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Growth and biochemical responses of *Chlorella pyrenoidosa* to glucose under mixotrophic conditions

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ABSTRACT

Glucose added as an organic carbon source has been shown to significantly influence the growth and biochemical composition of *Chlorella pyrenoidosa* under mixotrophic conditions. Variations in glucose concentrations led to changes in cell metabolic patterns, which were reflected in growth performance and the accumulation of key biomolecules. Glucose supplementation at a concentration of 15 g/L showed the most optimal response, with a more than threefold increase in biomass compared to the control without glucose. This condition also accelerated the specific growth rate and decreased the cell doubling time by approximately 30%, indicating that cells were able to utilize energy from photosynthesis and glucose respiration simultaneously to power the division process. The higher availability of glucose directed cell metabolism toward the formation of storage compounds. This was indicated by a decrease in protein levels to approximately 35% of the initial value in the control, indicating a diversion of metabolic resources from protein synthesis to the production of storage compounds. Conversely, lipid levels increased almost twofold at higher glucose concentrations, reflecting the microalgae's adaptive response of storing excess carbon in the form of fatty acids. Meanwhile, total sugar levels showed an increase pattern at low glucose concentrations, before decreasing again at high concentrations in line with the metabolic shift towards lipid formation. Overall, the results of this study confirm that the addition of glucose can increase growth efficiency and shift the metabolic direction of *Chlorella pyrenoidosa* towards the accumulation of high-value biomolecules. This provides a strong scientific basis for the use of glucose as an organic carbon source to optimize the production of biomass and important metabolites in mixotrophic microalgae culture systems.

Keywords: metabolism, nutrition, cultivation, carbon, productivity.

INTRODUCTION

As single-celled photosynthetic organisms, microalgae play a significant role in the development of modern biotechnology through their ability to produce various high-value compounds that can be utilized as sustainable food, feed, and bioenergy raw materials (Wang *et al*., 2024). In addition to their potential to provide an environmentally friendly alternative source of bioresources, microalgae are also attracting attention due to their ability to effectively utilize sunlight and nutrients, their rapid growth rate, and their high adaptability to various environmental conditions. One widely studied microalgae species is *Chlorella pyrenoidosa*, known for its rapid growth and high biochemical content, approximately 52.4% protein, 19.4% lipid, and 9.0% polysaccharide (Chen *et al*., 2022), making it potentially suitable for applications in the feed, pharmaceutical, and functional food industries.

The biochemical composition of *C. pyrenoidosa* is strongly influenced by environmental factors, particularly the availability of carbon sources in the culture medium. Under photoautotrophic conditions, microalgae growth depends on photosynthesis, which is often limited by the availability of inorganic carbon. Therefore,

mixotrophic systems that combine CO_2 uptake and organic carbon utilization have become an effective strategy for increasing growth efficiency (Yun, 2021). Zhang *et al*. (2014) reported that the addition of monosaccharides such as glucose and galactose significantly increased cell density and accelerated the exponential phase of *C. pyrenoidosa* compared to photoautotrophic cultures. Similar results were found by Yang *et al*. (2025), where the addition of acetate to Chlorella sorokiniana increased biomass by up to 48% and shortened the growth lag phase by activating the glycolysis and TCA cycle metabolic pathways.

Organic carbon sources not only function as electron donors in metabolic processes but also enhance photosynthetic activity and enzymatic efficiency, which play a role in biomass formation. Sharma *et al*. (2016) showed that mixotrophic Chlorella cultures supplemented with glucose, glycerol, or acetate increased lipid production by up to 3.5 times compared to control cultures without additional carbon. This demonstrates that microalgae have the flexible ability to utilize external carbon to accelerate growth and biomass accumulation.

The concentration of organic carbon in the culture medium can influence the growth and biochemical composition of microalgae (Sun *et al*., 2018). At optimum levels, the addition of glucose can enhance growth and biomass accumulation, but excess carbon can disrupt the metabolic balance of cells (Sharma *et al*., 2025). Variations in glucose concentration also influence the direction of metabolism, where increased carbon availability drives a shift from protein synthesis to the formation of energy-storing compounds such as carbohydrates and lipids (Kong *et al*., 2013). Increasing glucose concentration is known to decrease protein content and simultaneously increase carbohydrate and lipid accumulation in microalgae (Chai *et al*., 2018).

Thus, variations in glucose concentration not only affect the specific growth rate (SGR) and doubling time (DT), but can also alter the proportions of key biochemical components such as protein, lipids, and total sugars. Therefore, this study aims to analyze the effect of varying glucose concentrations on the growth, SGR, DT, and biochemical composition of *Chlorella pyrenoidosa*, including protein, lipid, and total sugar levels. The results are expected to provide a scientific basis for optimizing mixotrophic cultures to increase biomass productivity and high-value metabolite content in microalgae.

MATERIAL AND METHODS

Strains and cultivation media

C. pyrenoidosa was obtained from the Brackish Water Cultivation Center in Jepara, Indonesia. A 400 mL stock of microalgae was cultured in 500 mL Erlenmeyer flasks, with temperature and light intensity set at 25 °C and 5000 lux. The medium used for microalgae culture was BG-11 (Stainer, 1971).

Experimental culture conditions

The experiment was conducted using BG-11 base media with glucose addition as a carbon source at concentrations of 5, 10, 15, 20, and 25 g/L. *C. pyrenodoisa* (diluted to approximately 0.3 at OD 680, 30 mL) was inoculated into 300 mL of culture media in a 500 mL glass bottle. The light was set at 5000 lux and the light-dark cycle was 16:8 hours, and the temperature was set at 25 °C (Yun, 2021).

Growth analysis

The growth of *C. pyrenoidosa* cells was determined by measuring the optical density (OD) at a wavelength of 680 nm using a spectrophotometer (GENESYSTM 20 UV-Vis, Thermo Scientific, USA) every 24 hours (Fakhri *et al*., 2025). OD data were used to calculate the SGR and doubling time (Td) with the following formula:

$$SGR\ (\text{day}^{-1}) = \frac{\ln(N_t) - \ln(N_0)}{\Delta t} \quad (1)$$

$$T_d\ (\text{day}) = \frac{\ln 2}{\mu} \quad (2)$$

where: N_t – optical density at time t, N_0 – initial optical density, Δt – time interval between N_0 and N_t (day), μ – specific growth rate (day^{-1}), T_d – doubling time (day).

Biomass analysis

Biomass measurements were performed using GF/C filter paper (Whatman, 1.2 µm). A 25 ml sample of each microalgae culture was taken

and filtered using filter paper and rinsed twice with distilled water. The filter paper was dried at 105 °C for 2 hours and cooled in a desiccator for 30 minutes. The filter paper was weighed for its initial weight, and biomass was expressed in grams per liter (g/L) (Fakhri *et al.,* 2021).

Lipid analysis

Lipid observations were conducted using a modified method of Bligh and Dyer (1959). 30 mg of dry biomass was ground using a hammer mill and a mixture of chloroform: methanol (2:1, v/v) was added. 0.8 mL of 0.73% NaCl was used to separate the two liquid phases. Then, centrifugation was carried out for 2 minutes at 2000 rpm. Transfer the chloroform phase (bottom) to a weighed glass bottle. Repeated extractions were carried out to increase lipid yield. The chloroform solution containing lipids in the glass was dried using nitrogen gas (N2), then oven at 65 °C and a desiccator for 30 minutes. The following is the calculation formula for lipid content:

$$\text{Lipid content (\%DW)} = \frac{\text{M3} - \text{M2}}{\text{M1}} \times 100\% \quad (3)$$

where: M_1 – dry weight of the sample (mg),
M_2 – weight of the empty glass vial (mg),
M_3 – weight of the glass vial containing lipids after drying (mg)

Protein analysis

Protein content was analyzed using the method of Lowry *et al*. (1951) using reagents A (5% Na_2CO_3), B (1% $CuSO_4 \cdot 5H_2O$), C (2% $NaKC_4H_6O_6 \cdot 4H_2O$), and reagent D (a mixture of 50 mL A, 1 mL B, and 1 mL C). Microalgae samples (0.5 mL) were added with 0.5 mL of 1 N NaOH, heated for 10 minutes at 100 °C, and then cooled. After that, 2.5 mL of reagent D was added, homogenized and left for 10 minutes, then 0.5 mL of Folin–Ciocalteu reagent was added and left for 30 minutes at room temperature. Absorbance was measured at 750 nm, and protein content was determined based on a standard curve of BSA (2 mg/mL).

Total sugar analysis

Polysaccharide content was analyzed using the anthrone–sulfuric acid method according to Babich *et al*. (2024). A 0.5 mL sample was mixed with 2.5 mL of freshly prepared anthrone reagent (0.1 g anthrone in 100 mL of 98% concentrated H_2SO_4). The mixture was cooled for 10 minutes at 4 °C, then heated at 100 °C for 20 minutes in a water bath. After cooling to room temperature for 20 minutes, the absorbance was measured at 620–630 nm using a spectrophotometer.

Data analysis

The data obtained were analyzed statistically using the one-way ANOVA method, then continued with the Duncan test using SPSS version 25. Data that were significantly different were indicated by a p-value < 0.05.

RESULTS AND DISCUSSION

The effect of glucose on the growth of *C. pyrenoidosa*

The growth of *Chlorella pyrenoidosa* in BG11 medium with added glucose showed that the treatment with 15 g/L glucose resulted in the highest growth, with an OD value of 5.133 (λ 680 nm) and a dry biomass of 1.6 g/L. This value was significantly higher than the control (without added glucose), which only achieved an OD of 1.223 (λ 680 nm) and a biomass of 0.54 g/L. This significant difference indicates that glucose supply can enhance the growth of *C. pyrenoidosa* cells. Mixotrophic growth involves two distinct processes: photosynthesis and aerobic respiration. Photosynthesis is influenced by light intensity, and aerobic respiration is related to the concentration of organic substrate (glucose) (Kong *et al*., 2013) (Figure 1).

However, higher glucose concentrations do not always have a positive impact. At doses of 20 g/L and 25 g/L, growth rates and biomass accumulation decreased. This can be attributed to the excessive osmotic effect in the culture medium, thus inhibiting cell metabolic activity. The results obtained indicate an optimum glucose concentration limit to support the growth of *C. pyrenoidosa.* A concentration of 15 g/L proved to be the most effective dose, as it was able to increase biomass more than threefold compared to the control. Meanwhile, higher concentrations did not provide additional increases, but instead decreased productivity. This is because higher glucose concentrations reduce microalgae

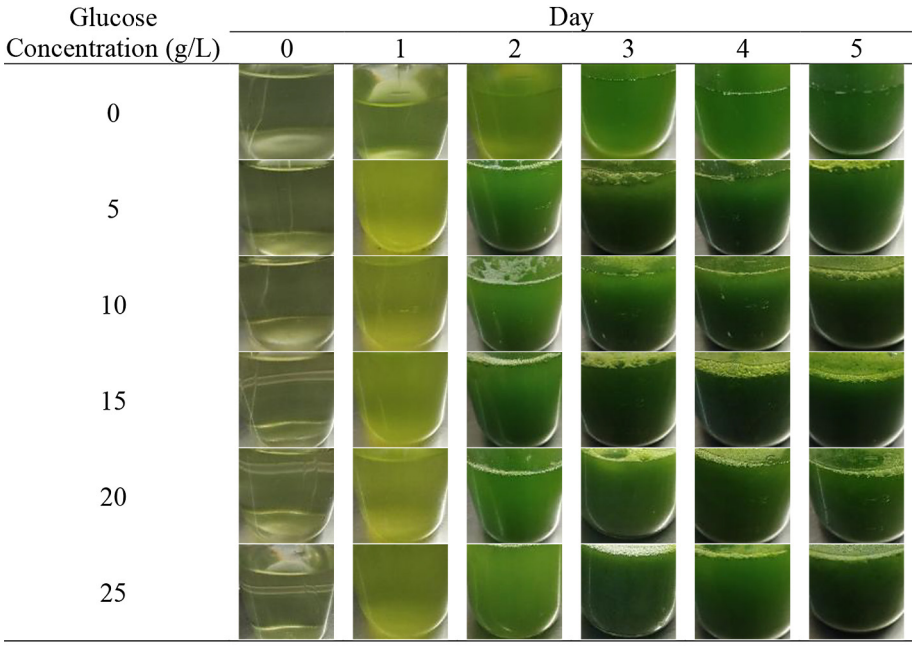

Figure 1. Comparative culture color of *C. pyrenoidosa* under different glucose treatments

growth, with a decrease in specific growth rate and photosynthetic pigment production (Maia *et al*., 2025) (Figure 2).

The specific growth rate (SGR) and doubling time (DT) of *Chlorella pyrenoidosa* at various glucose concentrations are shown in Table 1. In general, glucose supplementation in BG-11 medium significantly affected the microalgae's growth rate. The treatment with 15 g/L glucose supplementation showed the highest SGR of 0.98 days, with the fastest doubling time of 0.71 days, significantly higher than the control 0 g/L, which had an SGR of only 0.71 days and a doubling time of 0.98 days. This indicates that the supply of organic carbon in the form of glucose can accelerate cell division and increase the growth efficiency of *C. pyrenoidosa*.

The increase in SGR values in the 10–15 g/L treatment indicates optimal metabolic conditions in the mixotrophic system, where the microalgae utilize energy from photosynthesis and glucose respiration simultaneously. Increasing the organic carbon source has been shown to accelerate carbon assimilation and increase the rate of cell division to reach an optimum point (Zhang *et al*., 2014). However, at higher glucose concentrations 20–25 g/L, an unbalanced C:N ratio can cause osmotic stress and metabolic inhibitory effects that reduce growth rates (Lacroux *et al.,* 2021).

Research by Fakhri *et al*. (2025) showed that under phosphorus stress conditions, *Chlorella pyrenoidosa* experienced increased growth at optimum phosphorus levels (around 27.8 mg/L), but its growth declined when phosphorus levels were too high. Excess nutrients such as phosphorus or carbon can cause an imbalance in the C:N:P ratio, which results in decreased biomass formation and photosynthetic enzyme activity.

Protein level of *C. pyrenoidosa*

The results of the protein content analysis of *C. pyrenoidosa* at various glucose concentrations showed that protein levels decreased with increasing glucose concentration in BG-11 media. The highest average protein content was 49.53% in the treatment without glucose 0 g/L, while the lowest protein content was found in the 25 g/L glucose treatment, at 32.95%. In general, there was a significant decrease in protein levels from the 5 g/L to the 25 g/L treatment, with values ranging from 38.89–32,95%.

The decrease in protein levels at high glucose concentrations indicates that cellular metabolism shifts from protein formation to the accumulation of reserve compounds such as lipids and carbohydrates. Under conditions with excess carbon availability, cells utilize more energy and intermediate compounds to form fat as a reserve energy source (Ren *et al*., 2016). Furthermore, Fan *et al*. (2015) also explained that during the heterotrophic phase of *Chlorella pyrenoidosa*, the

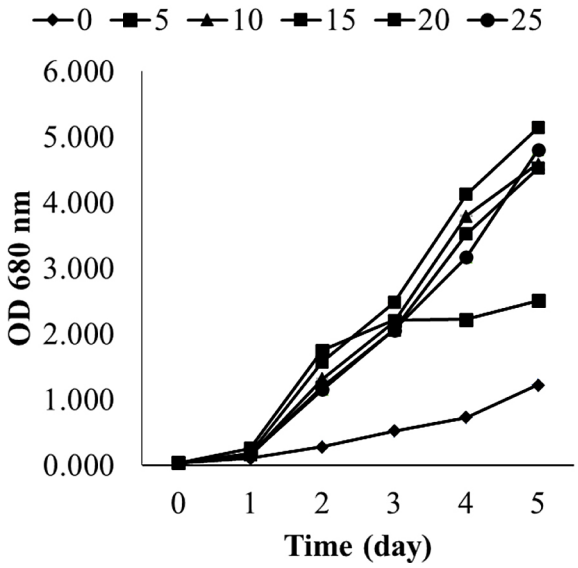

Figure 2. Optical density (OD_{680}) of *Chlorella pyrenoidosa* cultured for five days under different glucose concentrations

activity of genes involved in fatty acid synthesis and lipid metabolism increases, while pathways related to protein synthesis decrease. This suggests that excess glucose can trigger a shift in cellular metabolism toward the formation of energy storage compounds.

Total lipid levels of *C. pyrenoidosa*

The total lipid content of *Chlorella pyrenoidosa* increased with the addition of glucose to BG11 media. The results showed that the treatment with the highest glucose dose, 25 g/L, produced the highest total lipid content at 39.16%. The higher the glucose dose given, the higher the lipid content accumulated in the cells. This is in contrast to the control treatment that did not receive additional glucose, where the lowest total lipid content reached only 19.76%. The increase in lipids in the glucose treatment likely occurred due to the availability of an additional carbon source that can be used by the cells for the synthesis of fatty acids and secondary metabolites.

With the supply of glucose, *C. pyrenoidosa* cells were able to utilize additional energy to accelerate lipid accumulation, resulting in a higher lipid content compared to the control. This is because microalgae cultures in growth media supplemented with complex sugars can increase lipid yields (Okoro *et al.,* 2025). This is in line with the statement that the addition of glucose showed the highest increase in lipid content (31.87%), surpassing autotrophic conditions (24.80%) (Li *et al*., 2024).

Total sugar content of *C. pyrenoidosa*

The glucose treatment resulted in significantly different total sugar levels compared to the control, but no significant differences between glucose treatments. The results showed that the highest total sugar levels were found at the lowest glucose dose, 5 g/L, reaching 61.87 mg/g. Furthermore, carbohydrate levels decreased with increasing glucose doses. The lowest total sugar levels were found in the control without glucose addition, at 46.93 mg/g. In contrast to lipid levels, which increased with increasing glucose doses, the total sugar levels of *C. pyrenoidosa* showed a different pattern. This indicates that low-dose glucose addition can stimulate an increase in carbohydrates in cells, while at high doses, glucose is utilized more for lipid synthesis. This condition occurs because *C. pyrenoidosa* cells will direct carbon utilization according to their metabolic needs.

Too high a glucose dose tends to increase lipid accumulation, resulting in decreased carbohydrate levels (Costa *et al.*, 2021). Thus, low-dose glucose administration can increase carbohydrate content, while higher doses have a greater effect

Table 1. Growth and biochemical responses of *Chlorella pyrenoidosa* to glucose addition

Glucose concentration (g/L)	Biomass (g)	Specific growth rate (day^{-1})	Doubling time (day)	Protein (%)	Lipid (%)	Total sugar (mg/g)
0	0.54 ± 0.11^{a}	0.71 ± 0.02^{a}	0.98 ± 0.03^{b}	49.53 ± 4.46^{c}	19.76 ± 0.5^{4}a	46.93 ± 1.40a
5	0.84 ± 0.29^{ab}	0.80 ± 0.13^{a}	0.89 ± 0.16^{b}	38.89 ± 2.6^{4}b	25.30 ± 1.52^{b}	61.87 ± 1.9^{0}b
10	1.33 ± 0.30^{bc}	0.95 ± 0.02^{b}	0.73 ± 0.01^{a}	36.77 ± 2.66^{ab}	38.16 ± 1.3^{c}	60.67 ± 1.65^{b}
15	1.60 ± 0.34^{c}	0.98 ± 0.05^{b}	0.71 ± 0.04^{a}	36.24 ± 1.63^{ab}	38.50 ± 1.51^{c}	59.17 ± 3.55^{b}
20	1.36 ± 0.36^{bc}	0.95 ± 0.03^{b}	0.73 ± 0.02^{a}	33.68 ± 3.10^{ab}	38.83 ± 0.21^{c}	58.80 ± 2.50^{b}
25	1.38 ± 0.43^{bc}	0.97 ± 0.02^{b}	0.71 ± 0.02^{a}	32.95 ± 2.00^{a}	39.16 ± 1.3^{9}c	57.33 ± 3.01^{b}

Note: Different superscripts indicate significantly different treatments ($p<0.5$)

on increasing lipids. Therefore, changes in nitrogen concentration can affect growth rates, as well as protein, lipid, and carbohydrate synthesis in microalgae (Zarrinmehr *et al*., 2020).

CONCLUSIONS

Glucose concentration of 15 g/L resulted in the highest growth and lipid accumulation in *Chlorella pyrenoidosa* under mixotrophic conditions. Excessive glucose concentrations reduced growth and protein content due to a metabolic shift toward lipid and carbohydrate formation.

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