-Zavala, MA Ortiz-Cruz, G Mendoza-Hernández and R Moreno-Sánchez. (2010). Increased synthesis of α-tocopherol, paramylon and tyrosine by *Euglena gracilis* under conditions of high biomass production. *Journal of Applied Microbiology* 2010; **109(6)**, 2160-2172.

[57] F Matsuda, M Hayashi and A Kondo. Comparative profiling analysis of central metabolites in *Euglena gracilis* under various cultivation conditions. *Bioscience, Biotechnology, and Biochemistry* 2011; **75(11)**, 2253-2256.

[58] H Begum, FM Yusoff, S Banerjee, H Khatoon and M Shariff. Availability and utilization of pigments from microalgae. *Critical Reviews in Food Science and Nutrition* 2016; **56(13)**, 2209-2222.

[59] S Kakkar and S Bais. A review on protocatechuic acid and its pharmacological potential. *International Scholarly Research Notices* 2014; **2014(1)**, 952943.

[60] JC da Silva and AT Lombardi. *Chlorophylls in microalgae: Occurrence, distribution, and biosynthesis. In*: E Jacob-Lopes, MI Queiroz and LQ Zepka (Eds.). Pigments from microalgae handbook. Springer International Publishing, Cham, Switzerland, 2020, p. 1–18.

[61] S Liu. Bioprocess engineering: Kinetics, sustainability, and reactor design. 2nd ed. Elsevier, Amsterdam, Netherlands, 2016.

[62] Y Tanaka, T Ogawa, T Maruta, Y Yoshida, K Arakawa and T Ishikawa. Glucan synthase-like 2 is indispensable for paramylon synthesis in

Euglena gracilis. *FEBS Letters* 2017; **591(10)**, 1360-1370.

[63] D Siebert, J Altenbuchner and B Blombach. A timed off-switch for dynamic control of gene expression in *Corynebacterium glutamicum*. *Frontiers in Bioengineering and Biotechnology* 2021; **9**, 704681.

[64] T Erfianti, AYF Yusuf, S Handayani, BR Sadewo, BS Daryono, A Budiman and EA Suyono. Enhancing growth and metabolite profiles in indigenous *Euglena gracilis* through explorative light spectrum effect. *Egyptian Journal of Aquatic Research* 2024; **50(3)**, 318-331.

[65] SD Ramadhana, T Erfianti, D Aritonang and EA Suyono. Influence of nitrogen variation and 15% CO_2 injection on wax ester production in *Euglena gracilis* isolated from Dieng Peatland, Indonesia. *Applied Phycology* 2025; **6(1)**, 190-209.

[66] T Erfianti, BR Sadewo, BS Daryono, A Budiman and EA Suyono. Evaluating CO_2 concentration effects on growth kinetics and fatty acid composition in *Euglena gracilis*. *Applied Phycology* 2024; **5(1)**, 61-71.

[67] KQ Maghfiroh, T Erfianti, I Nurafifah, R Amelia, D Kurnianto, BR Sadewo, R Maggandari, BR Aji, A Budiman and EA Suyono. The effect of photoperiodism on nutritional potency of *Euglena* sp. Indonesian strains. *Malaysian Journal of Nutrition* 2023; **29(3)**, 453.

[68] M Cindiati, T Erfianti, R Adaranyssa, E Putri, D Kurnianto and EA Suyono. The effect of vinasse in various trophic conditions on the growth rate and metabolite contents of Euglena sp. with CO_2 aeration. *Molecular Biology and Biotechnology* 2026; **34**, 67-82.