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Mixotrophic culture of *Monoraphidium braunii* using glucose on the increase of biomass, protein, and lipid productivity

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ABSTRACT

In this study, the effect of exogenous addition of glucose on the growth, biomass, pigment content, protein, and lipid productivity of green microalgae *M. braunii* under mixotrophic culture was investigated. The microalgae were cultivated in a batch system under four different glucose concentrations, including 0 g L^{-1} (photoautotrophic conditions, control), 0.1 g L^{-1}, 0.3 g L^{-1}, and 0.5 g L^{-1} (mixotrophic conditions). The light intensity of 200 µmol photon m^{-2} s^{-1} under constant illumination was applied in this study. The obtained findings indicated that glucose-supplemented cultures under mixotrophic conditions produced higher growth and biomass productivity than the photoautotrophic culture. The maximum specific growth rate (0.64 d^{-1}) and biomass productivity (188.94 mg L^{-1} d^{-1}) were attained at 0.5 g L^{-1} glucose, which was 66.7% and 22.4% higher than the glucose-free cultures. The highest Chl *a*+*b* content was observed at 0.3 g L^{-1}, while carotenoid and protein content increased along with glucose concentration. The maximum protein productivity of 64.13 mg L^{-1} d^{-1} and lipid productivity of 33.92 mg L^{-1} d^{-1} were obtained at 0.5 g L^{-1} glucose, which was 1.5- and 1.3-times higher than the control. This study demonstrates that glucose supply under mixotrophic systems is an effective strategy to enhance the protein and lipid productivity of *M. braunii,* which could potentially be applied for large-scale cultivation.

Keywords: green microalgae, lipid, mixotrophic, photoautotrophic, protein, specific growth rate.

INTRODUCTION

Microalgae are unicellular microorganisms capable of fixing carbon dioxide into biomass and high-value chemical products, including pigments, lipids, and proteins (Udayan et al., 2022). Microalgae have attracted a lot of attention as a potential raw material for the production of bioenergy (Ananthi et al., 2021), pharmaceuticals, human food, and animal feed due to the increased demand for sustainable and renewable resources worldwide (Santin et al., 2021). Moreover, the ability of microalgae to capture carbon dioxide and then transform it into biomass as well as products of interest is greater than that of higher plants. They are also desirable for economic exploitation because of their lower needs for freshwater and arable land (Sun et al., 2018).

Low biomolecule content in microalgae frequently results from the conditions that promote high growth. Since they have a direct impact on the profitability of the microalgae-based industry, biomass, protein, and lipid productivity are important metrics in microalgal production (Thiviyanathan et al., 2024). Therefore, simultaneous enhancement of growth, biomass, protein, and lipid productivity is of great interest for improving the overall efficiency and sustainability of

microalgal biorefineries. Several strategies have been implemented to enhance the productivity of biomolecules, including metabolic engineering, improving culture conditions (Jaeger et al., 2017), and supplementation of organic carbon sources into medium (Fakhri et al., 2021).

Microalgae are mostly cultivated in a photoautotrophic culture, in which cells carry out the process of photosynthesis to produce biomass. However, a self-shading mechanism that reduces light penetration into culture media and low carbon fixation rates would ultimately restrict the growth and biomass production of microalgae (Cheirsilp and Torpee, 2012; Kim et al., 2013). An effective strategy for addressing low biomass production in autotrophic systems is cultivating microalgae under mixotrophic conditions. In a mixotrophic culture, microalgae simultaneously assimilate CO_2 and organic carbon as well as undergo anabolic and catabolic processes (Chojnacka and Marquez-Rocha, 2004). Some studies reported that mixotrophic systems enhance the growth and protein content (Morais et al., 2019), as well as increase the photosynthetic pigment (Deng et al., 2019) and lipid productivity of microalgae (Kong et al., 2013).

Glucose is one of the carbon sources extensively studied for its potential to increase microalgae productivity under mixotrophic conditions, because it is easily metabolized and generates higher energy per mole than other organic carbon sources such as acetate and glycerol (Sun et al., 2018). In addition to serving as an extra carbon source for biosynthesis and energy, glucose also functions as a signaling molecule that controls the metabolic processes related to the synthesis of proteins and lipids. Recent research has shown that a mixotrophic culture with glucose supplementation considerably increased the protein (Fakhri et al., 2021) as well as lipid content and productivity of green microalgae (Kong et al., 2013).

Monoraphidium braunii, a freshwater green microalga, has drawn the most attention among the many microalgal species because of its rapid growth, versatility under a range of cultivation conditions, and capacity to accumulate significant amounts of lipids and proteins (Bogen et al., 2013). Because of these traits, *M. braunii* is a potential choice for producing high-value bioproducts on a large-scale production. Due to limited research on the mixotrophic growth of *M. braunii* particularly the effect on the enhancement of both protein and lipid productivity remains poorly understood. Therefore, further research is needed to bridge this gap and determine the optimal parameters for the mixotrophic growth of *M. braunii*. While other substrates such as glycerol and acetate have also shown potential to enhance microalgal productivity (Nzayisenga et al., 2018; Joun et al., 2023), this study focused specifically on glucose to establish baseline information for *M. braunii*. In this study, the influence of glucose supplementation on the growth, biomass, and pigment content of *M. braunii* was evaluated. Importantly, it was determined whether glucose addition improves the protein and lipid productivity in *M. braunii*.

MATERIALS AND METHODS

Algal strain, medium, and pre-culture conditions

M. braunii SAG 48.87 was purchased from the Culture Collection of the University of Göttingen, Germany. The cells were maintained in the BG-11 medium (Stanier et al., 1971). For preculture, *M. braunii* was incubated in a 1.000 mL Erlenmeyer flask containing 700 mL of media at temperature, light intensity, and light:dark cycle of 25 °C, 200 µmol photon m^{-2} s^{-1}, and 24L:0D, respectively. The cultures were grown for 4 days to reach the logarithmic growth phase.

Experimental setup

Four glucose concentrations: 0 g L^{-1} (control, photoautotrophic culture), 0.1 g L^{-1}, 0.3 g L^{-1}, and 0.5 g L^{-1} were applied to the BG-11 medium to investigate the roles of glucose on the growth and biochemical profiles of *M. braunii*. All media were autoclaved at 121 °C for 15 minutes. The algal cells were grown under a batch system in a 500 mL bottle containing 350 mL BG-11 media with an initial optical density (OD_{730}) of 0.12 or an equivalent cell density of approximately 5 × 10^5 cells mL^{-1}. The cultures were incubated at 25 °C under continuous illumination (200 µmol photon m^{-2} s^{-1}) using white LED tube lamps. The cultures were shaken manually two times a day to prevent the accumulation of cells in the bottom part. The experiment was performed for 5 days. Samples were collected daily for growth analysis and at the end of the experiment for biomass, pigment, protein, and lipid analysis.

Determination of microalgal growth and biomass

M. braunii cultures were sampled daily under aseptic conditions for the quantification of microalgal growth. The absorbance (A) of samples was measured at 730 nm (OD_{730}) (Fakhri et al., 2024) using a spectrophotometer (GENESYS™ 20 UV-Vis, Thermo Scientific, USA). The specific growth rate (μ, d^{-1}) of microalgae was estimated using the following formula.

$$\mu(d^{-1}) = \frac{Ln(Nt) - Ln(N0)}{\Delta t} \quad (1)$$

where: Nt – optical density at 730 nm at the end of experiment, $N0$ – optical density at 730 nm at the beginning of cultivation, Δt – the duration of cultivation.

For biomass determination, 25 mL of algal samples were collected at the end of the experiment. After filtering the suspensions using a GF/C filter paper (Whatman, USA), they were washed twice with distilled water and dried for two hours at 105 °C until a constant weight was achieved. After cooling, the filter paper was placed in a desiccator for half an hour, and the weight was recorded. The dry weight of the microalgae was then calculated and expressed in g L^{-1} (Fakhri et al., 2021). Biomass productivity was calculated by the following formula.

$$Biomass\ productivity\ (mg\ L^{-1}\ d^{-1}) = \frac{W2 - W1}{\Delta t} \quad (2)$$

where: $W1$ and $W2$ are defined as the biomass at the beginning and at the end of cultivation, respectively.

Carotenoid and chlorophylls analysis

Carotenoids and chlorophylls (Chl *a*+*b*) were extracted using 90% methanol (*v*/*v*). The wet biomass from 2 mL of *M. braunii* cultures was collected by centrifugation for 15 min at 4,000 rpm. The sample was sonicated at 40 kHz for 10 min after 2 mL of 90% (*v*/*v*) methanol was added to the wet biomass. After 10 min of heating at 70 °C in a water bath, the tubes were centrifuged at 4.000 rpm for 15 min. The supernatant was measured spectrophotometrically (GENESYS™ 20 UV-Vis) at an absorbance of 665, 652, and 480 nm. The amount of Chl *a*+*b* and carotenoid were calculated based on the formula from (Ritchie, 2006) and (Kim et al., 2014), respectively.

Determination of protein

Microalgal cells were harvested at the end of the culture period. A 0.5 mL of *M. braunii* cells was used for protein analysis. The protein content of *M. braunii* was determined using the colorimetric method (Lowry et al., 1951). Bovine serum albumin was applied as a standard and the absorbance of standard and sample was measured at 750 nm. Protein concentration in the sample was expressed as μg mL^{-1}. Protein content and productivity were estimated according to the following formula:

$$Protein\ content\ (\%) = \frac{Protein\ concentration \times Volume\ of\ sample}{Microalgal\ biomass} \times 100\% \quad (3)$$

$$Protein\ productivity\ (mg\ L^{-1}\ d^{-1}) = Biomass\ productivity \times Protein\ content \quad (4)$$

Determination of lipid

The lipids from dried biomass were extracted as described in Bligh and Dyer (1959) with minor modifications. Initially, the dried material was crushed into a very smooth powder using a pestle and mortar. A 3 mL of extraction reagent (chloroform/methanol 2:1, v/v) was added to algal powder (30 mg) (M1). The samples were vortexed for 30 sec. To achieve a methanol:chloroform:water ratio of (2:1:0.8), 0.73% (w/v) NaCl (0.8 mL) was then added to the solution. Phase separation was facilitated by centrifugation at 2000 rpm for 2 min to form two layers. The upper layer was removed, and the bottom layer was placed into a constant-weight vial (M2). The extraction protocol was repeated, and the chloroform layer was combined with the initial extract. The solution was dried in an oven set at 65 °C after the collected supernatant was evaporated with N_2 gas. Finally, the vial was desiccated for 30 min (M3) until a constant weight was obtained (M3). Lipid content and productivity of *M. braunii* were calculated as follows:

$$Lipid\ content\ (\%\ DW) = \frac{M3 - M2}{M1} \times 100\% \quad (5)$$

$$Lipid\ productivity\ (mg\ L^{-1}\ d^{-1}) = Biomass\ productivity \times Lipid\ content \quad (6)$$

Statistical analysis

All treatments were performed in three replicates and data were analyzed using one-way analysis of variance and Duncan's test to evaluate

significant differences ($p < 0.05$) between treatments. Statistical analysis was evaluated using IBM SPSS version 27.

RESULTS AND DISCUSSION

Glucose addition stimulated growth and biomass productivity during mixotrophic culture

Figure 1A and Table 1 showed the OD_{730} values and specific growth rate of *M. braunii* cultured under different glucose concentrations under mixotrophic conditions, respectively. The results showed that increasing glucose concentrations positively influenced OD_{730} and the growth rate of *M. braunii*. The growth increased progressively in all treatments, with notable differences observed between the glucose-supplemented and glucose-free cultures. This alga grew slowly in the photoautotrophic cultures, with a low OD_{730} of 0.81 and a specific growth rate of 0.38 d^{-1}, after five-day cultivation. Specifically, the cultures supplemented with 0.5 g L^{-1} glucose exhibited the highest OD_{730} values (1.032) and specific growth rate (0.64 d^{-1}), indicating enhanced cell growth. This finding aligns with Morowvat and Ghasemi (2016), who reported that the growth of *Dunaliella salina* was remarkably higher in the media supplemented with glucose under mixotrophic culture than that of the photoautotrophic culture.

As shown in Figure 1B and Table 1, the trend for biomass yield and productivity was comparable to the growth profile. *M. braunii* showed little growth in the absence of glucose (0 g L^{-1}), and by the end of the cultivation period, its final biomass concentration and productivity were 0.82 g L^{-1} and 154.42 mg L^{-1} d^{-1}, respectively. These results are consistent with prior investigations that showed the availability of inorganic carbon sources in the medium limits the growth of microalgae in the autotrophic system (Stegemüller et al., 2024; Yang et al., 2024). On the other hand, the cultures that received glucose addition showed noticeably greater biomass yields. With

Table 1. Specific growth rate (μ) and biomass productivity of *M. braunii* under various glucose supplementation in mixotrophic conditions

Glucose (g L^{-1})	μ (d^{-1})	Biomass productivity (mg L^{-1} d^{-1})
0.0	0.381±0.181^{a}	154.42±6.02^{a}
0.1	0.427±0.074^{a}	159.10±11.34^{ab}
0.3	0.538±0.021^{ab}	173.87±4.22^{bc}
0.5	0.636±0.071^{b}	188.94±10.43^{c}

Note: All parameters were tested in triplicate. Different superscript letters indicate significant difference ($p < 0.05$).

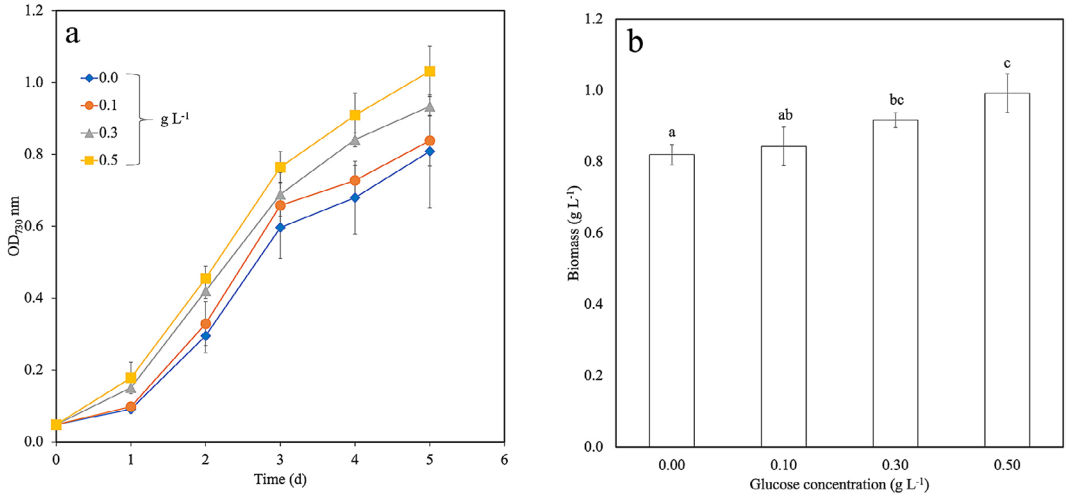

Figure 1. (a). Optical density at 730 nm (OD730); and (b). Biomass yield of *M. braunii* under various glucose concentrations. Error bars correspond to standard error of the mean. All parameters were tested in triplicates. Different letters indicate significant difference ($p < 0.05$) of biomass concentration

an average yield and productivity of 0.99 g L^{-1} and 188.94 mg L^{-1} d^{-1}, respectively, the highest biomass was recorded at 0.5 g L^{-1} glucose. This was significantly higher than that of the control ($p < 0.05$). This study highlights how important glucose is as a carbon source for improving the biomass of *M. braunii* under mixotrophic conditions. These results align with Deng et al. (2019) who reported that glucose addition induced biomass accumulation in *C. kessleri.* Similarly, Cheirsilp and Torpee (2012) found a significant increase in biomass yield of *Chlorella* sp. and *Nannochloropsis* sp. was observed when glucose was supplied in the medium.

Effect of glucose on photosynthetic pigment content

In this study, Chl *a+b* and carotenoid were analyzed to evaluate the influence of glucose addition on the photosynthetic pigment of *M. braunii*. As shown in Figure 2A, photoautotrophic culture produced the lowest Chl *a+b* content of 25.07 µg mL^{-1}, which was no significant difference ($p > 0.05$) with 0.1 g L^{-1} glucose. Interestingly, when 0.3 g L^{-1} glucose was added, the amount of chlorophyll enhanced significantly ($p < 0.05$), reaching a peak of 36.11 µg mL^{-1}. This result suggests that glucose is essential for chlorophyll synthesis in *M. braunii*. These results are in line with earlier research that showed glucose can increase the production of chlorophyll by supplying more carbon skeletons and energy for the manufacture of pigments (Shugarman and Appleman, 1966). The observed increase in chlorophyll content indicates that supplementing with glucose not only promotes growth but also improves the microalgal ability to photosynthesize. It is interesting to note that the Chl *a+b* content dropped by 11.4% when glucose was increased to 0.5 g L^{-1} as opposed to 0.3 g L^{-1}. These findings align with previous investigations that reported high glucose concentration inhibited the synthesis of chlorophyll in *Nannochloropsis* sp. (Cheirsilp and Torpee, 2012) and *C. vulgaris* (Kong et al., 2013). A change from autotrophic to mixotrophic metabolism, where light dependency diminishes, is suggested by the decrease in chlorophyll content at high glucose levels. Moreover, increased availability of organic carbon has been shown to limit photosynthetic activity, which in turn reduces the synthesis of pigments in microalgae (Kong et al., 2013).

Similar to chlorophyll, carotenoid concentrations increased significantly in the cultures treated with glucose, as seen in Figure 2B. The amount of carotenoid found in the 0.1 g L^{-1}, 0.3 g L^{-1}, and 0.5 g L^{-1} glucose concentrations was 4.12 µg mL^{-1}, 5.01 µg mL^{-1}, and 5.14 µg mL^{-1}, respectively, whereas the control treatment (0 g L^{-1} glucose) produced a carotenoid content of 3.84 µg mL^{-1}. Similarly, Anahas et al. (2024) discovered that adding glucose to the medium significantly increased the carotenoid content of *Anabaena sphaerica*. Higher levels of glucose result in an increase in carotenoid content, highlighting the

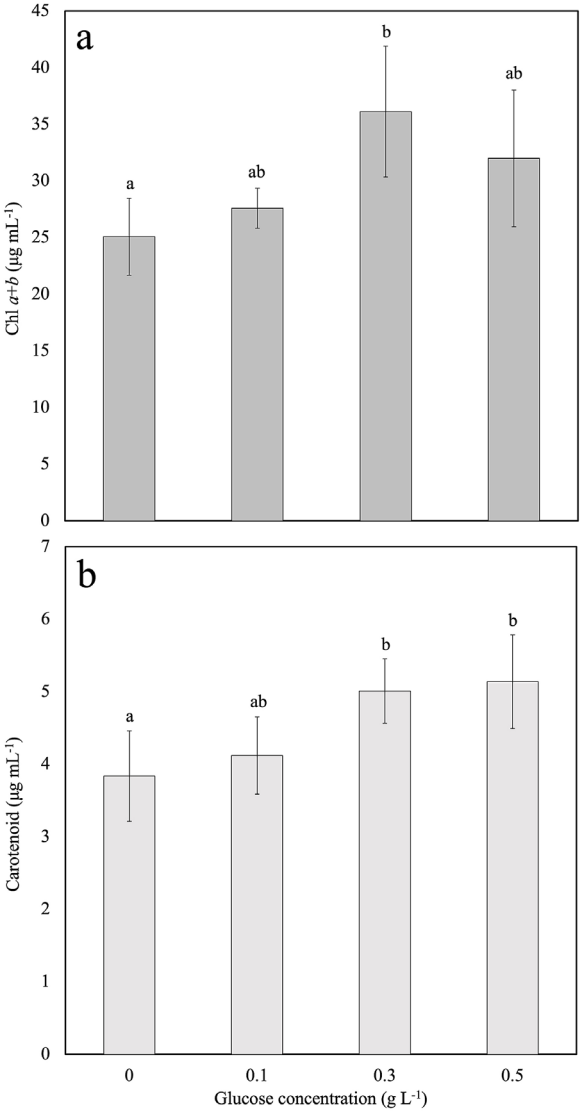

Figure 2. (a). Chl a+b; and (b). Carotenoid content of *M. braunii* under various glucose concentrations. Error bars correspond to standard error of the mean. All parameters were tested in triplicates. Different letters indicate significant difference ($p < 0.05$) of Chl a+b and carotenoid content

function of glucose in promoting the synthesis of secondary metabolites (Zhang et al., 2019). Ryu et al. (2004) suggested that glucose may increase pigment accumulation by upregulating the expression of genes involved in carotenoid production.

Effect of glucose on protein content and productivity

To assess how glucose availability in the presence of light affects cellular protein production, the protein content of *M. braunii* at various glucose concentrations was examined. Figure 3 showed that protein content positively correlated with increasing the amount of glucose. The cultures supplemented with 0.5 g L^{-1} glucose had the largest protein content of 34.01% ($p < 0.05$), while the photoautotrophic group (0 g L^{-1} glucose) had the lowest protein content of 28.11%. This study suggests that when glucose availability increases, protein synthesis gradually improves.

The conducted study also demonstrated that protein productivity increased significantly in the cultures treated with glucose, following a similar pattern to protein content (Figure 3). The protein productivity of the 0.1 g L^{-1}, 0.3 g L^{-1}, and 0.5 g L^{-1} treatments was 49.19 mg L^{-1} d^{-1}, 57.06 mg L^{-1} d^{-1}, and 64.13 mg L^{-1} d^{-1}, respectively. In contrast, the control treatment (0 g L^{-1} glucose) produced 43.40 mg L^{-1} day^{-1}. Increased glucose concentrations result in increased protein production, indicating the double function of glucose in promoting protein accumulation and biomass growth. These findings are in line with earlier research which found that supplementing microalgae with glucose increased their protein productivity under mixotrophic conditions (Fakhri et al., 2021). This result indicates that glucose can be utilized to maximize protein synthesis in *M. braunii*, which makes it a potential option for use in the food and feed industries.

Effect of glucose on lipid content and productivity

The lipid content and productivity of *M. braunii* cultured in various glucose concentrations are shown in Figure 4. The finding showed that the lowest lipid content of 16.89% DW was obtained in the photoautotrophic culture (0 g L^{-1} glucose). A slight increase was observed in the cells supplemented with glucose with the highest lipid content of 19.44% DW obtained at 0.3 g L^{-1} glucose, although no significant differences ($p > 0.05$) between the glucose-supplemented and glucose-free cultures. Similarly, Kong et al.

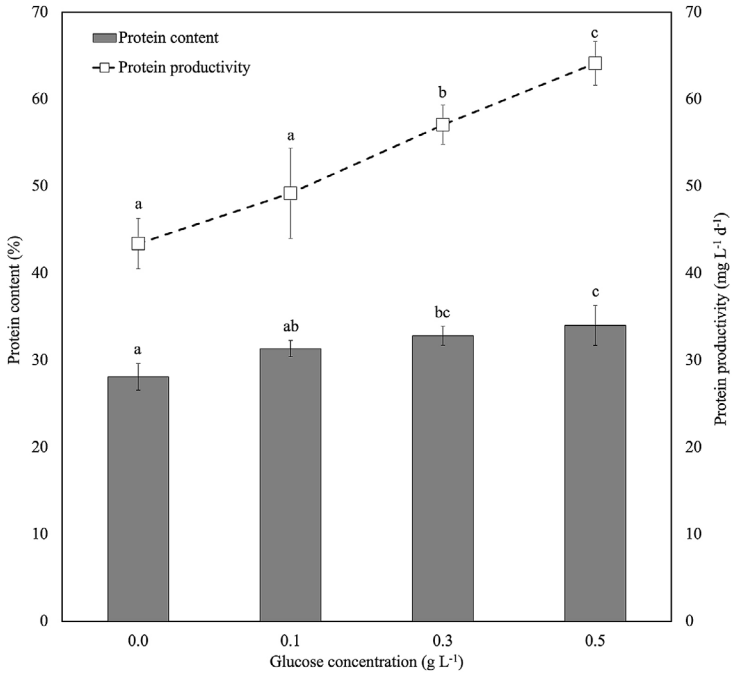

Figure 3. Protein content and productivity of *M. braunii* under different glucose concentrations. Error bars correspond to standard error of the mean. All parameters were tested in triplicates. Different letters indicate significant difference ($p < 0.05$) of protein content and productivity

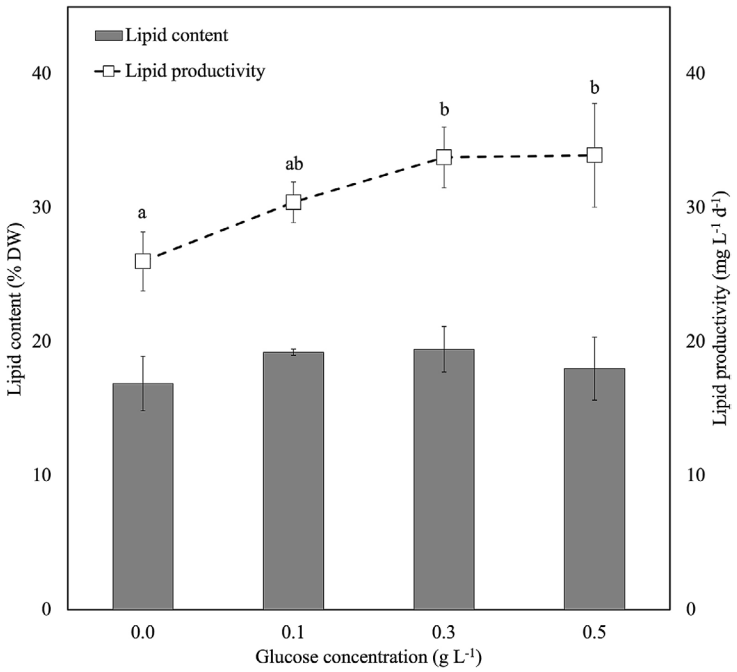

Figure 4. Lipid content and productivity of *M. braunii* at various glucose concentrations. Error bars correspond to standard error of the mean. All parameters were tested in triplicate. Different letters indicate significant difference ($p < 0.05$) of lipid productivity

(2013) reported that the supplementation of glucose in the medium did not support the accumulation of lipids in *C. vulgaris*. The obtained findings indicate that glucose might be used more for cell growth than converting carbon into lipids.

As illustrated in Figure 4, the lipid productivity of *M. braunii* significantly increased along with glucose concentrations. The availability of glucose had a favorable effect on algal lipid productivity. The 0.5 g L^{-1} glucose-supplemented cultures had the maximum lipid productivity of 33.92 mg L^{-1} d^{-1}, which was 30.5% greater than that of the control. Similarly, when glucose was added to the medium, Kong et al. (2013) and Anahas et al. (2024) observed a notable rise in the lipid productivity of *C. vulgaris* and *A. sphaerica*, respectively. Compared to the lipid content, the variations in lipid productivity generated by the effects of glucose addition were more noticeable. Since the organic carbon sources significantly increased the algal biomass content, glucose increased the lipid productivity of microalgae (Kong et al., 2013).

Although this study maintained constant illumination (200 µmol photon m^{-2} s^{-1}) and standard BG-11 nutrient ratios to specifically isolate the effects of glucose, it is recognized that environmental parameters such as light intensity, photoperiod, and C:N ratio could modulate the metabolic responses of microalgae (Li et al., 2014; Fakhri et al., 2017; Pereira et al., 2024). Future experiments combining glucose with varying light regimes and nutrient balances are therefore warranted to further optimize mixotrophic productivity in *M. braunii*.

CONCLUSIONS

Cultivating *M. braunii* in mixotrophic system with glucose as a carbon source has been shown to significantly boost biomass production, protein, and lipid productivity. The results of the study demonstrate that using glucose supplementation accelerates growth and increases metabolic activity, which leads to an increase in lipid and protein accumulation. The best glucose level of 0.5 g L^{-1} provided an excellent balance between growth, protein, and lipid productivity, with a biomass concentration of 0.99 g L^{-1}, a protein productivity of 64.13 mg L^{-1} d^{-1}, and a lipid productivity of 33.92 mg L^{-1} d^{-1}.

Glucose was selected due to its well-documented role in enhancing algal metabolism and ease of assimilation. However, future studies comparing alternative organic carbon sources such as glycerol or acetate would be valuable in determining the most cost-effective and efficient substrate for large-scale applications. The potential risk of microbial contamination in large-scale operations

also highlights the need for appropriate culture management strategies including sterilization or pasteurization of culture media and maintaining high algal cell densities to competitively suppress contaminants. On the basis of the enhanced protein productivity and moderate lipid improvement, the most promising applications of this research are in aquaculture feed and functional protein industries, with additional potential for integration into biofuel production within a biorefinery framework. Thus, this study provides both fundamental insights and applied perspectives for optimizing M. braunii cultivation under mixotrophic conditions.

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