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Exploration of Lipid Profile and Wax Ester Content from Local Strain *Euglena* sp. IDN33 Cultivated in Mixotrophic Condition Combined with Molasses Supplementation

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ABSTRACT

The transition from fossil fuels to sustainable renewable energy is currently growing rapidly. *Euglena* sp. can be the source of biofuel. Molasses, in addition to *Euglena* sp., can increase the levels of paramylon and wax esters produced. Therefore, this study aims to analyze cell growth, lipid profile, and wax ester content of *Euglena* sp. cultivated under mixotroph conditions with molasses. *Euglena* was cultivated under mixotrophic conditions with the addition of various concentrations of molasses (0, 10, 15, and 20 g/L) until the final log phase. Then, measurements were made on cell density, levels of lipids, proteins, and paramylon to measure the growth and metabolites profile of *Euglena* sp., along with lipid profile and wax ester analysis using GC-MS. The results of the study showed that 10 g/L molasses increased *Euglena* growth (SGR 0.1736± 0.0213 mg/ml) and carbohydrate (0.426 mg/ml) but reduced lipid accumulation (2.29 mg/ml). This treatment provides the best result before control. Molasses does not increase carbohydrates and protein but increases paramylon accumulation. The GC-MS assay detected more lipid profiles and concentrations of each lipid type in non-molasses-treated *Euglena* sp. compared to molasses-treated. *Euglena* was cultivated on molasses, which was dominated by palmitic acid and myristyl myristate with the absence of PUFA-type fatty acids in the cells, while the untreated ones were dominated by stearic acid and myristyl myristate. Thus, 10 g/L supplementation of molasses is the most effective treatment compared to others.

1. Introduction

Fossil fuels have significantly contributed to increased greenhouse gas emissions and air pollution worldwide. Emissions from fossil fuel combustion reached approximately 36.8 billion metric tons of carbon dioxide in 2023, underscoring the ongoing impact of these energy sources on the climate crisis (Poulter *et al*. 2023). Additionally, dwindling fossil fuel reserves threaten global energy security (Abas *et al*. 2015). Therefore, a transition to renewable energy sources is urgently needed. Microalgae have significant potential in various biotechnological applications, including biofuel production, pharmaceuticals, and environmental remediation, due to their efficient biomass generation and ability to utilize carbon dioxide (Khan *et al*. 2023). Microalgae can produce large amounts of biomass in a relatively short period and can be grown on cheap waste media (Gao *et al*. 2021). For instance, studies have shown that microalgal biomass yields can reach up to 40 g dry weight per square meter per day, which translates to approximately 145 metric tons per hectare per year (Li *et al*. 2022). Microalgae cultivation does not require fertile land and does not disturb food stocks (Chowdhury and Loganathan 2019). Several microalgal strains with high lipid content include *Aurantiochytrium* sp. and *Schizochytrium* sp., which contain up to 77.34% and 77% lipids, respectively

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(Park *et al*. 2017). *Porphyridium* sp. and *Isochrysis* sp. have lipid contents of approximately 6×10^{-6} g/cell and 4.5×10^{-7} g/cell (Anggraini *et al*. 2016). Microalgal lipids are advantageous for biofuel production due to their rapid growth, CO_2 utilization, and higher polyunsaturated fatty acids like DHA and EPA compared to vegetable or animal oils (Morabito *et al*. 2019).

Euglena is a freshwater microalgae with a lot of potential for biofuel production. This microorganism can live in acidic media, which reduces the risk of contamination, and does not have cell walls, which makes *Euglena* metabolites easier to extract (Inui *et al*. 2017). *Euglena* can produce various high-value substances such as paramylon and wax ester. Paramylon or β-1,3-glucan is a type of carbohydrate that can be used for multiple products such as nanofiber, dietary fiber, and supplements (Shibakami *et al*. 2013; Nakashima *et al*. 2018; Gissibl *et al*. 2019). *Euglena* can accumulate paramylon up to 78% dry weight under certain conditions (Wu *et al*. 2021). Apart from paramylon, a wax ester is an essential compound in *Euglena*. Wax esters, a class of lipids, possess the potential to serve as a precursor for biojet fuel. Nevertheless, wax esters derived from microalgae exhibit a freezing point of -32°C, necessitating the incorporation of an antifreeze agent to comply with the ASTM standard of -40°C (Bwapwa *et al*. 2018; Inui *et al*. 2017). These wax esters can be converted into fatty acid methyl esters (FAME) through a process called transesterification, which is essential for biodiesel production (Keng *et al*. 2009).

The paramylon accumulated by *Euglena* can be converted into wax ester through a fermentation process. Wax ester fermentation occurs in hypoxic and anoxia conditions without light. In this condition, paramylon levels in cells decrease significantly while wax ester levels increase because paramylon is converted into wax ester (Inui *et al*. 2017; Wu *et al*. 2021; Kim *et al*. 2022). Therefore, increasing paramylon levels in *Euglena* cells is essential to increasing wax ester yield. Kim *et al*. (2022) demonstrated a decrease in paramylon content and a concomitant increase in wax ester content during fermentation, supporting the conversion process. The molecular mechanisms underlying this process have been elucidated by Tomiyama *et al*. (2017), who identified wax ester synthase/diacylglycerol acyltransferase (WSD) isoenzymes as key enzymes in wax ester biosynthesis in *E. gracilis*. Consequently, increasing paramylon levels in *Euglena* cells can enhance wax ester production by providing a greater substrate pool for the enzymatic pathway involving WSD.

While microalgae hold immense potential as a sustainable alternative fuel source, several significant challenges hinder its widespread adoption. One of the primary challenges is the high cost of production. The cultivation, harvesting, and extraction of lipids from microalgae require substantial energy, water, and nutrients, making the production of microalgae-based biofuel more expensive compared to conventional fossil fuels. Additionally, the large-scale infrastructure and technological investments necessary for commercial production contribute to the overall costs (Hoang *et al*. 2022; Khan *et al*. 2023). Another major hurdle is energy efficiency. The production process itself is energy-intensive, requiring significant energy for both microalgal growth and lipid extraction. This can offset some of the environmental benefits of using biofuels if the energy used comes from fossil fuel sources (Doliente *et al*. 2020).

Euglena, a potential biofuel source, faces challenges in cultivation due to the high costs associated with medium preparation (Kottuparambil *et al*. 2019; Bansfield *et al*. 2021). To reduce costs, researchers are exploring alternative media, such as a byproduct of the sugar industry. Indonesia operates 63 sugar mills that produce 2.5 to 3.0 million metric tons of sugar annually, making it a major player in the global sugar refining industry. A significant number of byproducts, such as molasses, bagasse, and press mud, are produced during the sugar refining process. A byproduct abundant in fermentable sugars, molasses finds extensive application in a range of sectors, including animal husbandry, ethanol production, and the food and beverage industry. Previous research has demonstrated that molasses, rich in sugars, proteins, minerals, and urea, can serve as an effective carbon source for cultivating microalgae, including *Euglena*. While studies by Yan *et al*. (2011), Yew *et al*. (2020), and Rubiyatno *et al*. (2021) have shown that the nutrient-rich composition of molasses significantly enhances microalgal growth, no studies have yet optimized the optimal molasses concentration to enhance both growth and wax ester production in *Euglena*.

The research objectives of this study are as follows. First, we need to know the effect of adding molasses waste in mixotrophic cultivation on *Euglena* sp.'s

growth rate and biomass. Second, to determine the effect of molasses on the wax ester concentration in *Euglena* sp. cultivated as a mixotroph. Third, to determine the effect of molasses on the fatty acid methyl ester profile of *Euglena* sp., which is cultivated as a mixotroph.

2. Materials and Methods

This study was conducted to investigate the effect of molasses supplementation on the growth and biomass production of *Euglena* sp. strain IDN33 isolated from Dieng Peatland Water, Central Java, Indonesia. Molasses used in this research came from the Madukismo sugar factory in Yogyakarta, Indonesia. Four treatments with three replicates were used in this study, namely control (0 g/L molasses), 10 g/L molasses, 15 g/L molasses, and 20 g/L molasses. *Euglena* cultures were grown using the Cramer & Myers medium. Cell growth was measured by counting the number of cells using a hemocytometer, while biomass, lipid, protein, paramylon, and lipid profile were measured. Wax ester content was analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). Data were analyzed using one-way ANOVA followed by Duncan's test to determine significant differences among treatments. The analysis was conducted using IBM SPSS Statistics 26. Details of the experimental study with procedures are as follows:

2.1. Hydrolysis of Molasses

Molasses obtained from PT Madukismo Bantul Yogyakarta Special Region were subjected to pretreatment processes as described by Çalik *et al*. (2006). The molasses was initially diluted with distilled water to a concentration of 30%. Subsequently, the pH of the diluted molasses solution was adjusted to 3.5 using 5 M H_2SO_4. The acidified solution was then heated at 60°C for 2 hours to hydrolyze complex sugars. To remove impurities and concentrate the sugars, centrifugation was performed at 8000 g for 15 minutes. The resulting supernatant, containing a purified and concentrated molasses solution, is used as a substrate for microalgae cultivation.

2.2. Reducing Sugar Test on Hydrolysed Molasses

To determine the reducing sugars in hydrolyzed molasses, approximately 2 grams of the sample was dissolved in distilled water to form a homogenous solution, which was then transferred to a 50 ml volumetric flask. Twenty milliliters of this prepared sample, along with two boiling stones and twenty milliliters of Luff-Scholl reagent, were placed in a roundbottom flask, attached to a reflux condenser, and cooked for ten minutes. The mixture was quickly cooled after boiling, and then 15 ml of 20% KI solution and 25 ml of 26.5% H_2SO_4 were carefully added. Standardized 0.2 N $Na_2S_2O_3$ was used to titrate the solution, and a starch indicator was added near the titration endpoint. A blank test was performed without the sample to serve as a control for comparison, and the total reducing sugars in the sample were calculated using a formula that relates the volume of titrant used to the concentration of sugars present (Acosta *et al*. 2023).

2.3. Total Nitrogen Measurement

The total nitrogen content in molasses was determined using the Automatic Kjeldahl method. Approximately 1 g of the homogenized molasses sample was placed in a Kjeldahl digestion tube, and 3.5 g of K_2SO_4, 0.1 g of $CuSO_4 \cdot 5H_2O$, and 12 ml of concentrated H_2SO_4 were added. The mixture was heated in an Automatic Digestion Unit until a clear solution was obtained. The resulting digest was transferred to an Automatic Distillation and Titration System, where nitrogen content was quantified using standard reagents (H_3BO_3, NaOH, and HCl) (Saez *et al*. 2013).

2.4. Carbon Content Measurement

The carbon content in molasses was analyzed by weighing approximately 1 g of the homogenized sample in a porcelain crucible and heating it at 105°C for 3 hours to obtain a constant weight. The sample was then ashed at 350°C for 2 hours, followed by ashing at 600°C for 8 hours. The carbon content was calculated based on the weight differences before and after ashing (Anggelova *et al*. 2019).

2.5. Phosphor Content Measurement

Phosphorus content in molasses was assessed through acid digestion using HNO_3 and $HClO_4$ in a ratio of 1:1 to homogenize approximately 1 g of the sample. The solution was filtered and diluted to a final volume before measuring absorbance at a wavelength of 430 nm using UV spectrophotometry to determine phosphorus concentration (Ganesh *et al*. 2012).

2.6. Sulphur Content Measurement

Sulfur content in molasses was determined through acid digestion with HNO_3 and $HClO_4$, followed by spectrophotometric analysis. Approximately 1 g of the homogenized molasses sample was mixed with 10 ml of HNO_3:$HClO_4$ (1:1) and heated until a clear solution with white fumes formed. The solution was then filtered and diluted to 50 ml with distilled water.

For quantification, a calibration curve for sulfate ions ($SO4^{2-}$) was prepared using known concentrations ranging from 0 to 70 ppm, mixed with barium chloride solution to precipitate barium sulfate ($BaSO_4$). The absorbance was measured at 420 nm using UV spectrophotometry to determine sulfur concentration (Nair *et al*. 2020).

2.7. *Euglena* Strains and Culture Conditions

The microalgal strain used in this study was *Euglena* sp. strain IDN33, which was isolated from the Dieng Plateau by the UGM Microalgae Biorefinery Research Group. *Euglena* sp. was grown on CM media with the following composition as described by Cramer and Myers (1952): 1.8 g/L NH_4Cl, 0.6 g/L KH_2PO_4, 1.2 g/L $MgSO_4•7H_2O$, 0.02 g/L $CaCl_2•2H_2O$, 0.55 µg/L $Na_2EDTA•2H_2O$, 2.0 µg/L $Fe_2(SO_4)3$, 0.05 µg/L $CuSO_4•5H_2O$, 0.4 µg/L $ZnSO_4•7H_2O$, 1.3 µg/L $Co(NO_3)2•6H_2O$, 1.8 µg/L $MnCl_2•4H_2O$, 0.01 µg/L vitamin B1, and 0.0005 µg/L vitamin B12. The medium was added with molasses with concentrations of 0 g/L, 10 g/L, 15 g/L, and 25 g/L. These concentrations were chosen as the result of preliminary studies by authors on the limit of molasses concentration, in which *Euglena* sp. will still be able to show a complete growth curve. While the intervals were inspired by Thepsuthammarat *et al*. 2023. The pH value of the *Euglena* medium was carefully adjusted at 3.5 by gradually adding 2.5 mol/L NaOH and 1 mol/L L HCl (Cramer and Myers 1952). *Euglena* sp. was cultivated in liquid media with continuous agitation provided by air aeration and cultured in a culture room at 24°C under 24-hour photoperiod conditions with white light until the initial stationary phase, which typically occurs after several days of growth (Rubiyatno *et al*. 2021). In this case, cultivation time reaches 12 days.

2.8. Growth Rate Measurement

Optical density was measured daily using a spectrophotometric method at a wavelength of 680 nm (Almomani & Örmeci 2018). A total of 2 ml of sample was taken and put into a cuvette. Then, 1 ml of distilled water was put into the cuvette as a blank solution. Absorbance was measured using a spectrophotometer at a wavelength of 680 nm. The doubling time (td) calculation is based on the following formula:

$$td = \ln (2) / \mu \text{ where } \mu = (\ln(Nt) - \ln(N0)) / t.$$

Specific Growth rate calculated using the formula:

$$\mu = (\ln(Nt) - \ln(N0)) / t \text{ (Kadimpati } et\ al\text{. 2013).}$$

2.9. Lipid Measurement

Lipid levels were measured every three days using a modified Bligh and Dyer method (Bligh & Dyer 1959). Samples were centrifuged, and the resulting pellet was mixed with methanol and chloroform and then vortexed. After centrifuging again, the yellow lipid-containing layer was extracted, placed in a pre-weighed petri dish, and dried in a 40°C oven to evaporate the chloroform. The total lipid weight was then calculated as follows $x = (wt – wf) / v$ while lipid productivity counted ass follow: $\Delta x / t$ where wt = total weight, wf = weight of filter petri dish, Sample volume = v, Lipid weight (mg/ml) = x, t = days (Chen *et al*. 2018).

2.10. Carbohydrate Measurement

Carbohydrate levels were measured every three days until the stationary phase. Microalgae samples (5 ml) were centrifuged at 4,000 rpm for 10 minutes, and the supernatant was discarded. Then, 500 µl of 5% phenol and 1 ml of concentrated sulfuric acid were added, vortexed, and left for 30 minutes. A standard carbohydrate curve (25-250 mg/ml) was prepared with D-glucose, and absorbance was measured at 490 nm (Pooja *et al*. 2022). Carbohydrate productivity was calculated as:

$$\text{Carbohydrate Productivity (mg/ml/day)} = t\Delta x$$

Where:
Δx : carbohydrate concentration (mg/ml)
t : time in days

2.11. Measurement of Paramylon Content

2 ml of microalgae samples were centrifuged for 10 minutes at 7,500 x g., and then the pellets were dried. After that, it was washed once with distilled water and washed twice with 1 ml of ethanol. The resulting pellet was added with a 1 g/L trypsin solution in 0.1 M sodium phosphate buffer at pH 7.6 and then incubated overnight. Centrifugation was carried out, and the supernatant

was discarded. The pellets were added with 500 g/L urea solution. Centrifugation was carried out, and the resulting supernatant was discarded. Pellets were rinsed with distilled water. Then, it dried and weighed. Calculation of the ratio of paramylon to biomass is carried out using the formula: Paramylon per biomass ratio: (Weight of paramylon) / (weight of cell biomass) (Grimm *et al*. 2015).

2.12. Measurement of Wax Ester

Euglena sp. was cultivated with molasses concentrations that induced the most significant levels of paramylon. When *Euglena* has reached the final log phase, wax ester fermentation is carried out. A 50 ml sample was transferred to a 50 ml tube. Then, the tube is closed tightly to prevent air circulation and wrapped in aluminum foil. The tubes were incubated at 28°C in the dark for 48 hours without shaking (Kim *et al*. 2022).

2.13. Wax Ester Content and Profiling

GC-MS measured the wax ester profile and content. Lipids were extracted using the Bligh and Dyer method (Bligh and Dyer 1959). The oven temperature was set as follows: i) 50°C for 1 min, ii) heating to 175°C at a rate of 25°C/min, iii) heating to 200°C at a rate of 4°C / min. A sample volume of 1.00 µL was injected into the system using splitless injection mode to ensure optimal sensitivity and resolution. Nitrogen served as the carrier gas at a flow rate of 20 cm^3/min. The resulting chromatograms were analyzed to identify and quantify the fatty acid methyl esters (FAME) present in the samples. The mass spectral characteristics and retention times were compared with those of FAME standards from the Supelco™ 37 Component FAME Mix to ensure accurate identification and quantification of lipid components (Kim *et al*. 2022). This comprehensive analysis provides insights into the overall lipid composition, including various fatty acids and wax esters present in the samples. (Kim *et al*. 2022).

2.14. Statistical Analysis

All samples were performed in triplicate. The collected data was analyzed statistically using one-way ANOVA, and Duncan's Multiple Range Test (DMRT) was continued at a significance level of 95%. The analysis was conducted using IBM SPSS Statistics 26.

3. Results

3.1. Molasses Compositions Analysis

The Chemical Analysis results of molasses are as follows:

As shown in Table 1, the Chemical Analysis of molasses reveals important insights into its composition, highlighting its potential effects on *Euglena*'s cells. The reducing sugar content, which reached 445.300 mg/kg, plays a crucial role in providing a carbon source that could influence *Euglena* sp. growth and metabolic activity. This high sugar concentration is beneficial for energy production through cellular respiration, potentially promoting rapid cell growth.

Comparatively, the nitrogen content, measured at 8,400 mg/kg, is much lower than carbon that measured at 281,100 mg/kg but essential for protein synthesis and cellular structure development. Phosphorus, recorded at 323.16 mg/kg, is crucial for nucleic acid synthesis and energy transfer via ATP, while magnesium (1,031.80 mg/kg) and calcium (5,186.06 mg/kg) contribute to various cellular processes, such as enzyme activation and structural integrity. The significantly higher calcium content suggests a prominent role in stabilizing cell walls or membranes in Euglena, potentially improving its resilience. Potassium content, at 32,200 mg/kg, aids in regulating osmotic balance and enzyme activation, essential for maintaining cellular homeostasis. The sulfur concentration, at 3,753.41 mg/kg, also plays a

Table 1. Molasses chemical analysis results

Parameter	Result (mg/kg)	Methods
Sugar	445,300±30887.7	Luff Schroll
Carbon	281,100±18029.9	Gravimetry
Potassium	32,200±2613.1	SSA-flame
Total Nitrogen	8,400±654.0	Kjeldahl
Calcium	5,186±530.4	SSA-flame
Sulfur	3,753±286.4	Spectrophotometry UV-vis
Magnesium	1,032±113.5	SSA-flame
Phosphorous	323±20.6	Spectrophotometry UV-vis

role in protein synthesis, as sulfur is a key component of certain amino acids.

3.2. Cells Density

Euglena sp. cells divided along with their growth. The number of cell density help to determine the growth phase of the culture. Harvesting time is usually determined by the highest cell number obtained during the culture.

As presented in Table 2 and Figure 1, the concentration of molasses given could influence the cell density of *Euglena*. The highest cell density was obtained on day 8 of treatment at 10 g/L, reaching 1,476,041.67 cells/ml. These results were significantly different from those of other treatments on the same day. This result shows that the molasses concentration of 10 g/L is the optimal concentration to increase the growth of *Euglena* sp.

As depicted in Figure 1, the control treatment with 0 g/l molasses yielded a cell density of 1,256,597.22 cells/ml on the peak day, day 10. On the same day, the 15 g/L molasses waste treatment produced higher cell density results, specifically 1,402,777.78 cells/ml. While the density results were higher than the control treatment, they were not significantly different. The treatment with the lowest density results was 20 g/L molasses waste, generating 1,181,250 cells/ml. This result was notably different from the other treatments, indicating that increasing molasses concentration does not always lead to an increase in *Euglena* density.

Table 2. Cells density of *Euglena* sp.

Day	Control ($\times 10^4$ cell/ml)	10 g/L ($\times 10^4$ cell/ml)	15 g/L ($\times 10^4$ cell/ml)	20 g/L ($\times 10^4$ cell/ml)
0	(62.11±7.67)^{a}	(55.66±10.31)^{a}	(62.83±4.48)^{a}	(53.27±6.24)^{a}
1	(62.25±4.13)^{a}	(65.88±7.99)^{a}	(66.25±0.25)^{a}	(68.44±10.52)^{a}
2	(90±5.29)^{a}	(105±5.56)^{ab}	(104.88±22.75)^{ab}	(129.22±29.64)^{b}
3	(107.11±25.85)^{a}	(154.66±17.21)^{b}	(129±26.90)^{ab}	(153.66±16.28)^{b}
4	(108±21.54)^{a}	(172.33±50.76)^{b}	(153.08±21.34)^{ab}	(183.66±23.58)^{b}
5	(124±18.29)^{a}	(144.77±33.84)^{a}	(139.77±12.73)^{a}	(174±33.32)^{a}
6	(114.83±10.61)^{a}	(161.66±22.72)^{b}	(148.61±34.18)^{ab}	(174.22±8.95)^{b}
7	(152.5±53.39)^{a}	(206.33±72.74)^{a}	(182.16±74.65)^{a}	(188.33±10.12)^{a}
8	(134±7.54)^{a}	(236.16±28.22)^{c}	(178.33±15.60)^{b}	(189±14.17)^{b}
9	(153.66±34.50)^{a}	(179.66±25.54)^{a}	(186.47±21.31)^{a}	(184.83±34.04)^{a}
10	(201.05±26.89)^{bc}	(162.66±24.61)^{ab}	(224.44±39.52)^{c}	(125.5±19.48)^{a}
11	(155.63±14.42)^{a}	(125.16±23.93)^{a}	(128.33±19.030)^{a}	(143.83±38.97)^{a}
12	(75.5±13.25)^{a}	(85.66±13.86)^{a}	(76.66±24.70)a	(93.66±0.57)^{a}

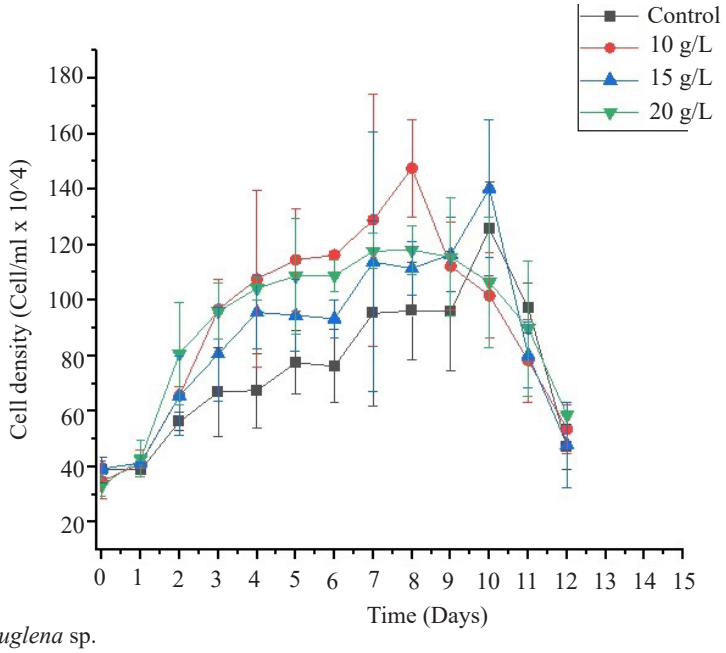

Figure 1. Cell density of *Euglena* sp.

This research shows that 10 g/L molasses provides optimal nutrition for increasing *Euglena*'s growth. This concentration produces the highest number of cells compared to other treatments and reaches the peak cell number most quickly. This result differs from the control and other treatments with higher concentrations, which provide lower cell density results, and the peak cell density is achieved longer.

3.3. Specific Growth Rate and Doubling Time

The growth of *Euglena* sp. was evaluated underdifferent molasses concentrations. As describe in Table 3, The 10 g/L molasses treatment exhibited the most favorable growth conditions, as indicated by the highest specific growth rate (SGR) of 17.36%±1.22% per day. In contrast, the control group had the lowest SGR of 13.6%±1.22% per day. Moreover, the doubling time was the shortest in the 10 g/L treatment at 4.0356±0.5238 days, while the 15 g/L treatment resulted in the longest doubling time of 4.8928±0.5238 days. These findings suggest that a molasses concentration of 10 g/L optimizes the growth of *Euglena* sp. under the experimental conditions.

3.4. Lipids

From the research that has been carried out, data on the response of *Euglena* lipid accumulation to variousconcentrations of molasses were obtained. As shown in Figure 2, The weight of *Euglena* lipids in all treatments on day 0 was not significantly different, but on the last day, namely day 12, there was an increase in lipid weight in all treatments. On day 12, the highest lipid weight was in the control treatment, weighing 5.03±2.53 mg/ml. This result significantly differed from all treatments except the 20 g/L treatment, which resulted in 2.89±0.06 mg/ml. The 10 g/l molasses treatment was the treatment that gave the lowest increase in lipid weight, namely 1.36±0.06 mg/ml. 15 g/L treatment gave higher results than 10 g/L, namely 2.29±0.40 mg/ml.

3.5. Paramylon

Based on the observations regarding the influence of molasses on the accumulation of paramylon in *Euglena* sp. as shown in the Figure 3, it was found that a molasses concentration of 10 g/L led to the fastest accumulation of paramylon, reaching its peak on day 9 with a value of 0.9748±0.09 mg/ml. In comparison, the other molasses concentrations required more time to reach their respective peak accumulation levels. These findings suggest that the addition of 10 g/L molasses is the most effective in promoting both the rapid and abundant accumulation of paramylon in *Euglena* sp., outperforming the other molasses treatments in terms of both speed and overall yield.

Table 3. Specific growth rate and doubling time of *Euglena* sp.

Treatment	Specific growth rate (μ/day)	Doubling time (day)
Control	(13.6±1.22)^{a}	(5.124±0.474)^{b}
10 g/L	(21.4±5.03)^{b}	(3.342±0.697)^{a}
15 g/L	(16.5±3.16)^{ab}	(4.309±0.763)^{ab}
20 g/L	(14.6±0.71)^{a}	(4.769±0.225)^{b}

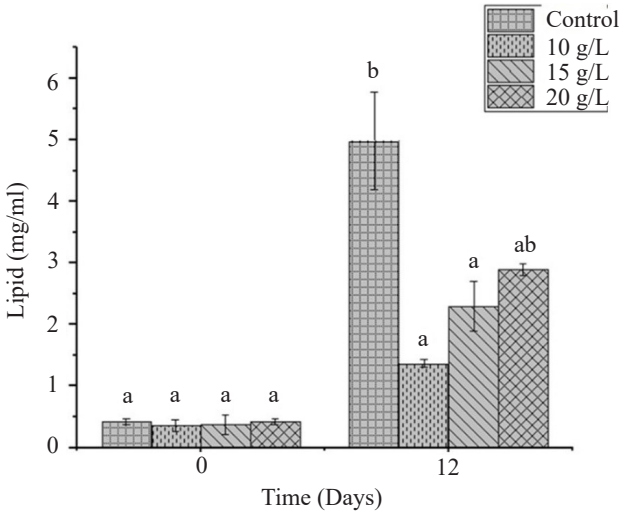

Figure 2. Lipid production of *Euglena* sp.

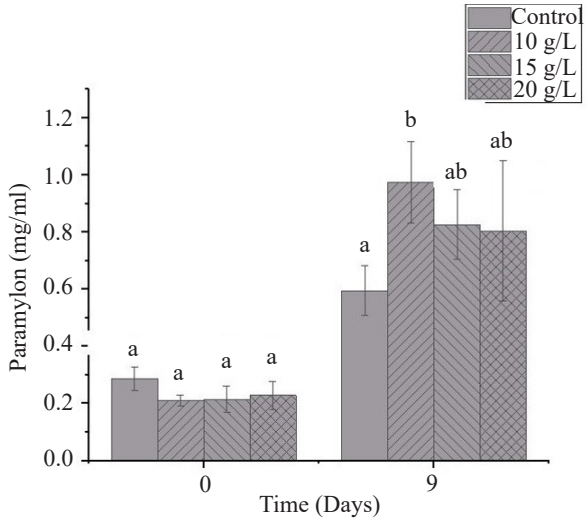

Figure 3. Paramylon production in *Euglena* sp.

3.6. Carbohydrates

As depicted in Figure 4, the results of research on *Euglena* carbohydrates in various molasses treatments showed that on day 0, carbohydrate levels were not different in all treatments, but on the last day, namely day 12, there were differences between treatments. The control treatment with 0 g/L molasses experienced the highest increase, namely 0.465±0.03 mg/ml, but based on statistical tests, the results of the control treatment were not significantly different from the 10 g/L and 20 g/L molasses treatments. A concentration of 20 g/L gave a result of 0.346±0.15 mg/ml, while a concentration of 10 g/l gave a result of 0.426±0.08 mg/ml. The 15 g/L molasses treatment was the treatment that experienced the lowest increase of 0.198±0.10 mg/ml.

3.7. Lipid Profile

After the first stage of cultivation, which was to determine the growth of primary metabolites, especially *Euglena* paramylon, in each treatment, the results showed that 10 g/L molasses produced the most optimal paramylon levels and growth. A molasses concentration of 10 g/L was used in the second stage culture for further fermentation for three days and continued with the GC-MS test. TThe results of the GC-MS test are shown in Table 4 and 5.

The higher the Similarity Index value, the more accurate the identification of the compound. Based on the peak data, similarity index, and retention time obtained, the identification of compounds detected in microalgae samples was carried out. In addition, data on the relative concentration of compounds detected in all treatments were obtained as shown in Table 6.

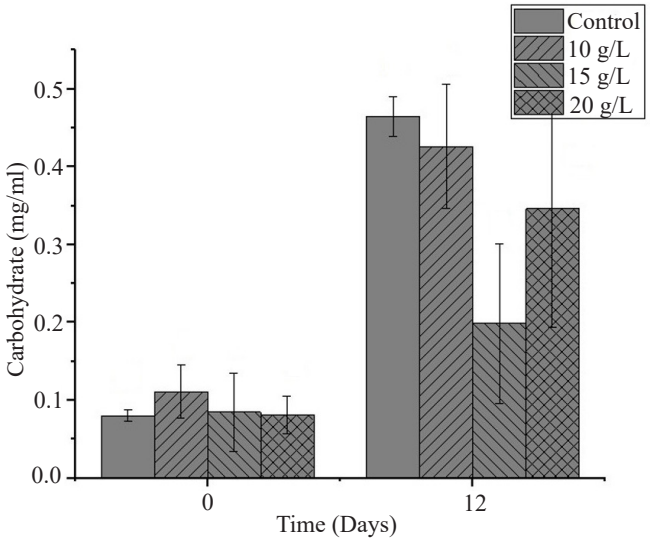

Figure 4. Carbohydrate production in *Euglena* sp.

Table 4. Lipid compounds detected in the control treatment

Compound name	Chemical formula	Lipid type	Similarity index	Ret. time
Linolenic acid	$C_{18}H_{30}O_2$	PUFA	90	27.90
Stearic acid	$C_{18}H_{36}O_2$	SOFA	91	28.23
Palmitic Acid	$C_{19}H_{40}O_2Si$	SFA	93	29.64
11,14,17-Eicosatrienoic acid, methyl ester	$C_{21}H_{36}O_2$	PUFA	88	31.15
Myristyl myristate	$C_{28}H_{56}O_2$	Wax Ester	88	42.13

Table 5. Lipid compounds detected in molasses treatment

Compound name	Chemical formula	Lipid type	Similarity index	Ret. time
Palmitic Acid	$C_{19}H_{40}O_2Si$	SFA	95	28.21
Myristyl myristate	$C_{28}H_{56}O_2$	Wax Ester	91	42.12

Table 6. Comparison of detected compound concentrations in each treatment

Detected compound	Lipid type	Consentration (%)	
		Control	Molasse
Linolenic acid	PUFA	4.17±0.65	N.d.
Stearic acid	SFA	9.53±2.57	N.d.
Palmitic Acid	SFA	(4.58±0.45)[a]	(10.45±5.7)[b]
11,14,17-Eicosatrienoic acid, methyl ester	PUFA	5.05±2.87	N.d.
Myristyl myristate	Wax Ester	(9.64±1.77)[a]	(8.9±1.57)[a]

Lipid compounds that were obtained is this research is shown in Figure 5. In the control treatment, five lipid compounds were obtained, namely linolenic acid, stearic acid, palmitic acid, 11,14,17-Eicosatrienoic acid, methyl ester, and myristyl myristate. The compound from the control treatment is myristyl myristate. In the molasses treatment, two lipid compounds were obtained, namely palmitic acid and myristyl myristate. The most abundant compound was palmitic acid. This compound dominates in *Euglena* samples cultivated using molasses.

Another compound that showed significant differences was palmitic acid. This compound is a type of saturated fatty acid with a C16 carbon chain. Palmitic acid is the most common fatty acid found in nature.

4. Discussion

The specific growth rate (SGR) is a mathematical parameter that quantifies microbial growth during the exponential phase. High SGR indicates rapid growth and high productivity, inversely related to doubling time, which measures the time for cell mass to double. High doubling time signifies slow microalgal growth

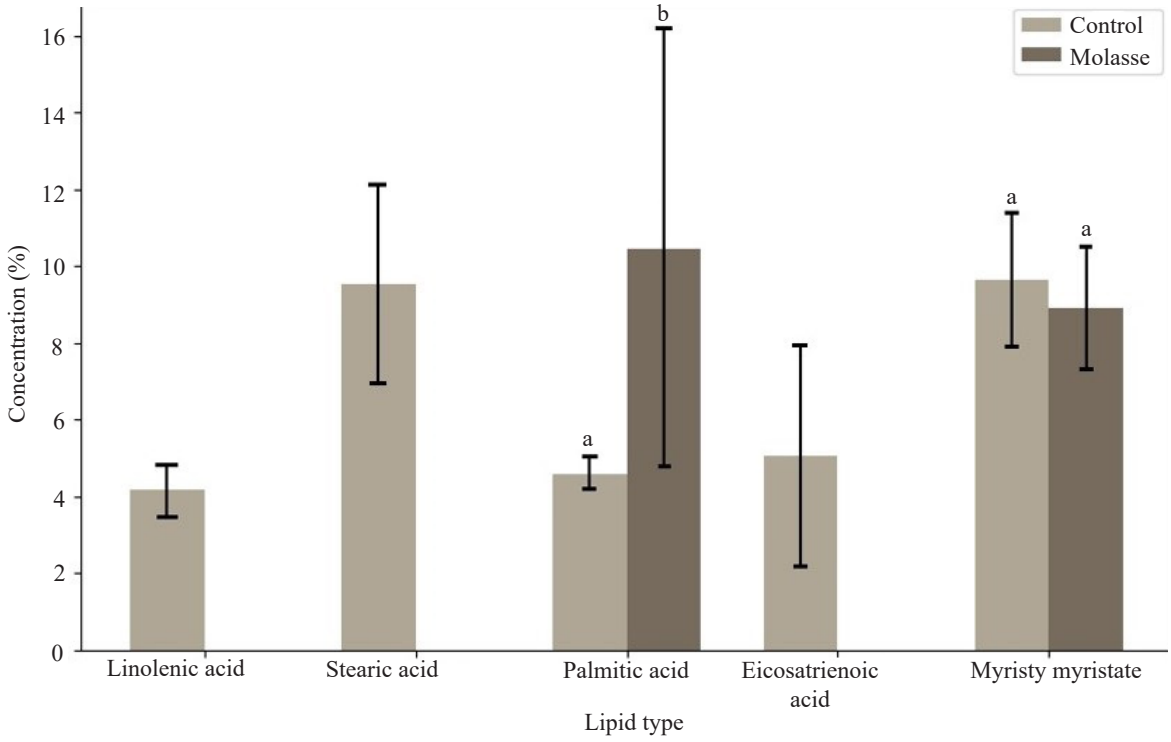

Figure 5. Graph of detected compounds and compound concentrations in treatments control and molasses

(Stanbury *et al*. 2017). This study demonstrates that molasses at a concentration of 10 g/L provides the best nutrition for *Euglena* growth paramylon cells with increased density, specific growth rate, and a shorter doubling time. This is because 10 g/L concentration is the amount generated by the highest cell from other treatments, and it reaches the fastest cell's peak density. The result of 10 g/L is different from the control and other treatments with a higher concentration of molasses.

Furthermore, it produces a low cell density, and the peak cell density is achieved longer. In contrast, a previous study by Ivusic and Santec (2015) demonstrated a different outcome. *Euglena gracilis* strain Z (Klebs SAG 1224-5/25), which was pre-adapted to molasses and corn step solid, exhibited the highest paramylon yield and biomass productivity when cultivated in a medium containing 30 g/L molasses and 30 g/L corn steep solid. Beyond this concentration, a notable decrease in cell growth was observed. The absence of a pre-adaptation step in our study likely explains the lower biomass and paramylon yields obtained. These findings underscore the importance of pre-adaptation as a strategy for optimizing the cultivation of *Euglena gracilis* in molasses-based media. Future studies should systematically investigate the mechanisms underlying the beneficial effects of pre-adaptation, such as changes in gene expression, enzyme activity, or cell morphology.

Based on this research, it was found that higher concentrations of molasses do not lead to increased cell density. Elevated molasses concentration likely contributes to greater medium turbidity, which in turn reduces the amount of light available for absorption by microalgae, ultimately hindering their growth. Previous studies on Scenedesmus dimorphus, and Spirulina platensis, reported lower cell densities in turbid media (Setyoningrum *et al*. 2014; Manzoor *et al*. 2020). Additionally, high molasses concentrations may be toxic to microalgae due to the presence of metal ions and colloidal substances, inhibiting their growth (Liu *et al*. 2012; Yin *et al*. 2019). These implications are significant for understanding the complex relationship between molasses concentration and cell growth.

The control treatment yielded the highest lipid accumulation, indicating that *Euglena gracilis* can naturally accumulate lipids without molasses. Lipid accumulation typically occurs under nutritional deficit conditions, while adequate nutrition allows energy allocation for cell division (Yew *et al*. 2020; Rubiyatno *et al*. 2021). Among molasses treatments, 20 g/L produced the most lipids, followed by 15 g/L, while 10 g/L resulted in the least. Increased turbidity from higher molasses concentrations likely obstructed light

penetration, inhibiting photosynthesis and creating stress (Manzoor *et al*. 2020). was confirmed by a decrease in growth rate at concentrations above 10 g/L.

Additionally, magnesium in molasses may enhance lipid accumulation, as shown in Chlorella studies (Kim *et al*. 2016; Ward and Rehmann 2019). Magnesium exerts significant regulatory control over fatty acid biosynthesis. As a cofactor for acetyl-CoA carboxylase and the pyruvate dehydrogenase complex, magnesium directly influences the flux of carbon towards fatty acid synthesis. Additionally, magnesium contributes to membrane stability, which is crucial for the intracellular transport of lipids and their precursors. The intricate interplay between magnesium concentration and enzymatic activity underscores its pivotal role in regulating lipid metabolism in microalgae (Nelson and Cox 2008). Although *Euglena* can naturally accumulate lipids without molasses, it can still enhance lipid production and increase paramylon accumulation, a starch-like compound convertible into valuable wax esters for biofuel production. This topic will be explored further in subsequent sections.

The addition of molasses did not affect carbohydrate accumulation in *Euglena* sp. in our study. A molasses concentration of 15 g/L gave the lowest results of all treatments and of the control. This is possible because the increase in medium turbidity due to the use of molasses can reduce the absorption of light for photosynthesis and the formation of carbohydrates. This finding aligns with previous research on the microalgae Scenedesmus dimorphus, which experienced growth inhibition when cultivated in a turbid bagasse medium (Manzoor *et al*. 2020). The high sulfur content in molasses can also cause a decrease in carbohydrate yield. In this research, tests showed that the molasses used contained high sulfur content. Previous research on the microalgae Chlorella and Scenedesmus showed that excess sulfur can inhibit cell growth (González-Camejo *et al*. 2017).

Euglena sp. cultivated in a molasses-based medium exhibited optimal paramylon production at a concentration of 10 g/L (Rubiyatno *et al*. 2021). Higher molasses concentrations led to decreased paramylon yield, potentially due to increased turbidity, which hinders photosynthesis (Manzoor *et al*. 2020). Additionally, the presence of toxic substances like sulfur in molasses may inhibit cell growth at elevated concentrations (Mera *et al*. 2014; González-Camejo *et al*. 2017). Ivušić and Šantek (2015) reported higher paramylon yields in *Euglena gracilis* strain Z pre-adapted to molasses and corn steep solid when cultivated in 30 g/L of each substrate. However, further increases in substrate concentration inhibited growth. Our study, lacking pre-adaptation, showed lower biomass and paramylon yields, highlighting the importance of this step for optimizing *Euglena gracilis* cultivation in molasses-based media.

In the control treatment, five lipid compounds were obtained: linolenic acid, stearic acid, palmitic acid, 11,14,17-eicosatrienoic acid, methyl ester, and Myristyl myristate. The compound from the control treatment is myristyl myristate. In the treatment group, two lipid compounds were obtained: palmitic acid and myristyl myristate. The most abundant compound was palmitic acid. This compound dominates in *Euglena* samples cultivated using molasses.

Euglena, when cultivated in a molasses medium, exhibited a distinct lipid profile compared to the control treatment (Yang *et al*. 2021). While linolenic acid and 11,14,17-Eicosatrienoic acid methyl ester, both polyunsaturated fatty acids (PUFAs), were detected in the control, they were absent in the molasses-treated *Euglena*. The absence of these PUFAs in the molasses-treated *Euglena* is likely attributed to the high sulfur content in molasses, which induces environmental stress (Yang *et al*. 2021). This stress leads to the formation of reactive oxygen species (ROS), which can degrade the double bonds in PUFAs, resulting in lipid peroxidation. Conversely, palmitic acid, a saturated fatty acid, was found at higher concentrations in the molasses-treated *Euglena*. This increase may be attributed to the increased energy demand associated with stress and the utilization of molasses as a carbon source (Zhukov 2015). These findings suggest that the use of molasses as a cultivation medium for *Euglena* can significantly alter its lipid profile, potentially impacting its suitability for various applications, such as biofuel production.

Euglena treated with molasses and control exhibited no significant difference in myristyl myristate or wax ester concentrations despite the increased paramylon accumulation observed in the molasses-treated group (Ogawa *et al*. 2022). This discrepancy may be attributed to the incomplete conversion of paramylon into wax ester during the fermentation process, which can be enhanced by the addition of specific chemicals (Ogawa *et al*. 2022). Moreover, the high sulfur content in molasses could be detrimental to wax ester production. *Euglena*, under sulfur-rich conditions, may produce hydrogen sulfide (H_2S) as a byproduct of cysteine

decomposition, a process aimed at obtaining additional energy (Giordano and Prioretti 2016; Yamada *et al*. 2019). H_2S can be toxic to *Euglena*, inhibiting wax ester production.

In conclusions, This research demonstrated that the addition of molasses significantly increased the growth of *Euglena* sp., particularly at a concentration of 10 g/L, which was identified as optimal for promoting cell proliferation. However, while molasses enhanced growth and paramylon accumulation, it did not lead to an increase in lipid accumulation or wax ester concentration. The lipid profile analysis revealed that molasses-treated cultures exhibited a reduced lipid profile compared to control conditions, with the absence of polyunsaturated fatty acids (PUFAs). These findings indicate that although molasses serves as an effective carbon source for enhancing biomass and paramylon production, further optimization is needed to improve wax ester yields. Future studies should explore additional strategies or nutrient combinations to balance growth and lipid production effectively, ensuring *Euglena* sp. can be utilized as a viable source for sustainable biofuel applications.

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