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THE GROWTH KINETICS AND TOTAL LIPID CONTENT OF *Thalassiosira* sp. UNDER MIXOTROPHIC CONDITIONS

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ABSTRACT

Conventional microalgae culture is challenged by issues of light limitation and cell self-shading. This study aims to evaluate the impact of different cultivation modes on the growth and lipid content of *Thalassiosira* sp. The diatom, *Thalassiosira* sp., was grown in autotrophic, mixotrophic-suspended, and mixotrophic-biofilm conditions until the stationary phase was reached. After four (4) days of culture, analysis of the cell densities revealed a significant difference between groups, with cell densities of 7.3×10^5 cells mL^{-1} for control, 1.1×10^6 cells mL^{-1} for mixotrophic-suspended, and 1.9×10^6 cells mL^{-1} for mixotrophic-biofilm cultures. Both treatments are significantly higher than the control. However, mixotrophic-biofilm culture achieved the highest cell density among all cultivation modes, 161.81% higher than the control. The specific growth rate of *Thalassiosira* sp. in mixotrophic-biofilm culture was highest among treatments, while the doubling time was significantly highest in the control. Moreover, mixotrophic-biofilm culture attained the highest biomass at 56 mg 100 mL^{-1}. *Thalassiosira* sp. cultured under mixotrophic-biofilm also recorded the highest lipid content at 9.89%. It is both significantly higher than the control (3.06%) and the mixotrophic-suspended culture (6.15%). The cell density, algal biomass, and lipid content of *Thalassiosira* sp. under mixotrophic-biofilm culture highlight this culture strategy's promising potential in improving microalgae growth and lipid content, ridding of light as an indispensable growth factor.

KEYWORDS: biofilm; lipid content; mixotrophic growth; suspended culture; *Thalassiosira*

INTRODUCTION

Microalgae are unicellular photosynthetic microorganisms typically found in freshwater and marine environments. Their excellent potential for use as animal feed, bioactive compound, bioremediation agent, fertilizer, and fuel production feedstock has gained immense attraction in various industries (Abu Hasan *et al*., 2021). In aquaculture, microalgae are used as live feeds for zooplankton, bivalves, crustaceans, and other fish species. Their ability to biosynthesize long-chain polyunsaturated fatty acids (LC-PUFAs) has attracted interest for their use as an omega-3 source in aquaculture (Shishlyannikov *et al*., 2014).

The marine diatom *Thalassiosira* sp. is one of the many microalgal species commonly used in commercial aquaculture settings for its small size, fast growth, and high nutritional content (Kaspar *et al*., 2014). *Thalassiosira* sp. is often used as feed for larvae and juveniles of commercially important shellfish species. Aside from being used as feed for shellfish, it is also fed to rotifers fed to late larvae, juvenile finfish, and crustaceans to enhance their nutritive values (Brown, 2002; Jannah *et al*., 2019). Marine diatoms such as *Thalassiosira* are well-known for their high concentrations of biologically essential highly unsaturated fatty acids (HUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Becker, 2004) indicating their role as a potential primary and sustainable source of omega-3 fatty acids in aquaculture. Recently, a species of *Thalassiosira* was incorporated into the diet of seawater-tilapia as a feed additive and improved its omega-3 fatty acid content (Huervana *et al*., 2022).

Currently, microalgal production is done autotrophically, where the microalgae utilize light energy for growth and biomass production. Autotrophic cultivation, however, severely limits growth, biomass, and lipid production due to light limitations and cellular self-shading (Cheirsilp & Torpee, 2012). In addition, technological and economic challenges, such as high harvesting costs in microalgae production, must be overcome (Ribeiro *et al*., 2019). This signifies the need for sustainable culture strategies that

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could boost microalgae growth, biomass, and lipid production while reducing production costs.

An alternative culture strategy to autotrophic culture is mixotrophic culture. Mixotrophic culture is another culture condition where microalgae can use light and organic carbons for growth, maximizing their use of resources and overcoming problems associated with light limitation, resulting in higher growth rates and increased lipid production (Roostaei *et al*., 2018). For instance, in a recent study, results show that the lipid composition of *Phaeodactylum tricornutum* increased by 4.6-fold when grown mixotrophically with glucose as a carbon source without reducing biomass productivity (Wang *et al*., 2012). Positive results were also shown in another study wherein the lipid productivity of *Chodatella* sp. increased by about 5.6-fold under mixotrophic cultivation (Li *et al*., 2014). Studies have demonstrated the feasibility of growing microalgae under mixotrophic conditions for higher biomass and lipid productivity. However, the mixotrophic suspended culture of microalgae still faces high production costs and stress susceptibility issues.

Interestingly, research into mixotrophic microalgae biofilms is scarce. There is also limited research examining the impact of mixotrophic-biofilm cultivation on diatoms' growth and lipid content. The integration of mixotrophic culture into biofilms could overcome challenges associated with conventional microalgae culture technology. Hence, this study focused on examining the impact of the different modes of cultivation (suspended autotrophic, mixotrophic, and mixotrophic-biofilm) on the growth and lipid content of *Thalassiosira* sp.

MATERIALS AND METHODS

Experimental Setup

The study was conducted at the multi-species hatchery of the Institute of Aquaculture, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miagao, Iloilo. The axenic stock culture of *Thalassiosira* sp. was obtained from the Southeast Asian Fisheries Development Center, Aquaculture Department (SEAFDEC/AQD). The growth, biomass yield, and lipid content of *Thalassiosira* sp. were compared under suspended (autotrophic and mixotrophic) and attached (mixotrophic-biofilm) culture conditions.

Culture Conditions

For the suspended culture, 200 mL of *Thalassiosira* sp. (4.60×10^5 cells mL^{-1}) was cultured in a 750 mL glass bottle containing 450 mL chlorinated and UV-filtered seawater with a salinity of 30 g/L. Meanwhile, for the mixotrophic-biofilm treatment, the same volume of algal stock and water were added to 3-L glass jars with roughened coupon polycarbonate (PC) roofing sheet (L x W: 13.21 cm 6.35 cm). The biofilm carriers were oriented in a slanted direction and submerged in the culture medium. Two mL of glycerol (99.5%, reagent grade) was added for the mixotrophic culture. F-medium (Guillard & Ryther, 1962) composed of 1 mL of each technical grade reagent (Na_2SiO_3, $FeCl_3$, $NaNO_3$ and NaH_2PO_4, Na_2EDTA, trace metal, and vitamin stock) was used as an enrichment medium.

The cultures were maintained under a salinity of 30 g/L, water temperature of 25°C, with continuous mild aeration (0.2 L air min^{-1}) and illuminated under a light intensity of 100 µmol photons m^2 s^{-1} . The autotrophic condition served as the control in this study. All test cultures were performed in triplicates and were arranged in a completely randomized design (CRD).

Cell Density Monitoring and Growth Rate

Ten mL of samples from triplicates in each suspended culture were collected daily to monitor microalgal growth. At the end of the experiment, approximately 1 cm^2 was used as a measuring area for the microalgal biofilm and was randomly scraped. Algal cells were resuspended in 1 mL of sterile marine water and counted for microalgal growth.

Cell density was determined by counting using a Neubauer chamber or Hemocytometer and a compound microscope. The specific growth rate (SGR) and doubling time were determined using the formula by Bhattacharya and Shivaprakash (2005).

Biomass Determination

All treatments was harvested after it reached the stationary phase on the fourth day of culture and their cell density and dry cell biomass were determined and analyzed. Harvested algal cells were centrifuged at 4,000 rpm and vacuum filtered through the Whatman GF/C glass microfiber filter (47 mm). The filtered biomass was then transferred to a Petri dish and weighed gravimetrically to obtain the wet weight. The Petri dishes containing the biomass were oven-dried at 60°C overnight. Dried biomass was then weighed to obtain the algae's dry weight.

Lipid extraction

Algal lipids were extracted using the modified Bligh & Dyer (1959) method. Dried algal biomass was pulverized using a mortar and pestle, and half a gram (0.50 g) of the algal sample was weighed and transferred to a 125 mL Erlenmeyer flask for extraction.

The samples were then moistened and stored overnight in a refrigerator. The next day, samples were added with ten (10) mL of chloroform: methanol (1:1) mixture, taken for an hour of sonication, and again stored in a refrigerator overnight. Next, samples were added with another fifteen (15) mL of chloroform: methanol (1:1) mixture and sonicated for an hour. The samples were then vacuum filtered, and the liquid phase was recovered and transferred to a separatory funnel. After transferring to a separatory funnel, five (5) mL of distilled water was added to the sample, and the bottom phase was recovered for rotary evaporation. Extracted lipid from samples was transferred to a pre-weighed vial and was air-dried overnight. The vials were subsequently reweighed to determine the extracted lipids as a percentage of dry weight.

Statistical Analysis

Statistical analysis was performed using the IBM SPSS 26.0 software. The data obtained were subjected to a one-way analysis of variance (ANOVA) to determine whether there is a significant difference in means of the cell density, biomass, and lipid content of *Thalassiosira* sp. under the different cultivation methods. Mean values from various test cultures were compared using Tukey's multiple comparison tests at a 0.05 significance level.

RESULTS AND DISCUSSION

Growth Performance in Mixotrophic Conditions

The growth patterns of *Thalassiosira* sp. under different culture conditions were nearly identical on the first two days. In the following days, however, an exponential increase in cell densities was observed in glycerol-treated samples, indicating the algae's utilization of the organic carbon for growth. Mixotrophic conditions (suspended and biofilm) resulted in higher cell densities than controls, with 1.1×10^6 cells mL^{-1} for suspended culture and 1.2×10^6 cells mL^{-1} for biofilm culture (Figure 1). A significant difference in cell densities ($p < 0.05$) was observed between the culture conditions on day 3 and day 4. On these days, statistical analysis of the cell densities of cultures revealed that cell densities in the mixotrophic-biofilm culture were significantly different from the control ($p < 0.05$) but were not significantly different from the mixotrophic-suspended culture ($p > 0.05$).

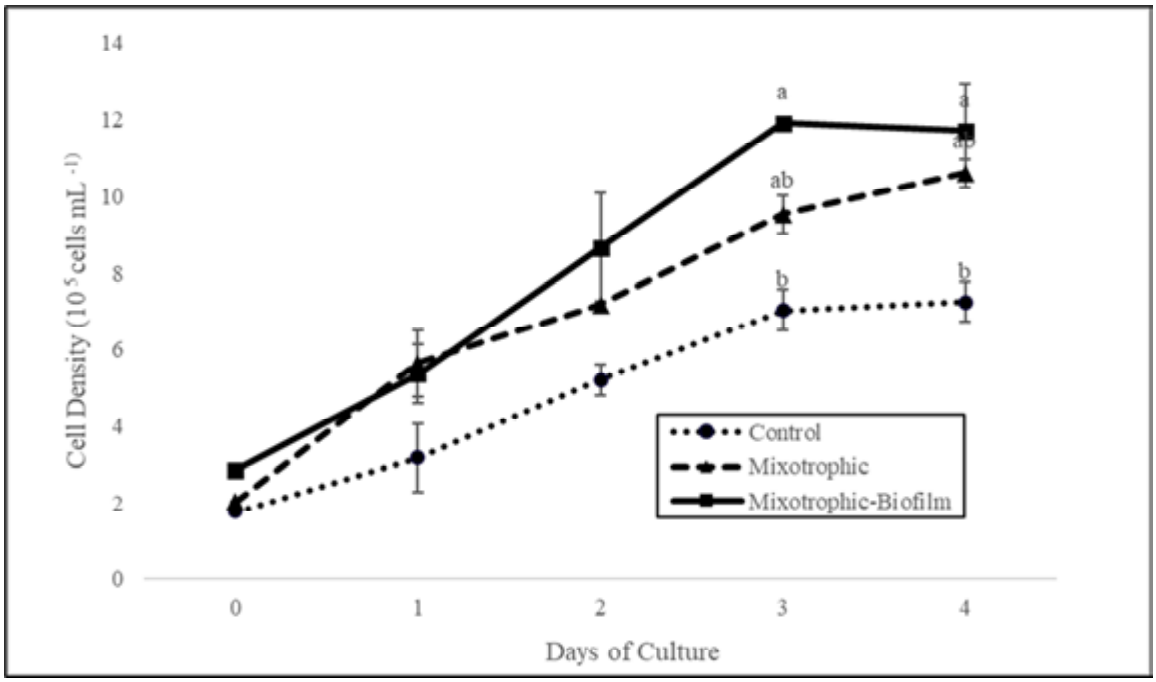

Figure 1. Growth curve of *Thalassiosira* sp. under different cultivation modes (suspended). Values are the means of the replicates ± SEM (n=3). Points with different superscripts indicate significant differences between groups ($p < 0.05$).

Algal growth can be affected by the mode of cultivation. Generally, algal cultivation modes can be divided into autotrophic, heterotrophic, and mixotrophic. Mixotrophic was considered the best mode of cultivation among the three as it effectively integrates the benefits of autotrophic and heterotrophic modes while mitigating the drawbacks associated with both cultivation methods (Meng *et al.*, 2020). In this study, adding glycerol in sample treatments resulted in higher cell densities. In au-

totrophic conditions, light will always be an indispensable growth factor (Cheirsilp & Torpee, 2012). This is because algal species possess a chloroplast to capture light energy and use it for their growth. However, light utilization is affected by self-shading in dense algal cultures, significantly limiting algal growth (Li *et al*., 2019). As observed in the study, the autotrophic culture had lower cell densities and SGR and high doubling time, suggesting a limitation in the growth of *Thalassiosira* sp.

Meanwhile, if cell densities recorded from the substrate of the mixotrophic-biofilm culture were added to the cell densities recorded from its liquid phase, significantly higher cell densities from both the control and mixotrophic-suspended culture were recorded ($p < 0.05$). A total cell density reaching up to 1.9×10^6 cells mL^{-1} was recorded for the mixotrophic-biofilm culture, 161.81% higher than the control (Figure 2). Furthermore, the SGR of *Thalassisosira* sp. in mixotrophic conditions was significantly higher than in autotrophic conditions (Table 1). Particularly, the mixotrophic-biofilm culture exhibited the highest SGR among treatments ($p < 0.05$). The doubling time was significantly lower in mixotrophic conditions than in the autotrophic condition ($p < 0.05$). These results indicate that rearing *Thalassisosira* sp. in mixotrophic conditions improved its growth performance.

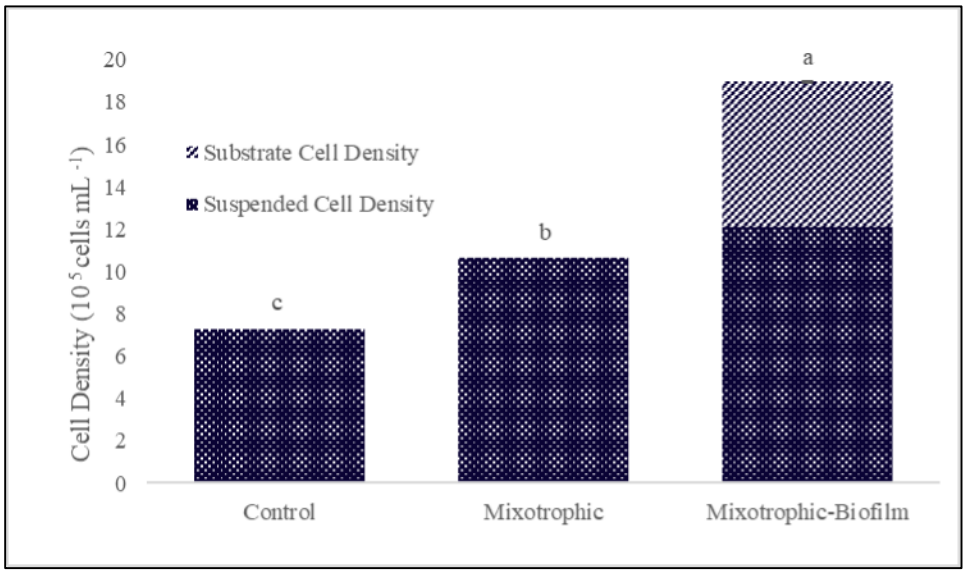

Figure 2. Total cell density (substrate + suspended) of *Thalassiosira* sp. under different cultivation modes. Values are the means of the replicates ± SEM (n = 3). Bars with different superscript letters are significantly different ($p < 0.05$).

Table 1. Growth performance of *Thalassiosira* sp. reared at different culture modes (substrate + suspended)

	Control	Mixotrophic	Mixotrophic-biofilm
Specific growth rate (day $^{-1}$)	3.30 ± 0.02^{c}	3.42 ± 0.01^{b}	3.45 ± 0.00^{a}
Doubling time (day)	0.21 ± 0.00^{a}	0.20 ± 0.00^{b}	0.19 ± 0.00^{c}

Values are the means of the replicates ± SEM (n = 3). Rows with different superscripts indicate significant differences between treatments ($p < 0.05$)

The addition of organic carbon substrates like glycerol in algal cultures allowed the algae to assimilate organic and inorganic carbon (CO_2) substrates, ridding the dependency of the algae on photosynthesis while sustaining optimal or even improved growth. This statement was consistent with the findings of Jiao *et al*. (2018), where the addition of organic carbon substrate (glucose and glycerol) in the culture promoted the optimum growth of *Porphyridium purpureum*. Similarly, higher cell densities were also observed in the mixotrophic culture of *Chlorella* sp. and *Nannochloropsis* sp. (Cheirsilp & Torpee, 2012). Su *et al*. (2020) also reported a 1.6-fold increase in cell densities of *Phaeodactylum tricornutum* under mixotrophic conditions compared to autotrophic conditions. The low light sensitivity of mixotrophic

cultivation is useful in growing microalgae as it could promote higher cell densities, eliminating the influence of light as a limiting factor for growth (D'Imporzano *et al*., 2017).

Comparing the growth between the mixotrophic-suspended and mixotrophic-biofilm cultures, the total cell densities of cultures under mixotrophic-biofilm are significantly higher than those in mixotrophic-suspended. ($p < 0.05$). Cultures under mixotrophic-biofilm attained the highest cell density during the study. In the mixotrophic-biofilm cultures, some algal cells attached to the biofilm carrier (solid phase), and some remained in suspension (liquid phase). The higher cell density recorded for mixotrophic-biofilm compared to other culture modes may be owed to the presence of the biofilm carrier PC on top of the advantage provided by the organic carbon substrate. In this study, adding PC as the biofilm carrier in mixotrophic-biofilm did not limit the growth of *Thalassiosira* sp. Similar to the study of Lin-Lan *et al*. (2018), biofilm carriers added extra space and extra attached friction for the algae to grow, resulting in higher cell densities compared to the suspended cultures.

As observed in Figure 1, algal growth in the mixotrophic-suspended culture on the first day is higher, although not statistically significant with the mixotrophic-biofilm culture ($p > 0.05$). The slow growth of mixotrophic-biofilm on the first day of culture could be attributed to the trapping of cells in the supporting material upon its addition, causing the slow growth of cells in the liquid phase of mixotrophic-biofilm. However, increased growth in mixotrophic-biofilm culture was observed on the following days compared to mixotrophic-suspended culture. After the short-term entrapment of cells, light may find it difficult to reach the solid phase, leaving the cells in the liquid phase of the mixotrophic-biofilm culture an advantage in obtaining and utilizing the growth sources. The same observations were recorded in the studies of Hamano *et al*. (2017) and Lin-Lan *et al*. (2018), where microalgae under suspended-solid culture have better growths than in single suspended culture. Nevertheless, cultures under mixotrophic-biofilm integrated the advantages of suspended and attached algal culture, thus recording the highest growth among all cultivation modes ($p < 0.05$).

Microalgal Biomass in Mixotrophic Conditions

In addition to cell densities, dry weight biomass between the different culture conditions was also compared. Mixotrophic-suspended and mixotrophic-biofilm have significantly higher dry-weight biomass compared to the control at 46.85 ± 0.60 mg 100 mL^{-1} and 56.4 ± 0.31 mg 100 mL^{-1}, respectively ($p < 0.05$). Recorded biomass for cultures under mixotrophic conditions (suspended and biofilm) are 193% and 133% better than the control (Figure 3). Specifically, *Thalassiosira* sp. reared in mixotrophic-biofilm culture had the significantly highest dry-weight biomass among treatments.

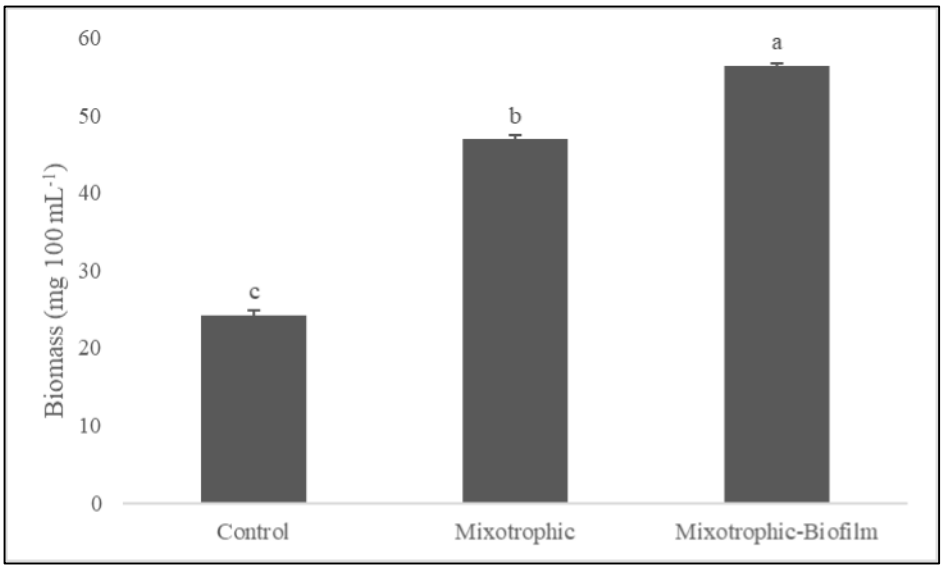

Figure 3. Dry weight biomass of *Thalassiosira s*p. under different cultivation modes. Values are the means of the replicates ± SEM (n=3). Bars with different superscript letters are significantly different ($p < 0.05$).

As microalgae like *Thalassiosira* can utilize organic and inorganic carbon sources, the addition of glycerol to the mixotrophic culture (mixotrophic suspended and mixotrophic biofilm) has significantly enhanced the biomass yield. Glycerol is considered one of the great sources of organic carbon for microalgae culture, which promotes growth and maximizes biomass production, resulting in higher biomass yield (Yun *et al*., 2021). This is supported by the study of Kong *et al*. (2013) where treatments with carbon sources like glycerol and glucose in the culture medium have superior biomass production and yield than the autotrophic control. Compared to autotrophic cultures, where light is the sole energy source for optimum growth, mixotrophic cultures have two energy sources, i.e., carbon and light energy. Thus, problems like photoinhibition during culture can be overcome, leading to high biomass yields, as observed in treatments with glycerol (Pereira *et al*., 2021).

Microalgal Lipid Content in Mixotrophic Conditions

Stress conditions, e.g., environmental or nutrient stress, are often employed to enhance the lipid content in many microalgal species. However, this method usually leads to low growth rates and biomass yield. In this study, glycerol was added to the growth medium of the diatom, *Thalassiosira* sp., cultured in different cultivation modes in an attempt to enhance its lipid content without compromising the biomass.

As seen in Figure 4, glycerol-treated samples have better lipid contents than the control. *Thalassiosira* sp. cultured under mixotrophic-suspended condition attained an average of 6.15% lipid content (100.98% higher than the control). Meanwhile, *Thalassiosira* sp. cultured under mixotrophic-biofilm condition attained an average of 9.89% lipid content (223.20% higher than the control). By comparison, *Thalassiosira* sp. cultured under mixotrophic-biofilm condition achieved the highest lipid content, which was significantly higher than the control and the mixotrophic-suspended culture conditions ($p < 0.05$).

Similar results were obtained in the study of Choi & Lee (2015), where the mixotrophic culture of *Neochloris oleabundans*, *Botryococcus braunii,* and *Dunaliella* sp., with glycerol as the carbon source, yielded a 2–13% higher lipid content compared to the autotrophic culture. Results in this study are also consistent with the findings of Wang *et al*. (2012), where a 2.8- to 4.6-increase in lipid content was observed in *Phaeodactylum tricornutum* under mixotrophic culture with glucose, starch, and acetate sodium as organic carbon source. The addition of organic carbon sources like glycerol in the growth medium drives the microalgae to utilize the organic carbon source as an energy source together with light (Meng *et al*., 2020), providing additional energy and material for biosynthesis, improving the microalgae's growth and lipid content (Wan *et al*., 2011). Although not thoroughly studied in diatoms, organic carbon sources like glycerol in microalgae can generally be used as a backbone for TAG synthesis after being converted to glycerol-3-phosphate (G3P). In addition, glycerol can also be degraded to dihydroxyacetone phosphate (DHAP), which is converted into glyceraldehyde phosphate (GAP) and subsequently to 3-phosphoglycerate. The conversion of DHAP to the mentioned metabolic intermediates is biochemically sig-

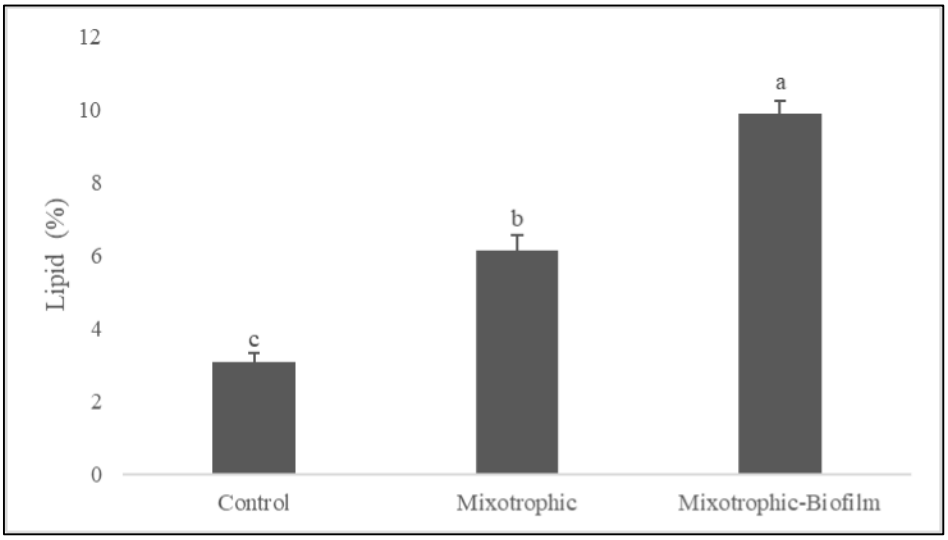

Figure 4. Lipid content of *Thalassiosira* sp. under different cultivation modes. Values are the means of the replicates ± SEM (n=3). Bars with different superscript letters are significantly different ($p < 0.05$).

nificant to lipid synthesis as they indirectly contribute to this process (Baldisserotto *et al*., 2021; Cecchin *et al*., 2018; Villanova *et al*., 2017; Yang *et al*., 2000). For instance, l-serine, required to synthesize lipids, is derived from the glycolytic intermediate 3-phosphoglycerate (Yamasaki *et al*., 2001). Generally, regardless of the organic substrate used, in mixotrophic microalgal culture, the production of intermediates essential for lipid metabolism, such as Acetyl-CoA and NADPH, are enhanced, thus triggering lipid synthesis (Wan *et al*., 2011).

In addition to the high lipid contents observed in samples treated with glycerol compared to the control, the highest lipid content was observed in *Thalassiosira* sp. cultured under mixotrophic-biofilm condition. Usually, algal biofilms yield lower or unchanged lipid contents due to over-shading and nutrient limitations, and there are also instances where regions of "thick" biofilms are not exposed to light, thus limiting their ability to synthesize lipids (Roostaei *et al*., 2018; Schnurr, 2016). However, with the addition of glycerol to the growth medium, issues of overshading and nutrient limitation, especially of the carbon source, are addressed. Carbon is a crucial nutrient in growing algal biofilms. Algal growth plummets once the carbon utilization exceeds its supply, resulting in lower biomass quantity, quality, and lipid content (Patwardhan *et al*., 2022). With the presence of another carbon source, i.e., glycerol, microalgal growth continues to be supported even with the depletion of carbon dioxide (CO_2). The presence of organic carbon may also promote the release of extracellular polysaccharides, which are beneficial for algal biofilm growth (Roostaei *et al*., 2018). Although both the mixotrophic suspended and mixotrophic biofilm has glycerol added in their medium, the mixotrophic biofilm yielding the highest DW biomass among all treatments explains why it also produced the highest lipid content as a high biomass production and yield equates to enhanced lipid production.

CONCLUSION

The addition of glycerol in the culture improved the growth, dry-weight biomass, and lipid content of *Thalassiosira* sp. The addition of biofilm carriers to the mixotrophic mode of cultivation further improved the cell density, dry-weight biomass, as well as lipid content of *Thalassiosira* sp. Utilizing the mixotrophic-biofilm culture in microalgae production proved to be a promising method for improving growth, biomass yield, and lipid content, eliminating the challenges posed by conventional microalgae culture technologies.

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